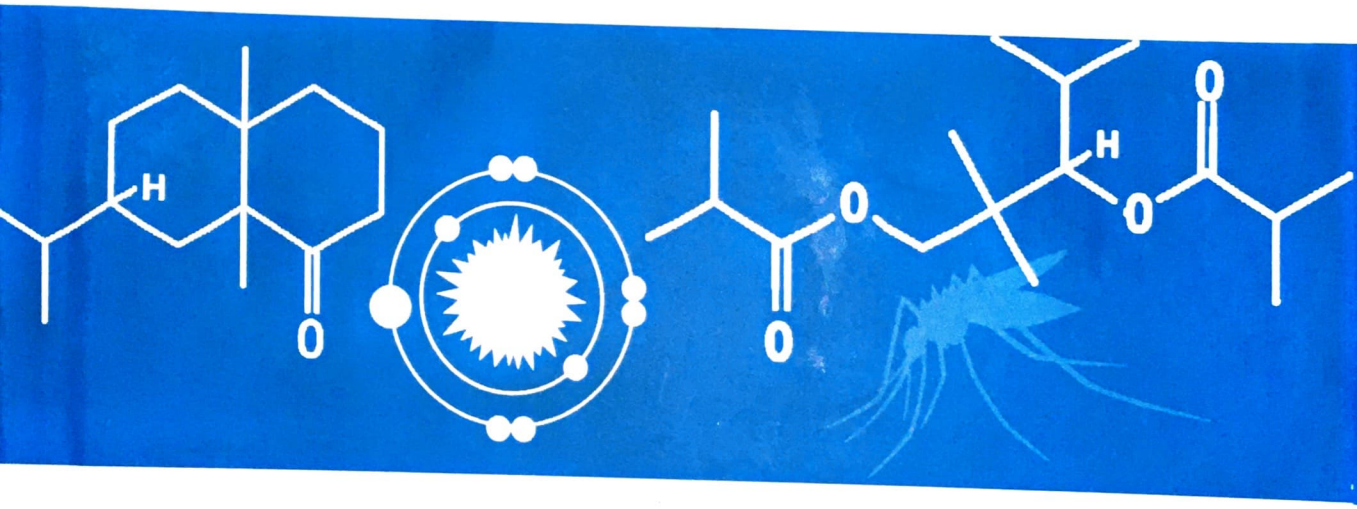
LARVICIDAL EFFICACY OF A FEW BIOACTIVE COMPOUNDS FROM TRADITIONALLY USED MEDICINAL PLANTS AND THEIR ACTION ON A FEW TARGET ENZYMES IN *AEDES AEGYPTI* (L), A DENGUE FEVER VECTOR

ANOOP KUMAR A.N.

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the faculty of science, University of Calicut



Co- Guide

Dr. SUDHIKUMAR A. V.
Assistant professor
Research and Post-Graduate
Department of Zoology
Christ collge, Irinjalakuda

Under the supervision of

Dr. ANEESH E. M.
Assistant professor
Communicable Disease Research Laboratory [CDRL]
Department of Zoology
St. Joseph's college, Irinjalakuda

CHRIST COLLEGE
(AUTONOMOUS) IRINJALAKUDA
THRISSUR, KERALA, INDIA 680125

CDRL
Communicable Disease Research Laboratory [CDRL]
DEPARTMENT OF ZOOLOGY, ST.JOSEPH'S COLLEGE,
IRINJALAKUDA, THRISSUR, KERALA, INDIA

LARVICIDAL EFFICACY OF A FEW BIOACTIVE COMPOUNDS FROM TRADITIONALLY USED MEDICINAL PLANTS AND THEIR ACTION ON A FEW TARGET ENZYMES IN *AEDES AEGYPTI* (L), A DENGUE FEVER VECTOR

ANOOP KUMAR A.N.

Thesis submitted in partial fulfilment of the requirements for the Degree of DOCTOR OF PHILOSOPHY IN ZOOLOGY
Under the faculty of science
University of Calicut

Under the supervision of

Dr. ANEESH E.M.

Assistant professor
Post-Graduate Department of Zoology
St. Joseph's College, Irinjalakuda, Thrissur

CO-GUIDE

Dr. SUDHIKUMAR A.V.

Assistant professor
Research and Post-Graduate Department of Zoology
Christ College, Irinjalakuda, Thrissur

CHRIST COLLEGE, IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

OCTOBER 2020

DECLARATION

I, ANOOP KUMAR A.N, hereby declare that the work embodied in the thesis "**Larvicidal efficacy of a few bioactive compounds from traditionally used medicinal plants and their action on a few target enzymes in *Aedes aegypti* (L), a dengue fever vector**" submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by me under the supervision of Dr. Aneesh E.M, Assistant professor, Post-Graduate Department of Zoology, St. Joseph's college, Irinjalakuda, Thrissur and Dr. Sudhikumar A.V, Assistant professor, Research and Post-Graduate Department of Zoology, Christ College, Irinjalakuda, Thrissur as Co-guide and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

Irinjalakuda
October 2020

Mr. Anoop Kumar A.N.

Research and Post-Graduate Department of Zoology
Christ College Irinjalakuda

CHRIST COLLEGE, IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

October 2020

CERTIFICATE

This is to certify that the thesis entitled "**Larvicidal efficacy of a few bioactive compounds from traditionally used medicinal plants and their action on a few target enzymes in *Aedes aegypti* (L), a dengue fever vector**" submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is an authentic record of the work carried out by Mr. ANOOP KUMAR A.N, under my supervision of Dr. Aneesh E M in Communicable Diseases Research Laboratory (CDRL) and Research and Post-Graduate Department of Zoology, Christ College, Irinjalakuda, University of Calicut and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

Dr. SUDHIKUMAR A.V.
(Co-guide)

Dr. SUDHIKUMAR A.V.
ASSISTANT PROFESSOR & RESEARCH SUPERVISOR
CENTRE FOR ANIMAL TAXONOMY & ECOLOGY
DEPARTMENT OF ZOOLOGY
CHRIST COLLEGE, IRINJALAKUDA, KERALA - 680 125

Dr. ANEESH E.M.
(Guide)

Dr. ANEESH E.M.
Assistant Professor & Research Supervisor
Department of Zoology
St. Joseph's College (Autonomous)
Irinjalakuda, Kerala - 680 121

Research and Post-Graduate Department of Zoology
Christ College Irinjalakuda

CHRIST COLLEGE, IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

October 2020

CERTIFICATE

This is to certify that the thesis entitled "**Larvicidal efficacy of a few bioactive compounds from traditionally used medicinal plants and their action on a few target enzymes in *Aedes Aegypti* (L), a dengue fever vector**" submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is an authentic record of the work carried out by Mr. ANOOP KUMAR A.N, in the Research and Post-Graduate Department of Zoology, Christ College, Irinjalakuda, University of Calicut and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

Head in-charge
Department of Zoology

Dr. SUDHIKUMAR A.V.
ASSISTANT PROFESSOR & RESEARCH SUPERVISOR
CENTRE FOR ANIMAL TAXONOMY & ECOLOGY
DEPARTMENT OF ZOOLOGY
CHRIST COLLEGE, IRINJALAKUDA, KERALA - 680 125

CERTIFICATION OF SUPERVISING GUIDE

This is to certify that all the corrections/suggestions from the adjudicators have been incorporated in the thesis entitled "**Larvicidal Efficacy of a Few Bioactive Compounds from Traditionally used Medicinal Plants and their action on a Few Target Enzymes in *Aedes Aegypti* (L), A Dengue Fever Vector**", carried out by Mr. ANOOP KUMAR.A.N. under my supervision. The contents in the CD and thesis are the same.

Dr. Sudhikumar A.V.

Co-Guide

Dr. SUDHIKUMAR A.V.
ASSISTANT PROFESSOR & RESEARCH SUPERVISOR
CENTRE FOR ANIMAL TAXONOMY & ECOLOGY
DEPARTMENT OF ZOOLOGY
CHRIST COLLEGE, IRINJALAKUDA, KERALA-680 125

Dr. Aneesh E.M.

Research Supervisor

Dr. ANEESH E.M.
Assistant Professor & Research Supervisor
Department of Zoology
St. Joseph's College (Autonomous)
Irinjalakuda, Kerala - 680 121

CHRIST COLLEGE, IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

October 2020

CERTIFICATE

This is to certify that Mr. ANOOP KUMAR A.N has completed the research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis "**Larvicidal efficacy of a few bioactive compounds from traditionally used medicinal plants and their action on a few target enzymes in *Aedes Aegypti* (L), a dengue fever vector**" embodies the results of his investigations conducted during the period at which he worked as a research scholar. I recommend the thesis to be submitted for the evaluation for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

PRINCIPAL

In-charge of Principal
Christ College (Autonomous)
Irinjalakuda

ACKNOWLEDGEMENTS

Undertaking this Ph.D. has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

First and foremost, I want to thank my supervisor **Dr. ANEESH E.M**, Assistant Professor Department of Zoology, St. Joseph's College, Irinjalakuda; Director, Communicable Diseases Research Laboratory (CDRL). I appreciate all his contributions of time, and ideas to make my Ph.D. experience productive and stimulating. The joy and enthusiasm have for his research were contagious and motivational for me, even during tough times in the Ph.D. pursuit. Moreover, his exemplary guidance, monitoring, and constant encouragement during the course of research will linger in my memory throughout the journey I am about to embark. Your wealth of knowledge in the field of mosquito vector control, Phytochemistry, Microbiology, and Molecular Biology in particular is inspiring. Thank you for giving me the opportunity to grow in this field of research.

I am grateful to my Co-guide Dr. Sudhikumar A.V, Assistant Professor, Research and Post-Graduate Department of Zoology, Christ College, Irinjalakuda for his immense guidance, suggestions and support.

I would like to express my deep sense of gratitude to Dr. Prof. Nasser M, Pro-Vice Chancellor, and Director of DOR, University of Calicut for the constant support and motivation throughout my research.

My deep appreciation goes out to Dr. Mathew Paul, Principal (Retd.), and Rev. Dr. Jolly Andrews, Principal-in-Charge, Christ College, Irinjalakuda for providing sufficient facilities for the successful completion of work.

I also extend my sincere gratitude to former principal Fr. Dr. Jose Thekkan C M I (Late) for his timely support and blessings.

I express my immense gratitude to the Dr. Sr. Asha. Therese, Principal, St. Joseph's College, Irinjalakuda for the facilities provided.

My deep appreciation goes out to Dr. C O Joshi, Head, Department of Zoology, Christ College, Irinjalakuda and Head, Department of Zoology, St. Joseph's College, Irinjalakuda for providing research facilities.

I extend my sincere thanks to teaching and non-teaching staff of Christ College, Irinjalakuda and St. Joseph's College, Irinjalakuda for their kind support and encouragement.

The financial assistance from E-Grantz, Government of Kerala is gratefully acknowledged.

I owe special thanks to Dr. Pushpalatha, Head, University of Calicut and members of the teaching and non-teaching staff of the University of Calicut for their co-operation during the period of my study.

The members of the Communicable Diseases Research Laboratory (CDRL) have contributed immensely to my personal and research work. The CDRL community has been a source of friendships as well as good advice and collaboration. I am also grateful to Dr. Sharrel Rebello, Assistant Professor, Department of Applied Microbiogy and Forensic Science, St. Joseph's College, Irinjalakuda for her valuable advice. The work could not have gone ahead without the support of my colleagues, Ms. Lekshmi jayakrishnan, Mr. Sreedev Puthur, Mrs. Fathibi, Mrs. Asha Amabadath, Mrs. Lekshmi K V, Mrs. Elsa, Ms. Kavya Raj, Ms. Sini Francis, Ms. Aby Mol, and Ms. Alisha. I am obliged them for their valuable comments, suggestions and timely support throughout my study. I am much indebted to Dr. Siva Prasad M.S, Assistant Professor, Kerala Police Academy, Thrissur, India, whose constant support has helped me a lot.

A very special thank you to Lt Litty chacko, Head, Department of Malayalam, St. Joseph's College, Irinjalakuda for her constant support and encouragement.

In addition to this, I am greatly indebted to Dr. Jisha M S, Professor, School of Biosciences, MG University for her support.

I express my immense gratitude to Ms. Edna, School of Biosciences, MG University for her immense support.

I express my immense gratitude to Dr. Binu Ramachandran, Department of Zoology, Christ College, Irinjalakuda for his timely support and valuable suggestions and cooperation during the way of my investigation.

I also utilize this opportunity to express my thanks to all the Research Scholars of Department of Zoology, Christ College, Irinjalakuda for their support and encouragement during the course of the study.

I would like to express my deepest appreciation to Noble A. Paliath, Baby. Pushkaran, Kuttamman and Vinurajan P.K. ,

My special thanks to Mr. Jithin Mathew, Christ College, Irinjalakuda. I am greatly indebted to him for the completion of this work in the specified period.

I would like to extend my sincere thanks to my best friends; Mr. Suresh Kumar B, Mr. Sudhin P P, Mr. Binu George, Mr. Praveen and other friends in men's hostel for their support.

Completion of the thesis, of course, ultimately due to the encouragement and support of my family. For my parents who raised me with a love of science and supported me in all my pursuits. I am grateful to them for their immense love, patience, care and understanding in making the years of my research unforgettable.

I also utilize this opportunity to express my thanks to my dear friends and people from Pothumoola, Thirunelly, Wayanad for their co-operation and giving helps.

Besides the above said persons, I express my profound gratitude to all those unknown persons who helped me in some way or the other in the course of my research.

Above all, I thank the Almighty God from the bottom of my heart, who is the constant source of strength, wisdom, love, guidance and inspiration and for giving me immeasurable blessing, for without him this could not be possible.

Thank you

Mr. Anoop Kumar A.N

Acronyms and Abbreviations

ACT	Artemisinin-based Combination Therapy
CNS	Central Nervous System
CYTP450	Cytochrome P450
DDT	Dichloro Diphenyl Trichloroethane
DEET	N,N-diethyl-meta-toluamide
DEM	N,N-diethyl mendelic acid amide
DENV	Dengue Viruses
DHF	Dengue Hemorrhagic Fever
DMP	Dimethyl phthalate
DNA	Deoxyribonucleic acid
FTIR	Fourier-Transform Infrared Spectroscopy
GBS	Guillain-Barré Syndrome
GC-MS	Gas Chromatography–Mass Spectrometry
GIS	Geographic Information System
GPS	Global Positioning System
GSTs	Glutathione S-transferases
HCH/BHC	Hexachlorocyclohehane/ Benzenehexa chloride
IRS	Indoor Residual Spraying
ISDP	Integrated Disease Surveillance Project
KPO_4	Potassium phosphate
NIST	National Institute of Standards and Technology
NMEP	National Malaria Eradication Programme
NMR	Nuclear Magnetic Resonance Spectroscopy
NVBDCP	National Vector Borne Disease Control Programme
PAGE	Polyacrylamide Gel Electrophoresis
PNS	Peripheral Nervous System
QPAR	Quantitative Property–Activity Relationship
QSAR	Quantitative Structure-Activity Relationship

ROS	Reactive Oxygen Species
RR	Resistance Ratio
SD	Standard deviation
TLC	Thin Layer Chromatography
TMBZ	3, 3`, 5, 5` tetramethyl benzidine
WHO	World Health Organization
WHO-SEA	World Health Organization-South East Asia

Definition of key terms

The present study used the terms "CC7" (Jatamansone), "EE5" (2,2,4- Trimethyl-1,3-pentanediol diisobutyrate), and "PP4" (3-hydroxy- 2,2,4- trimethylpentyl isobutyrate) for respective isolated compounds.

CONTENTS

List of Figures

List of Tables

Screening of Aromatic Plants against *Aedes aegypti*

Abstract

The control of dengue fever vectors has arrived a major phase of challenge to the research community due to the enhanced risk of resistance to synthetic insecticides in *Aedes aegypti* mosquitoes. About 2 million of the human population has been severely affected by arthropod-borne diseases, especially by dengue fever every year. The frequent and routine use of synthetic insecticides for mosquito control is allied with several challenges like resistance, environmental hazards, and toxic effects to non-target organisms. A research based on the development of eco-friendly strategies that would enlighten the existing method is significant for killing the aforementioned challenges. Therefore, this objective is intended to screen at least fifty locally available and traditionally important medicinal plants against *Aedes aegypti*, using organic solvents of increasing polarity. The mean percentage of mortality recorded after 24 hours of continuous exposure of extracts has exposed that out of 50 traditionally used medicinal plants, 21 plants exhibit more than 41% of toxicity, four plants show 31-40 of toxicity, and eight plants show 20-30% of mortality. Despite the fact that there exist several studies on medicinal plants as an effective larvicide against mosquitoes, none of them have revealed the isolation, and characterization of bioactive elements from three medicinal plants such as *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius*. The aforementioned three plants have shown a prominent range of larvicidal potential against *Aedes aegypti* fourth instar larvae. This study suggested that the medicinal plants showing a prominent range of toxicity against *Aedes aegypti* could be recognized as a ground-breaking notion for the development of natural insecticide.

Identification of Bioactive Compounds

Abstract

Novel insecticides and mosquito control strategies are needed to broaden the horizons of the chemical arsenal for preventing the threats from emerging infectious agents, such as Dengue virus (I-V). Medicinal plants are known to produce a wide range of phytochemical constituents with potential larvicidal activity. Therefore, significant consideration has been given to research on a plethora of traditionally used medicinal plants for outlining their larvicidal potential against the dengue fever vector *Aedes aegypti*. The present objective delivers the first evidence that the three isolated bioactive elements such as Jatamansone, 3-hydroxy-2,2,4-Trimethylpentyl isobutyrate, and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate isolated from *Jasminum brevilobum*, *Pogostemon auricularius*, and *Aglaia edulis* respectively exhibited sturdy larvicidal efficacy against *Aedes aegypti* fourth instar larvae. The identification and the structural characterization of the aforementioned compounds were accomplished using Thin-layer chromatography, Column chromatography, Fourier-transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR) spectroscopy, and Gas Chromatography-Mass Spectrometry (GC-MS). For statistical analyses, Statistical Package for the Social Sciences (SPSS), and R software were used. Among the three isolated bioactive elements, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate exhibited more toxicity than the other two with an LC_{50} 1.724 (0.834-2.479). The findings of this objective have shown extensive effectiveness of the isolated bioactive elements for mosquito control. This investigation advocates that future research aims at determining the chance of emerging insecticide resistance against the *Aedes aegypti* mosquitoes.

Susceptibility Status of 4th Instar Larvae of *Aedes aegypti* towards the Plant Isolates

Abstract

During the past few epochs, the emergence of resistance against various synthetic insecticides among dengue fever vector *Aedes aegypti* has posed a drastic challenge in many countries including India. One of the most effective ways to reduce the chance of insecticide resistance is the use of bioactive elements for vector control instead of using synthetic insecticides. Therefore, modern vector control strategies have a habit of using less polluting and more selective bio-derived products for mosquito control. The natural insecticides have a reputation for being expensive; hence, makes the public choose synthetic insecticides for vector control. In context with the aforementioned concepts, the present objective analyzed the susceptibility status of 4th instar larvae of *Aedes aegypti* towards the plant isolates such as Jatamansone, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, and 3-Hydroxy-2,2,4-trimethylpentyl isobutyrate isolated from *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius*. This objective has verified that the three selected bioactive elements such as Jatamansone, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, and 3-Hydroxy-2,2,4-trimethylpentyl isobutyrate don't instigate a threat towards mosquito control in terms of insecticide resistance since their higher concentration causes a moderate range of resistance in *Aedes aegypti* fourth instar larvae. The results obtained in this study could be considered as a platform to prevent the challenges confronted in the frequent use of natural insecticides for mosquito control.

Qualitative and Quantitative Analysis of a Few Detoxifying Enzymes in Susceptible and Phytochemical Selected Lines of *Aedes aegypti*

Abstract

For the effective control of *Aedes aegypti* mosquitoes, monitoring the emergence of resistance against the toxic compounds is vital. The earlier objectives have verified the toxic potential of three bioactive elements; however, their mode of action, and mechanism behind the emergence of a moderate level of resistance need to study for the development of natural insecticides. Therefore, the present objective has verified the qualitative and quantitative changes of certain detoxifying enzymes such as glucose 6-phosphate dehydrogenase (G6PD), α-esterases (A-est), β-esterases (B-est), and cytochrome P450 with special inference on the extermination of microbial inhabitants and phytochemical prompted reactive oxygen species. All the isolated bioactive elements and the plant extracts were exhibited a prominent range of toxicity against the tested microbial consortia. The bioactive elements such as Jatamansone, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, and 3-Hydroxy-2,2,4-trimethyl pentyl isobutyrate were considered as a double-edged weapon since the excessive production of free radicals has instigated the oxidative stress in the target insect. In context with the aforementioned results and due to the low level of esterases and dehydrogenases activity, the bioactive elements such as Jatamansone, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, and 3-Hydroxy-2,2,4-trimethylpentyl isobutyrate can be recognized as an effective raw material for the preparation of natural insecticide against *Aedes aegypti* mosquitoes in future, and draws a conclusion of the investigation.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Novel repellents and insecticides are essential to extend the diversity of our chemical arsenal for preventing the rapid proliferation of emerging pathogens through the mosquito vectors (Procópio et al., 2015). Emerging evidence in vector control research with special inference on unselective toxicity, insect systems, and environmental persistence suggests that the insecticides of botanical origin have been in the core of interest as source phytochemical compounds for the discovery of novel insecticides (Aiub, Coelho, Sodré, Pinto, & Felzenszwalb, 2002; Amer & Mehlhorn, 2006a; Melo-Santos et al., 2010; Ocampo, Salazar-Terreros, Mina, McAllister, & Brogdon, 2011). The plant-based protection measures have been used in traditional practice against host-seeking arthropod vectors, specifically mosquitoes. Traditional knowledge on repellent plants is considered as a valuable resource to counter various mosquito-borne diseases by developing novel natural insecticides (Kantheti, Alapati, & Sulthana, 2018; Maia & Moore, 2011; Youmsi et al., 2017).

Today, repellent products comprising plant-based constituents have extended aggregating popularity among customers, as these products are usually ascertained as “harmless and safe” in contrast to previously developed synthetic products (Maia & Moore, 2011). The use of insecticides of synthetic origin to prevent the proliferation of mosquitoes of epidemiological significance is becoming increasingly difficult. Problems including product residues, unacceptable threats to non-target organisms, adverse environmental persistence, and withdrawal of

active ingredients together with pest resistance are among those stimulating vector control research to develop alternative strategies. The plant-derived products are considered as significant candidates with low mammalian toxicity, and diminutive environmental persistence (Miresmailli, Bradbury, & Isman, 2006). The increased preference of traditional medicinal plants has subsequently propelled the exploration of vector control strategies against mosquito vectors, specifically *Aedes aegypti* (Linnaeus, 1762), a dengue fever vector. Despite rigorous efforts to counter arboviral diseases, the illness remains to be one of the most serious threats faced by endemic regions of the world (Cook, Diallo, Sall, Cooper, & Holmes, 2005; Ravi, 2006; Reiskind & Lounibos, 2009). The chemical control of mosquito vectors has challenged severe complications such as unselective toxicity of frequently used synthetic insecticides and environmental persistence followed by the emergence of resistant strains (Aiub et al., 2002; Melo-Santos et al., 2010; Ocampo et al., 2011; Vontas et al., 2012). One of the reasonable approaches to mosquito vector control is the search for insecticides of botanical origin as they are biodegradable in nature and minimize the risk of resistance development (Vontas et al., 2012).

India has a diverse culture of traditional medicinal practices for healthcare. In spite of significant advancements in modern therapeutic methods, medicines based on traditional aspects have been still recognized as a primary form of treatment against a large number of abnormalities, diseases, and wounds in developing countries including in India. Hence, in India, a wide variety of decoctions, plants, pastes including plant extracts have principally been used for the treatments against wounds, burns, and cuts (Kumar, Vijayakumar, Govindarajan, &

Pushpangadan, 2007). The number of people using traditional medicine as alternative medicines is promptly increasing day by day all over the world. Well established knowledge regarding the effect of plants on human physiology and the metabolic process have extensively enlarged the significance of medicinal plants in the current scenario (Dey, Ota, Srikanth, Jamal, & Wanjari, 2012). Remarkably, the demand for medicinal herbs in the market is still reported as high (Li, 2000). The prominent compounds in traditional medicinal plants constitute a wide range of active chemical elements that can interfere with the certain biological process of the insect vectors, thus disrupting their life cycle and diminishing the risk to animals and humans (Fallatah & Khater, 2010). In tune with these perspectives, a number of attempts have been made to combat mosquito-borne disease by targeting their larvae as an effective vector control approach. The various forecasting factors such as less chance of developing resistance, target-specificity, higher acceptability, reduced number of applications, appropriateness for rural regions and eco-safety are considered as additional benefits in choosing insecticides of botanical origin (Govindarajan, Mathivanan, Elumalai, Krishnappa, & Anandan, 2011). However, very few attempts have been made on the practical approaches of traditionally used plant-based approaches for the control of mosquito vectors (Innocent et al., 2014). Studies on these aspects can also provide significant results essential for the discovery of novel compounds with insecticidal toxicity.

Today, the majority of research in vector control and pharmacological aspects is focused on the killing effects of plant-based products against various mosquito species. Aromatic plants specifically those in the families of Rutaceae, Labiate,

and Graminae are the most widely used for insect control. In addition, previous studies have reported that several plant species from Oleaceae, Lamiaceae, and Meliaceae families have already been investigated and verified as an effective alternative for synthetic insecticides against *Aedes aegypti*, a dengue fever vector (Candido, Cavalcanti, & Beserra, 2013; Kweka et al., 2008; Misni, Nor, & Ahmad, 2016).

Why *Aedes aegypti*?

Dengue infection, clinical manifestations, pathogenesis, public threats, and significance

Dengue infection is primarily considered as one of the major viral infections in human populations, transmitted by *Aedes* mosquitoes. The extensive distribution of dengue infections includes 3.6 billion people from 124 countries and the risk of infection on each year has now reached around 500 million people (Villanes, Griffiths, Rappa, & Healey, 2018). The severe form of infection is believed to be instigated by a number of aspects, specifically including the host immune status and virus genotype (Rico-Hesse et al., 1997). Five closely linked but antigenically different dengue virus serotypes (DENV-I to DENV-V) have been characterized (Mustafa, Rasotgi, Jain, & Gupta, 2015).

The level of infection rate in the tropical and subtropical regions all over the world has rapid upsurge in these years. One of the probable reasons for the afore-mentioned state is the enhanced number of travelers between the endemic regions (Ito et al., 2004). Besides its burden to the public health systems, dengue-related disorders were responsible for substantial economic losses in tourism, and the

dengue viruses may have been found to grow owing to such significant factors as global warming, and human population size growth together with international transportation systems (Klungthong, Zhang, Mammen, Ubol, & Holmes, 2004). Clearly, as a consequence of the impact of increased population density by the development of international transportation systems and urbanization, the exposure incidence of humans to arthropod vectors and the international mobility of human beings have prominently increased (Gould & Higgs, 2009).

Establishment of new forest-farmland margins, deforestation, uncontrolled urbanization, primitive irrigation systems together with vector populations breeding in sewage systems and aquatic ecosystems, have been implicated in the invasion of domestic animals and human beings into new arthropod vectors habitat. The increased encroachment of human beings into the forest areas which was formerly a habitat of arthropods and animals have consequently resulted in certain adaptive processes also. One of the reasons for the aforementioned adaptation (domestication of insects) is the recurrent exposure of arthropods against the modern human environment. In addition, increased long-distance transportation of livestock, followed by the new routing of bird migrations has caused the bearing of viruses and arthropods to a new region (Gibbs, 1981; Oluwayelu, Adebiyi, & Tomori, 2018). The domestic dogs that share the human environment have usually facilitated the exchange of infectious agents between arthropod vectors, animals and humans. This has validated that domestic animals can act as reservoirs of several zoonotic diseases. It has also revealed the blood-feeding patterns of dengue fever vector *Aedes aegypti* from Singapore, Puerto Rico, Thailand, and the United States of America with special reference to

domestic dogs that may be infected with flaviviruses (Thongyuan & Kittayapong, 2017).

The principal clinical manifestations of dengue infections include plasma leakage and organ impairment together with bleeding (Verma, Sahu, & Holla, 2014). However, classical dengue fever is regarded as one of the serious threats by a number of severe symptoms such as headache, myalgia, arthralgia, retro-orbital pain followed by fever. The severe forms of dengue fever have also been termed as dengue shock syndrome and dengue hemorrhagic fever (DHF). Apart from the general clinical manifestations of infection, several neurological manifestations such as Guillain-Barré Syndrome (GBS), encephalitis, myositis, and myelitis have been well characterized as dengue infection. The complications associated with neurological manifestations were supposed to be the outcome as a result of the multisystem derangement resulting in encephalopathy (Cam et al., 2001).

The infection has been endemic for the past two centuries in India. The first epidemic of dengue in India has been reported during 1963-64 from Kolkata (Bandyopadhyay, Jain, & Datta, 1996). The rapid upsurge in the frequency of dengue outbreaks reported from Kerala, India during 2017-18, indicates the significance of the current study (Anoopkumar, Puthur, Rebello, & Aneesh, 2019; Anoopkumar, Puthur, Varghese, Rebello, & Aneesh, 2017a). As per the statistical analysis of the Kerala Health Department under the Integrated Disease Surveillance Project (ISDP), 8888 confirmed dengue infections were reported from Kerala from January 1st to June 30th, 2017. In addition to this, 56 suspected, and 15 confirmed dengue fever deaths were reported in 2017 from Kerala, India

(Anoopkumar et al., 2017a). It is therefore foreseen that the studies concerning the annihilation of mosquito vectors of epidemiological significance will brighten the imminent prospect and enhance the knowledge of natural vector control strategies and also decrease the arboviral disease transmission level in the human population.

Common methods for vector control

The traditional aspects of vector control strategies have mainly pivoted on a large number of synthetic insecticides. Destruction of mosquito breeding sites by environmental management together with microbiological larvicides and pupicides has got much more importance in areas where endemic arthropod-borne diseases occur (Amer & Mehlhorn, 2006b). The introduction of synthetic insecticides such as organophosphates, pyrethroids, carbamates, and various other insecticides of botanical origin during the 1960-1980s has contributed effectively to vector control (Aktar, Sengupta, & Chowdhury, 2009). The development of insecticide resistance in arthropod vectors, severe toxic effects on non-target organisms, and damages induced in the environment has forced to regulate the use of synthetic insecticides (Ranson & Lissenden, 2016).

Chemical methods for vector control

Synthetic insecticides

The frequent use of synthetic insecticides, especially carbamates, pyrethroids, and organophosphates can cause adverse effects on human health. Personal protection against arboviral diseases can encompass several strategies including the use of mosquito repellents such as N,N-diethyl mendelic acid amide (DEM), N,N-

diethyl-meta-toluamide (DEET), and dimethyl phthalate (DMP); as well as phytochemical constituents, clothing that covers the maximum part of the body, and sleeping using mosquito nets. However, sleeping under the nets has only prevented the bite from mosquitoes during the night (Benelli, Jeffries, & Walker, 2016). Previous studies have reported that the use of insecticide-treated bed nets reduced the spread of *Plasmodium falciparum* and this has resulted in the incidence of vector-borne diseases fall from 2010 to 2015.

The use of the afore-mentioned insecticides may primarily focus on the various stages of the vector life cycle including its larval and adult forms (Goldberg & Margalit, 1977). In addition, indoor residual spraying (IRS) has been used as an effective vector control strategy to prevent various mosquito-borne diseases, especially malaria. IRS involves the spraying of insecticides on the walls of a home or building with an alert (Kolaczinski, Kolaczinski, Kilian, & Meek, 2007). Previous studies have reported that the effective use of DDT has successfully eradicated the yellow fever mosquito *Aedes aegypti* from 19 countries during 1947 and early 1960s. However, the resistance developed against DDT has forced the public health system to phase out these kinds of compounds. Similarly, the development of mutation in the voltage-gated sodium ion channel together with the neurotoxic effect by pyrethroids have limited their use in vector control programmes (Weeratunga, Rodrigo, Fernando, & Rajapakse, 2017). In this regard, the agencies for environmental protection have banned the practical assumptions of synthetic insecticides; as for the reason that they induce several adverse effects on nature and the environment including humans. The norms by the governmental agencies together with the high cost of synthetic insecticides

have forced the manufacturers to withdraw their products too (Collins & Blackwell, 2000).

Today people are using various substitutes for these synthetic insecticides for getting rid of the nuisance, biting from mosquitoes, and infectious diseases followed by high-pitched buzzing (Table 1.1; Page No. 82). The aforementioned important synthetic substitutes are aerosols, mosquito coils, and vapourizers. Most of all the synthetic insecticides are thermostable and therefore have been principally used for the preparation of mosquito coils (Sharma, 2001). While burning liquids and various coils that are available in the market against mosquitoes, the compounds like allethrin, s-bioallethrin 1.9%, d-allethrin 0.2 to 0.3% w/w, and years 4% become vapourized without disintegration at high temperature (up to 400°C), and can produce effective repellent action against them (Rahman, 2013). However, the afore-mentioned toxic compounds are reported with several health issues in human beings such as eye irritation, asthma, bronchial irritation, pain in the throat, skin reaction, pain in the ear, cough, running nose, cold, breathing problems accompanied with headache, fever, and sneezing (Rahman, 2013). Some of the afore-said compounds including acetaldehyde, and formaldehyde (about 55%) can cause sturdy annoyance on the respiratory tract of humans. The frequent use of Organophosphate insecticides, a derivative of phosphoric acid, usually inhibits the action of cholineesterases resulting in paralysis, and death. This has validated its toxic action over the enzyme acetylcholinesterase found in the Central Nervous System (CNS), and Peripheral Nervous System (PNS) in synaptic junctions. The rapid hydrolysis of acetylcholine has caused the repolarization of the basal plate found in

neuromuscular networks, preparing for the influx of new impulses. The synthetic insecticides have found to be generated strong covalent bonds against acetylcholinesterase, the inhibition of enzyme happened, resulting in the deposition of acetylcholine in the synaptic region followed by the disruption of normal nerve impulses transmission (Rey Vega, 2011). However, due to the insecticide resistance induced by organophosphates, and carbamates in mosquitoes, they are no longer effective in the current scenario.

Due to the toxic nature of organophosphate pesticides on the vertebrate nervous system, many of them are banned in several countries. However, temephos is the unique organophosphate pesticide that is still used for vector control (Fortin, Maire, & Leclair, 1987). Moreover, the carbamate pesticides exhibit resemblance towards the organophosphates in their mode of action since both of them inhibit the cholinesterase enzyme. Therefore, the impacts of carbamate poisoning in insects are usually similar to those faced with organophosphates. The carbamates block acetylcholinesterase enzyme by carbamylation in the muscle region, which is recognized as a reversible reaction; hence, the carbamate poisoning in human beings is easily recoverable than the organophosphate intoxication (Roberts & Reigart, 2013).

Some of the synthetic insecticides including pyrethrins are known to exhibit relatively low levels of toxicity to humans; although, it may also instigate dizziness, nausea, and headache in male sprayers. The burning of mosquito coils may generate many volatile organic compounds (VOCs), including suspected carcinogens. Many of the toxic elements from the smoke may reach the

respiratory tract, and prompt a plethora of drastic effects. The gas-phase of the burning coil constitutes certain carbonyl compounds and the emission of the same from one coil is equivalent to burning 51 cigarettes. Moreover, the emission of such non-selective toxic constituents from the smoke and long-term exposure has been previously reported to induce persistent wheeze and asthma in humans (Rahman, 2013). The fact that many phytochemical constituents are irritants to hematophagous insects including mosquitoes could be recognized as an evolutionary relict, and this irritant response is well conserved in the ancestry of Diptera, and we can choose this loophole to counteract against them. Of note, studies that exploit the afore-mentioned perspectives can achieve the following facts that cannot be gained from synthetic insecticide-based approaches; i) target-specificity, ii) relatively non-toxic nature, iii) as an affordable tool in an environmental perspective. Therefore, the traditional knowledge and prominent use of medicinal plants as forecasting natural insecticides would be considered as prerequisites for the development of standardized natural pesticides. Previously, homemade natural insecticides were prominently used by human populations in low-income countries; and from the literature, it was evident that expensive and limited availability of some of the synthetic insecticides is the driving force behind this circumstance (Dougoud, Toepfer, Bateman, & Jenner, 2019). However, there exists a chance of emerging questions concerning the practical implications and scientific evidence of such an excellent eco-friendly approach. This investigation hence provides focus on the scientific evidence for such natural approaches. In light of the prohibitive cost along with limited availability in the village provinces of low-income countries, the use of botanicals with scientific

evidence could be considered as a valid alternative to chemical insecticides. While we consider the hazardous effects linked with the practical implications of synthetic insecticides, the formulations and preparations of synthetic insecticides have also allied with the extinction of natural enemies of *Aedes, Culex* and other epidemiologically important mosquito vectors. Such situations may augment the prominent use of natural insecticides in vector control. In agreement with the afore-mentioned perspective, the local communities from different provinces over the world, especially from African countries, adapt a plethora of approaches to repel mosquito vectors. Burning the plant material, spraying plant extracts, sprinkling and hanging the leaves at home are the major prominent strategies for the previously mentioned approaches (Kishore, Mishra, Tiwari, Tripathi, & Lall, 2014).

Owing to the benefits of plant-based insecticides and deleterious effects of synthetic products on human health and the environment, it is imperative to diminish the use of synthetic insecticides by using safe and eco-attractive alternatives. The residues of synthetic pesticides in food and animal feed including the environment resulted from its frequent use are considered as one of the eminent threats in the current scenario. Most of them cannot be degraded from the natural source easily and thus remain for years and can lead to bioaccumulation. The non-target nature of synthetic insecticides has also led to the extinction of beneficial organisms, loss of biodiversity, and the emergence of insecticide resistance (Zikankuba, Mwanyika, Ntwenya, & James, 2019). The major following factors that are allied with synthetic products have a greater tendency to pollute the environment including the natural water resources; and are

a) poor storage, b) inappropriate disposal, c) cocktail application (single spray using several insecticides), d) use of unlabelled products, e) continuous exposure, and f) obtain illegal varieties of registered products.

The excessive use of synthetic insecticides is recognized as a foremost matter of modern concern since it is assessed that around 2.5 million tons of insecticides are used every year, creating hazards tantamount to $100 billion annually. Some of the other major drawbacks that sprung up when chemical insecticides were frequently used are (a) the generation of legal complications and risk; (b) destruction of advantageous species by toxic effects to non-target organisms; (c) reappearance of previously treated populations; (d) expensive rate of materials, equipment followed by labour; and (e) development of insecticide resistance (Koul, Walia, & Dhaliwal, 2008). Moreover, the toxic properties of synthetic insecticides together with waste formed in crops, food, soil and water that influence the public health threat are additional motives to search for novel alternatives for the problems induced by the synthetic insecticide (Koul et al., 2008).

Biorational insecticides

The previous studies have indicated that the use of biorational insecticides in recent years gained much more importance than conventional strategies since they possess low toxicity to the environment. Methoprene is reported as one of the most commonly used insecticides which has the ability to regulate insect growth together with a wide spectrum of action that impedes the insect lifecycle (Klowden & Chambers, 1989). Likewise, the biorational insecticide, Spinosad

that was made by using spinosyns A and D, has principally acted on the GABA receptors and postsynaptic nicotinic acetylcholine receptors. The new generation insecticides coming under biorational insecticides (eg: pyriproxyfene), tested against larval and adult mosquitoes have caused decreased egg and sperm production followed by a reduced mating activity and blood-feeding in insects (Iwanaga & Kanda, 1988).

Spinosad is recognized as one of the important biorational insecticides produced by the fermentation of actinomycete. The presence of effective spinosyn neurotoxins in the spinosad offers high toxicity to insects of the Diptera family. The low toxicity of spinosad to vertebrates has been observed as an additional advantage to approve the same as a mosquito larvicide in drinking water (WHO, 2010). Spinosad has been found to have an adverse impact on the normal immature aquatic life cycle stages of development of the vector mosquito species, specifically *Anopheles gambiae, Anopheles albimanus, Anopheles pseudopunctipennis, Culex quinquefasicatus, Culex pipiens, Aedes albopictus* and *Aedes aegypti* (Hertlein et al., 2010). Studies based on biorational insecticides in practical assumptions are scanty in the current scenario. However, Marina et al. (2012) have compared the larvicide potential of spinosad against VectoBac and temephos granules based on their practical assumptions from Southern Mexico. They have also discussed the influence of sunlight over the larvicidal potential and confirmed that the half-life of the spinosad in the wet environment has been assessed at less than three days once exposed to sunlight (Perez et al., 2007).

Biocontrol to prevent the proliferation of the mosquito population

Use of traditionally used medicinal plants

Mosquito-borne diseases are still an imminent risk to the world population, especially in tropics and subtropics regions. The research community around the world prominently seeks an innovative solution to fight against them (Ikram & Simonsen, 2017). The richness of traditional medicinal plants in India, that have been used for the past years can be exploited as a diverse chemical space for novel biological vector control strategy (Mohanraj et al., 2018). It is remarkable to note that the Indian herbal formulation with traditional aspects is usually based on the empirical knowledge of the traditional medicinal practitioners rather than the mechanical understanding of the effective bioactive elements.

The Ayurveda Materia Medic is an important book that buried the important formulations of traditionally used medicinal plants from India. Owing to the non-digital nature of the traditional knowledge on medicinal plants, the digitization and database development in the afore-mentioned aspects has enabled the recent research to develop computational strategies (Dash & Kashyap, 1999; Jensen, Panagiotou, & Kouskoumvekaki, 2014; Pandey, Rastogi, & Rawat, 2013).

The extensive availability of medicinal plant information and their bioactive elements can help develop various strategies for the public health system. In this direction, the analysis of the phytochemical elements from edible, herbaceous and traditionally used medicinal plants will help the public health system to explore the chemistry of phytocompounds from Indian medicinal plants, that has provided

the potential applications of bioactive elements in vector control strategies (Govindarajan & Benelli, 2016).

The various databases such as TCM@Taiwan, CVDHD, Nutrichem, KNAPSACK, TCMID, TCM-Mesh, and Phytochemica can be used to explore the virtual screening of potential bioactive elements (Mohanraj et al., 2018). As per the statistical analysis of the World Health Organization (WHO), approximately 80% of the world's population follows traditional medicines to cure various diseases as a primary healthcare requirement. The use of herbal medicinal plants denotes a long history of effective interactions between the environment and humans. Previous studies have verified the beneficial biological activity of traditional medicinal plants; and it includes antioxidant, wound healing, anticancer, antidiarrheal and antimicrobial potential together with larvicidal efficacy (Govindarajan & Benelli, 2016; Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011).

The higher plants could offer a rich source of the novel bioactive element with pharmacological benefits, which are essential for the development of new drugs and products including the antimalarial agent artemisinin and anticancer drugs taxol and vinblastine. The careful selection of medicinal plants with special emphasis on their chemotaxonomic data and field observation is essential for the success of research on the aforementioned concepts. The bioactivity-guided fractionation and isolation of novel metabolites are the main strategies to target the bioactive elements (Queiroz, Wolfender, & Hostettmann, 2009).

The discovery of artemisinin, a plant-based drug highlights the significance of choosing medicinal plants as a prominent source of phytochemical elements for mosquitocidal properties. Subsequently, in 2015, Professor Youyou Tu has received the Nobel Prize in Physiology or Medicine for her extensive contribution to saving millions of lives infected with malaria (Su & Miller, 2015).

Perspectives of secondary metabolites from medicinal plants in context with Artemisinin and Nobel Prize in Physiology or Medicine

The discovery of artemisinin by Professor Youyou Tu has intensely made alterations in the landscape to fight against malaria thereby causing a paradigm shift in the development of antimalarial drugs. The incidence rates of malaria were dramatically decreased to 25% during 2000-2012 over the globe and the mortality rate was diminished to 42% throughout the same period since the Artemisinin and its by-products have displayed a significant role. In addition, the practice of artemisinin combination therapies (ACTs) has averted 22% of the 663 million clinical cases (Bhatt et al., 2015).

The Artemisinin was isolated from the medicinal plant *Artemisia annua*. One of the drawbacks of the isolation of Artemisinin from *Artemisia annua* is the low productivity and this might lead to the scarcity of global supply. It's complex chemical structure has prevented us to synthesize Artemisinin using chemical strategies. Therefore, till date, the scientific community has chosen *Artemisia annua* as a significant contributor to the commercial production of the same (Ikram & Simonsen, 2017; Pulice, Pelaz, & Matías-Hernández, 2016).

Based on the chemical structure, the Artemisinin is having a specific endoperoxide structure deprived of nitrogen comprising a heterocyclic ring. World Health Organization in 2006 has recommended the artemisinin as a foremost choice to combat malaria (WHO, 2015). However, the emergence of drug resistance against the compound has drawn attention to developing artemisinin-based combination therapy (ACT). The primary substance for the ACT is the Artemisinin (WHO, 2015). The afore-mentioned concepts have indicated that there must be an extensive and consistent effort needed to augment the production of artemisinin over the globe. Many biotechnological strategies followed by certain plant-breeding approaches have also been employed for the cultivation of *Artemisia annua*. Efforts in these concepts have been considered as a challenging threat since *Artemisia annua* possesses heterozygous nature (Jewitt, Agarwal, Weaver, Mutchler, & Larson, 2013). However, the combinations of many elicitation and cultivation strategies are now being geared for the large-scale production of artemisinin through the root of *Artemisia annua*. Developments in synthetic biology and plant engineering have prominently augmented the alertness of using herbs as production hosts thereby leading to great efforts in the enrichment of artemisinin and other bioactive elements production for the public health (Ikram & Simonsen, 2017).

Metabolites in plants

Plants are able to synthesize diverse chemical elements usually referred to as secondary metabolites which have unique carbon skeleton structures. It is thus a matter of attention to exploring the phytochemical elements to develop a novel vector control strategy. The isolation and characterization of bioactive elements

from plants are feasible today, but it necessitates specific knowledge of the herbs and its phytochemical elements (Atangwho et al., 2009). The formation of phytochemical elements is usually organ and cell-specific and they differ from each other in respect to their types and amounts (Kliebenstein & Osbourn, 2012). They offer protection against plants from both biotic (insects, bacteria, nematodes, grazing by animals, and fungi) and abiotic stresses (Shading, heavy metal exposure, moisture, injury).

The great economic value of the secondary metabolites made them an excellent resource for the development of fragrances, drugs, dyes, and insecticides (Akula & Ravishankar, 2011; Verpoorte, van der Heijden, & Memelink, 2000). Based on the biosynthesis origin, three different groups of secondary metabolites such as polyketides, terpenoids, and phenypropanoids are classified. Alkaloids are recognized as another class of secondary metabolites usually characterized as nitrogenous organic molecules (Croteau, Kutchan, & Lewis, 2000).

The primary metabolites perform vital metabolic tasks by participating in reproduction and nutrition. However, it is tough to indiscriminate the primary metabolites from secondary metabolites since some of the compounds exhibited both primary and secondary roles in several metabolic processes (Croteau et al., 2000). Moreover, based on the chemical nature, there are three kinds of plants secondary metabolites has been recognized and are, i) Phenolics, ii) Terpenes, iii) S (sulphur) and N (Nitrogen) containing compounds (Pagare, Bhatia, Tripathi, Pagare, & Bansal, 2015). Previous studies have reported that approximately

2,50,000 known herbs have proceeded for phytochemical studies. But the fact is that only 5% have been investigated for their biological activities.

Alkaloids

The alkaloids are recognized as a significant group of secondary metabolites found in more than 20% of plant species all over the world. The difference in structure (indole alkaloids) or the presence of common precursor (pyrrolizidine, purine alkaloids, tropane, and benzylisoquinoline) are the main criteria to distinguish them from other chemical elements (Zulak, Liscombe, Ashihara, & Facchini, 2006). The prominent use of alkaloids as medicine stretches back approximately 5000 years and these chemical elements have paid the majority of the traditional psychedelics, neurotoxins, poisons, and certain social drugs such as cocaine, methamphetamine, opiates, and caffeine used by humans (Zenk & Juenger, 2007). In terms of their important roles in ecological aspects, they are principally represented as toxic elements to insects. The alkaloids are directly linked with the nervous system as antagonists and agonists to a diverse neurotransmitter system including ion channel function, neurotransmitter metabolism and signal transduction (Wink, 2000). This indicates that the alkaloids isolated from plants have been found to influence the physiological systems of mammals and other vertebrates as well as in insect vectors. Previous studies have mentioned their toxic effects on insects too (Saxena, Dixit, & Sukumaran, 1992; Schneider et al., 1982).

Liu, Liu, Du and Deng (2012) reported the larvicidal potential of three isolated alkaloids such as rutaecarpine, wuchuyuamide, and evodiamine against *Aedes*

albopictus. It has been also studied that the *Ipomoea cairica* comprises alkaloid, which significantly serves as an insecticide of botanical origin against the mosquito population (Talontsi, Matasyoh, Ngoumfo, & Chepkorir, 2011). The most common alkaloids which are used as insecticides are ryanodine, anabasine, and nicotine. Ghosh, Chowdhury and Chandra (2012) reviewed that the mode of action of the afore-mentioned elements on insect vectors may differ by the structure of the alkaloids; however, they are principally noted to inhibit the sodium channel and acetylcholinesterase (AChE). In addition, Ghosh et al. (2012) reported that the inhibition of AChE by the alkaloid is considered as one of the significant physical disruptions on insects since it functions as a major enzyme responsible for the disruption of nerve impulse transmission. Congruently, the insects (mosquitoes) exposed with constituents encompassing AChE will eventually cause paralysis and death.

Terpenes

The terpenes are another class of elements constituting approximately 30,000 compounds. They exhibit a broad range of toxic effects in the central nervous system of insects. The neurotoxic action of several terpenes in insect vectors have been shown to include a link with the cholinesterase inhibition, direct and allosteric binding to $GABA_A$, multiple direct interactions, allosteric binding to $GABA_A$ receptors, followed by blockade of GABA-gated chloride channels (Rattan, 2010). The wide range of ecdysteroids formed from the plants can also play a wide range of defensive roles in the herbivorous insects by interfering with the metamorphoses, delaying pupation and molting (Céspedes, Salazar, Martínez, & Aranda, 2005). However, one of the major problems associated with the

terpene compounds is the dual nature. That is some of them can act as both attractants and deterrents. 1,8-cineole, a monoterpene is an excellent example of the afore-mentioned condition since it acts as an effective toxin to some species of flies and beetles. At the same time, it is harmless to honey bees since it acts as a fragrant attractant for pollination (Kennedy et al., 2011).

It is important to explore that several terpenoids possess a significant range of toxicity to insects but extremely low to mammals (Rattan, 2010). The afore-mentioned groups of phytochemical constituents are existing in nutrition that forms indispensable elements of our healthy diet. The major example of this includes β-carotene (Kennedy & Wightman, 2011). Terpenoids generally show a complex structure and they are also able to provide synergistic action (Wink, 2003). Dória, Silva, Carvalho, Alves and Cavalcanti (2010) verified the larvicidal potential of *β*-caryophyllene against the yellow fever mosquito *Aedes aegypti*. Similarly, the other chemical elements such as sabineno, *β*-Pinene, 3-carene and eucalyptol have possessed a significant range of larvicidal potential against mosquito vectors of epidemiological significance.

A study conducted by Kumar et al. in 2012a has proposed that the larvicidal potential of terpenes and other chemical elements in *Calotropis gigantea* (Linnaeus, 1811) is due to the ability to destruct the sterol carrier protein (AeSCP-2). The major function of AeSCP-2 in insects is the intracellular cholesterol transport. The compounds which have the ability to inhibit the AeSCP-2 protein have a high impact as an effective vector control agent since the larvae has depended on cholesterol for the synthesis of certain steroids and their derivatives

(Kitamura, Kobayashi, & Okada, 1996; Larson, Wessely, Jiang, & Lan, 2014). Andrade-Ochoa et al. (2018) verified the afore-mentioned inhibition activity of compounds using the crystal structure of AeSCP-2 in docking to develop a homologous enzyme from *Culex quinquefasciatus*.

The previous studies have verified the significance of terpenoids, phenylpropanoids, and terpenes through in vitro evaluation with special inference to molecular perspectives. In spite of many studies concerning the larvicidal potential of essential oils and their active ingredients, little is known on the mode of action of phenylpropanoids and terpenoids on mosquito larvae. This has augmented the studies on structural modulation and reactivity of chemical constituents to reduce the effort for the development of novel compounds with significant larvicidal efficacy (Andrade-Ochoa et al., 2018).

The prediction of the biological potential of the phytochemical constituents using computer-assisted technologies is now a common strategy. Quantitative Property–Activity Relationship (QPAR) and Quantitative Structure-Activity Relationship (QSAR) studies can offer significant information to find out the bond between biological activity and the chemical structure. In addition, the molecular docking provides information on the free energy values based on the strength of protein-ligand interaction (Yuriev, Agostino, & Ramsland, 2011). Andrade-Ochoa et al. (2018) reported the larvicidal efficacy of terpenes and other related compounds of essential oils against *Culex quinquefasciatus*.

Phenolics

Till date, approximately 10,000 phenolic compounds have been identified from plants. In most of the conditions, the phenylpropanoid pathway plays a significant role in the synthesis of phenolic compounds. As a broad group, the phenolics represent a wide range of compounds from low-molecular-weight (coumarins, benzoic acid derivatives, and phenylpropanoids) to more complex compounds (tannins, flavonoids, and stilbenes). Among them, flavonoids characterize the most diverse group and constitute approximately more than 6,000 compounds. Based on the alterations of the basic structure, the flavonoids can be divided into flavonols, isoflavones, chalcones, flavan-3-ols, chalcones, and anthocyanins (Bowsher, Steer, & Tobin, 2008; Dutta, Dey, & Chaudhuri, 2014).

Previous studies based on epidemiological aspects suggested that there are strong associations between phenolic-rich foods and many diseases including neurologic disorders (AD/dementia), stroke and cardiovascular diseases (Vingtdeux, Dreses-Werringloer, Zhao, Davies, & Marambaud, 2008). It has been also reported that flavonoid-rich foods have restricted the neurodegeneration. Resveratrol, curcumin, and EGCG are important examples of phenolics compounds (Spencer, 2010). Nowadays, studies on the mosquitocidal potential of known alkaloids with special emphasis on the search for 'unknown novel alkaloids' remains an objective of many researchers (Masi et al., 2017). Masi et al. (2017) isolated four alkaloids such as sarniensine, lycorine, 3-epimacronine and tazettine with a significant range of toxicity. The essential oil and volatile elements including phenols from medicinal plants are highly efficient against insect vectors of epidemiological

significance. Various previous studies have documented and verified the acute toxic effects of phenolics against mosquitoes (Pavela, 2011).

The phenolic compounds constitute a hydroxy (-OH) group, usually attached with aromatic ring structures or benzene ring, eg., resorcinol or catechol, and pyrogallol. They have provided anti-ovipositional, growth inhibitory and antifeedant potential. In spite of the afore-mentioned fact, studies concerning the insecticidal efficiency of phenols based on their chemical structures are scanty. Such studies can provide significant information for the development of novel botanical insecticides for vector control (Pavela, 2011). For instance, thymol and other chemical elements present in the medicinal plants have exhibited significant toxicity against larvae and adult mosquitoes (Pavela, 2007). The hydrophilic nature of tannin coupled with a strong affinity to bind with proteins made them an abundant phytochemical element for vector control strategies (Rey, Cuany, Pautou, & Meyran, 1999).

The rotting vegetation in aquatic ecosystem leads to the sudden release of biologically active tannin that is antagonistic to the development of mosquitoes. The tannins released in the water from leaf litter may found to constitute both deterrents as well as nutrients in the water habitat of mosquitoes. Approximately, 800 g/m2 of tannins have been produced from the forest every year and the toxic effects induced by them on larvae of mosquitoes appear to be more acute than conventional strategies using other insecticides. However, some of the mosquito vectors developed P-450-associated detoxification to tannins. The addition of piperonyl butoxide (PB) as a typical P-450 inhibitor has revealed potent

synergistic effects against mosquitoes (Rey et al., 1999). Therefore, the studies based on the link between chemical messengers and invertebrates with special inference on phytochemical constituents will allow the scientific community to develop insecticides of botanical origin.

Saponins and Glycosides

The best-known sources of saponins include soybeans, peas, and some herbs. their chemical structure revealed a polycyclic aglycone linked with sugar side chains. The aglycone part of saponins is either triterpene (C30) or steroid (C27). The combination of hydrophilic sugar part and hydrophobic sapogenin caused them to form foams. Previous studies have verified the larvicidal efficacy of saponins against the mosquito vector *Culex pipiens*. In addition, the larvicidal potential has also been exhibited by saponins as confirmed by the study by Chapagain, Saharan and Wiesman (2008), Ghosh and Chandra (2006), and Pelah, Abramovich, Markus and Wiesman (2002) wherein saponins displayed a significant range of larvicidal efficacy against mosquito vectors such as *Anopheles stephensi* and *Aedes aegypti*.

The saponins are greatly soluble in both water and organic solvents, and they can have the ability to disarrange the cuticle membrane of the mosquito larvae, which is recognized as the probable mode of action of tannin in mosquito larval death (Del Carmen Recio et al., 1995). A wide range of other impeding effects such as increased mortality levels, retardation, lowered food intake, decreased reproduction, and disturbances in the development induced by them made saponins effective insect control agents. They block the uptake of sterol by the

genesis of certain insoluble complexes and make the insect as less attractive to food.

Saponins are also able to induce deleterious effects in the digestive system of host insect as well as molting problems (Silva, Silva, Santos, Rodrigues Filho, & Elias, 2004). The glycosides especially cyanogenic glycosides have exhibited toxicity to different kinds of insect vectors by developing inhibiting effects on certain respiratory enzymes and cytochrome oxidase (Yu, 2015). The iridoid glycosides exhibit anti-feeding potential in some insect species (Bowers & Puttick, 1989). Due to the insecticidal activity exhibited, the cyanohydrins can be used as an effective fumigant against a wide range of insect vectors. Similary, Dave and Lediwane (2012) reported that the bioassay-guided fractionation of anthraquinones from the medicinal plant *Cassia species* revealed potent insecticidal and antimalarial activity (Cunha et al., 2017). Phytochemical studies based on the afore-mentioned concepts in *Cestrum nocturnum* (Linnaeus 1753) confirmed the presence of phenol glucosides, flavonol glycosides, calcinogenic glycosides, and steroidal saponins (Gao & Wang, 2006; Mimaki, Aoki, Jitsuno, Kiliç, & Coşkun, 2008).

Matsuo, Akagi, Hashimoto, Tachikawa and Mimaki (2013) have reported five known and four novel steroidal glycosides from *B. elegans*. The glycosides comprise a huge group of phytochemical elements usually distributed in plants. They are structurally diverse and the studies reflect on the traditional aspects, possible adverse effects, mechanisms of action, toxicity, followed by prospects and trends in analyzing natural glycosides gained much more significance in the

current scenario. Post-modification of the secondary metabolites by glycosyltransferases is recognized as the principal process involved in the synthesis of glycosides in plants (Bruneton, 1996; Dembitsky, 2004). Frequently, further modification such as acylation, oxidation followed by degradation has also taken place. The extraction of glycosides from medicinal plants is typically accomplished with the aid of polar solvents (Yu, Sun, & Yang, 2012).

As medicines

The term "Ethno-botany" depicts a significant field that made studies on the historical and current therapeutic uses of medicinal herbs. It is of extensive importance for the conservation of traditional medicinal values, as well as for determining the changes in the culture and history. It also provides the sustainable use of traditionally used medicinal plants thereby paid special inference for their conservation of natural resources (Gemedo-Dalle, Maass, & Isselstein, 2005; Lewu & Afolayan, 2009).

The advantageous therapeutic effects of traditionally used medicinal plants generally result from the combinations of afore-mentioned phytochemical constituents in the herbs. The therapeutic effects of medicinal plants are species-specific and their phytochemicals possess a defensive role towards the pathogen attack as well as inter-plant competition. They also offer an attractive role towards the insect species including symbionts or pollinators (Auwal, Atiku, Wudil, & Sule, 2012). Moreover, previous studies have confirmed their potential role at the cellular levels as modulators of gene expression and plant growth regulators (Briskin, 2000; Gupta, Bisht, Kukreti, Jain, & Brahmachari, 2007).

The phytochemical constituents can have a diverse functional role in herbs. It is likely that a significant role in ecological concerns may have some manner on enormous medicinal effects. For instance, the phytochemicals concerned with the plant defense processes through cytotoxicity towards the microbial consortia could verify as an antimicrobial agent in humans (Gibbons, 2004). Similarly, secondary metabolites and their derivatives involved in plant defense could have advantageous effects as antidepressants, anesthetics, sedatives and muscle relaxants in humans. In this respect, most of all the phytochemical constituents and their derivatives may exert their activity by resembling hormones, ligands, neurotransmitters, metabolites and signal transduction molecules and thus have an advantage on humans owing to the similarities in their target sites such as endocrine system and central nervous system (Briskin, 2000).

In contrast to the single chemical-based synthetic pharmaceuticals, various natural medicines exert their advantageous impacts through the synergistic action of several secondary metabolites acting at a multiple target site linked with physiological activities. Of the diverse number of traditional medicinal plants used in the non-Western medical methods, only a few numbers have gained considerable research interest over the past few years (Houghton, 1995). However, the past years have perceived a great resurgence in the research interest and the use of plant products for medicinal purposes in North America. Statistical analysis on the medicinal plants that are used as a primary health care concern by the American public health has revealed that the usage was gradually increased from 3% to 37% from 1991 to 1998 (Briskin, 2000).

Research based on phytochemical constituents has led to the development of novel drugs and paying to the local economy. Till date, millions of people rely on traditionally used medicinal herbs as a primary health care strategy since it provides cultural development and economic benefits. The significance of traditionally used medicinal plants is greatly augmented in areas where hospital facilities and services are weak (Pierce & Laird, 2003).

Antioxidant potential and Oxidative Stress linked resistance

Till date, much attention has been paid to the development of pharmaceutical products with sturdy antioxidant potential. One of the basic principles for the antioxidant potential of medicinal plants is the free radical scavenging activity of the afore-mentioned phytochemical constituents such as phenolic terpenes, polyphenols, flavonoids and tannin in plants (Shetty, Udupa, & Udupa, 2008).

The free radicals are formed instantaneously during metabolic activities of biological systems and can induce extensive damages to biomolecules as well as tissues leading to several severe impeding effects associated with neurodegenerative disorders and diabetes mellitus (Yazdanparast, Bahramikia, & Ardestani, 2008). Though a large number of drugs of synthetic origin are known to defend oxidative impairment, a major disadvantage owing to their frequent use is the impeding effects induced by them in the target organism. Consumption of traditional medicines and food supplements containing natural antioxidants constitute an alternate solution to the afore-mentioned drawbacks (Ghosh et al., 2013).

The free radical scavenging potential of many traditionally used medicinal plants is rationalised by previous studies on *Pentadesma butyracea*, *Rumex abyssinicus*, *Paullinia pinnata*, *Hibiscus asper*, *Psorospermum febrifugum*, *Origanum compactum*, *Tectona grandis,Pentadesma butyracea*, *Dichrostachys glomerata*, and *Rumex bequaertii* (Bouhdid et al., 2008; de Dieu Tamokou et al., 2013).

Londhe, Devasagayam, Foo and Ghaskadbi (2008) mentioned that polyphenol constituents of the traditional medicinal plant *Phyllanthus amarus* exhibit the significant potential to scavenge free radical (pulse radiolysis generated $ABTS^{+}$ radical). Likewise, many authors have verified the medicinal properties of plants with special inference to their great antioxidant potential. A large number of well-known medicinal plants belong to the family Lamiaceae, in which most of them are well studied for their biological potential. Several studies have explored the diverse activities of these herbs. The Ayurvedic medicine system in India has mentioned several medicinal plants and their extensive uses for the treatment of various diseases such as rheumatoid arthritis, diabetic mellitus and cardiovascular diseases (Shetty et al., 2008). There are still a great number of medicinal plants that are not studied with respect to their pharmacological and larvicidal potential (Cetin & Yanikoglu, 2006; Matkowski & Piotrowska, 2006). Therefore, there is growing attention paid to natural antioxidants and bioactive compounds present in the traditional medicinal plants that might aid attenuate damage caused by free radicals (Sharififar, Dehghn-Nudeh, & Mirtajaldini, 2009). Siraichi et al. (2013) have used Liquid Chromatography joined to Diode Array Detection (LC–DAD) together with Electro Spray Ionizationtandem Mass Spectrometry (LC–ESI-MS/MS) to determine the various phenolic bioactive

compounds (luteolin, scutellarein, hispidulin, isoscutellarein, apigenin, and 6-hydroxyluteolin) with potent anti-oxidant activity from the traditional medicinal plant *Arrabidaea chica*. The significant potential of the *Arrabidaea chica* could be attributed to the presence of the afore-mentioned phenolic compounds. Multiple studies have confirmed the antioxidant potential of medicinal plants which is significantly linked with their phenolic constituents (Sharififar et al., 2009).

The presence of hydroxyl group in their chemical configuration has enhanced their scavenging ability. This has been studied comprehensively and was recurrently summarised in previous investigations concerning the determination of scavenging of superoxide anion, hydroxyl, DPPH, nitric oxide, and superoxide radicals. It is important to note that the derivatives of diphenyl sulfone as a potent antioxidant are used in the treatments of rheumatoid arthritis (Diaz-Ruiz et al., 2008).

Diphenyl sulfone was found to be predominant in extracts of *Gentiana glauca* (Pall, 1788). There are very few reports on its natural occurrence, most noteworthy being in the case of plant-like *Myriactis humilis*. It is significant to note that diphenyl sulfone and its derivatives have been reported as antioxidants and are also used in the treatment of various collagenoses of rheumatoid nature like rheumatoid arthritis as well as antioxidants. The presence of well-known pesticide Diphenyl sulfone in the medicinal plant *Galphimia glauca* underpin its significance in mosquito vector control strategies as a natural and economic approach (Ghosh et al., 2013). The afore-mentioned concepts have indicated that

the redox reactions induced by the free radicals have played a critical role in the metabolic processes of insects. Moreover, it is important to note that the balance of redox homeostasis is vital during the redox shift induced by the fluctuations in numerous biological contexts (Champion & Xu, 2018). That is, if the amount of free radicals/reactive oxygen species (ROS) is increased to an excess level in the cellular regions, oxidative stress to cellular constituents occurs. This may comprise the damages in aminoacids, nucleic acids, other cellular constituents, and proteins including DNA (Sies, 1997; Wang & Hai, 2016).

The previous studies have mentioned the link between insecticide resistance and oxidative stress. For instance, the higher mitochondrial free radical production was observed in the permethrin resistant strain of *Anopheles gambiae*, indicating that ROS has a prominent influence in diminishing longevity in the insecticide-free condition thereby sustaining the insecticide resistance linked detoxification capacity (Oliver & Brooke, 2016). The longer lives of female mosquito vectors are more likely to reach and exceed the normal incubation period of pathogens; therefore, minute changes in longevity can provide a substantial impact on vector competence (Garrett-Jones & Shidrawi, 1969). The insecticide resistance and adult vector longevity in *Aedes* mosquitoes are recognized as the important phenotypes in terms of dengue epidemiology and the former can harmfully influence the vector control interventions if not properly managed (Belinato, Martins, & Valle, 2012).

Insecticide Resistance in Mosquitoes

Studies on insecticide resistance in vector mosquitoes have thrived during the 1950s, once the first report of insecticide resistance to chlorinated-hydrocarbon was issued. This has alarmed the research community to elucidate the various prominent mechanisms influencing the insecticide resistance development, a vibrant initiative toward the development of novel, more effective approaches to prevent insecticide resistance and diminish the incidence of mosquito-borne diseases (Gjullin & Peters, 1952).

Ever since the publication of first research article on insecticide resistance in mosquitoes to chlorinated-hydrocarbon insect repellent, the effort focussed on understanding the emergence of insecticide resistance mosquito vectors has been strong, with a large number of original research and review articles published in well-established scientific journals (Liu, 2015). The insecticide resistance is considered as one of the foremost obstacles in the efforts to prevent the rapid proliferation of mosquito vectors of epidemiological significance and it can interfere with the vectorial capacity (Martins, Bellinato, Peixoto, Valle, & Lima, 2012). Vector control inventions based on the frequent use of synthetic insecticides has resulted in an increased incidence of insecticide resistance in mosquito populations, a prominent condition that made weakening or complete failure of mosquito control. Resistance to pyrethroid, temephos, DDT as well as to other synthetic insecticides have already been studied in *Aedes aegypti* populations all over the world (Brengues et al., 2003). The pyrethroid resistance in mosquitoes has been associated with two significant major mechanisms; i)

increased the rate of metabolic detoxification of the target insecticide by monooxygenase activity, and ii) variations in the target site sensitivity (Bergé, Feyereisen, & Amichot, 1998).

Interactions between the genes allied with resistance provide a hint concerning exactly how this increased level of resistance may happen in insects, especially in mosquitoes. Gene overexpression, extensive mutation followed by amplification in coding regions that are resulted in alterations in proteins have recurrently been interlinked to insecticide resistance in mosquito vectors, while transcriptional overexpression of genes in resistant strains found to be a foremost determining event in the resistance development (Liu, Liu, Zhu, & Zhang, 2007; Nabeshima et al., 2004). In addition, the elevated levels of glutathione-S-transferases and specific esterases have also been noted to confer resistance against pyrethroids (Vulule et al., 1999).

The voltage-gated sodium channel is the primary target site for DDT and pyrethroids and the sensitivity of this target site has been linked with *kdr* (knockdown resistance (kdr) mechanisms) associated mechanisms in mosquitoes (Brengues et al., 2003). Previous studies have mentioned shreds of evidence of the specific role of acetylcholinesterase in insecticide resistance against carbamates and organophosphates. Irrespective of the link between the enzyme activities and range of insecticide resistance, the α-esterases, and β-esterases enhanced activities together with acetylcholinesterase and glutathione-S-transferase in *Culex quinquefasciatus* (Malaysian region) verified the insecticide

resistance incidence towards malathion, permethrin, propoxur and DDT (Low et al., 2013).

An elevated level of both acetylcholinesterase, glutathione-S-transferase, and oxidases in *Culex quinquefasciatus* has been formerly studied (Low et al., 2013). The enhanced level of enzyme activities is really placing a drastic burden on present and future vector control approaches. Studies based on these aspects have highlighted the significant need for suitable resistance management methods to be applied in mosquito control programs all over the world (Low et al., 2013).

Taken all, the afore-mentioned concepts suggest that insecticide resistance in mosquitoes not only happened through multiple resistance mechanisms but also through the interaction between the resistance genes and regulatory genes. The resistance genes usually encode proteins that are linked with insecticide resistance. In recent years, a vast number of novel, exciting, advanced technologies and techniques including double-stranded-RNA-mediated gene interference (RNAi), high-throughput sequencing, single nucleotide polymorphism determination, whole-genome sequencing have become widely existing, enabling the research community to identify resistance genes involved in insecticide-resistance mechanisms. The afore-said technologies have prominently helped researches into insecticide resistance and have directed to substantial progress in illustrating the roles of the genes and the communication between the corresponding regulatory elements, as well as proteins, pathways and other significant factors involved (Deletre, Martin, Duménil, & Chandre, 2019; Liu,

2015). Therefore, the studies focussed on the underlying mechanisms of resistance in *Culicidae* gained much more significance in the current scenario.

Owing to the limited foraging range of *Aedes Aegypti*, it is more likely to spread than other mosquitoes since it may be unintentionally transported as dried eggs. By horizontal transmission, the *Aedes* female mosquito vectors become infected and it is capable of transmitting the infectious agent when the extrinsic incubation period (EIP) is over. Moreover, some of the infectious agents from the infected parent mosquitoes have been transferred to the offspring during the oviposition period, indicating the role of vertical transmission in the rapid proliferation of mosquito-borne diseases transmitted by *Aedes aegypti* (Rosen, 1987). The afore-mentioned concepts have verified that the synthetic insecticide-based mosquito control has been confronted by the emergence of insecticide resistance to frequently used synthetic insecticides reported all over the world for the order *Diptera*. Consistently, the development of resistance in mosquitoes against a vast number of insecticides has been reported to befall in urban and village provinces where the mosquito population are living close to the human environment (Hamid, Prastowo, Ghiffari, Taubert, & Hermosilla, 2017; Naqqash, Gökçe, Bakhsh, & Salim, 2016).

The resistance to various synthetic insecticides like pyrethroid, in specific, and resulting cross-resistance to other ones are life threatening-issues and have led to the re-emergence of mosquito-borne diseases all over the world. The increased resistance by mosquito species has been reported in more than 60 nations,

influencing all of the epidemiologically important mosquito vectors and all classes of synthetic insecticides (WHO, 2016).

The resistance related issues become more challenging in the current scenario since the number of commercially developed new insecticides are scanty. Controlling mosquitoes is the only effective way to fight vector-borne diseases when appropriate vaccines for mosquito-borne diseases are not yet developed. Since many years, a large number of international and national agencies have been fighting against *Aedes Aegypti* mosquitoes as major vectors of dengue fever, yellow fever, chikungunya and probably Zika fever. However, with partial victory so far due to associated aspects, other struggles carried on being the development of resistance against synthetic insecticides (Anoopkumar et al., 2019; Hamid et al., 2017; Puthur, Anoopkumar, Rebello, & Aneesh, 2019).

Detoxification of Insecticides in Mosquitoes

The detoxification of synthetic insecticides in mosquito vectors is threatening mosquito control programs all over the world. Glutathione S-transferases (GSTs), cytochrome P450s and esterases are known to possess significant roles in resistance, permitting resistant strains to metabolize the synthetic insecticides at maximum level. In addition, the Glucose-6-Phosphate Dehydrogenase (G6PD) is recognized as the key enzyme involved in the oxidative pentose phosphate pathway. This pathway converts the nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH (reduced form) and simultaneously form ribulose-5-phosphate, pentose sugar, RNA, DNA, and ATP. The NADPH is important for defense against oxidative stress (Peters & Noorden, 2009).

The overproduction of cytochrome P450 monooxygenases, mixed-function oxidases, together with elevated levels of esterases has been frequently linked with insecticide resistance to organophosphates, organochlorines, pyrethroids, and carbamates in mosquitoes (Brewer & Keil, 1989). P450s are important haem-thiolate-comprising enzymes that exist in insects and are involved in the various metabolic processes (Chandor-Proust et al., 2013). Among the diverse P450s found in mosquitoes, the CYP6Zs has been noted as the important ones allied with pyrethroid resistance (Marcombe et al., 2009).

The analysis of biochemical mechanisms associated with insecticide resistance in mosquitoes can be performed using enzyme assay since it is simple, rapid and sensitive to other approaches (Lee, 1990). Irrespective of the links between the degree of enzyme activities and insecticide resistance, the augmented activities of α-esterases and β-esterases in *Culex quinquefasciatus* verified the prevalence of insecticide resistance towards malathion, permethrin, and DDT (Low et al., 2013). However, studies based on the afore-mentioned aspects of isolated bioactive compounds from traditionally used medicinal plants from India against the dengue fever vector *Aedes aegypti* are scanty in the current scenario.

Altogether, natural insecticide-based mosquito control is a key tactic in the prevention of mosquito-borne disease, especially the dengue, which results in 390 million infections every year. Low-cost insecticides like dieldrin and DDT, used during the global malaria eradication campaign as an indoor residual spray in the 1950-1960s, have initially been very effective in several countries. However, the emergence of insecticide resistance in the epidemiologically significant vectors

has limited their sustained use in mosquito vector control programs. Therefore, monitoring the insecticide resistance in mosquitoes in a systematic and sophisticated way is vital for the vector control programmes. New inventions to tackle the adverse effects of synthetic insecticides are urgently required. Keeping in mind that end, an effective analysis of the factors influencing the probable mode of action of isolated bioactive compounds from traditionally used medicinal plants with special inference on the processes governing the insecticide resistance and oxidative stress in *Aedes aegypti* is vital.

The present investigations were made to undertake the following objectives:

1. To screen at least fifty locally available and traditionally important medicinal plants against *Aedes aegypti*, using organic solvents of increasing polarity.
2. To isolate and identify the bioactive principle from at least three plants.
3. To evaluate the susceptibility status of 4^{th} instar larvae of *Aedes aegypti* towards the plant isolates by employing the standard larval bioassay procedure prescribed by WHO.
4. To investigate the qualitative and quantitative changes of certain detoxifying enzymes such as esterases, dehydrogenases, and monooxygenases if any, due to the effect of botanicals.

The objectives are discussed in separate chapters as follows:

Chapter 1: Screening of aromatic plants against *Aedes aegypti*.

Chapter 2: Identification of bioactive compounds.

Chapter 3: Susceptibility status of 4^{th} instar larvae of *Aedes aegypti* towards the plant isolates.

Chapter 4: Qualitative and quantitative analysis of a few detoxifying enzymes in susceptible and phytochemical selected lines of *Aedes aegypti.*

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The first exact report of yellow fever appears to be described in the year 1495 after the Vega Real by Columbus. Based on the report by da Roch-Lima (1912), the first emergence of yellow fever in South American provinces happens during 1658. The appearance of yellow fever in New York, Boston and Philadelphia took place in 1668, 1691, and 1669 respectively. Although, due to the extensive military expeditions and the services in passenger routes, rigorous epidemics of yellow fever broke out. Similarly, during 1779 and 1780, the other important mosquito-borne disease dengue has been reported from the three continents of Africa, North America and Asia (Hirsch, 1883).

The basic report found to date is in the encyclopedia of disease symptoms from China published from 265 to 420 A.D (Chin Dynasty). It was edited in 610 A.D. and 992 A.D. (Nobuchi, 1979). The dengue outbreak could also have occurred in the French West Indies and Panama in 1635 and 1699 respectively. However, before the 18^{th} century, the dengue or dengue associated infections had an extensive geographic distribution all over the world. It is indeterminate whether the epidemics in Cairo, Egypt, Indonesia and Batavia that happened during 1779 were dengue. However, it is fairly confirmed that the epidemic occurred in the Philadelphia province, was dengue (Carey, 1971).

The ecologic disturbance in the Pacific and Southeast Asia regions by World War II has developed the convenable environmental conditions for mosquito vectors thereby causing increased dengue epidemic transmission (Monath, 1988). The

incidence of dengue fever in India was first reported in Chennai (Madras-1780) during the 1780s. The epidemics of dengue fever has been very complex in the Indian subcontinent and has significantly updated over the past decades in terms of severe effects, different strains and wide geographical distribution (Ramakrishnan, Gelfand, Bose, Sehgal, & Mukherjee, 1964).

De Rèaumur in 1738 described the life history of *Culex pipiens*, and today, he would have been renowned as an ecologist. According to him, all the living things are interconnected; so that he opened a new perspective on the historical aspects of entomology. In 1741, Malaga faced five epidemics which led to the death of more than 5,000 individuals, and Spain paid high price against the incidence. Carl Linnaeus employed his binomial classification system in 1758 towards the mosquitoes. Dr. Finlay's wrote ten papers from 1865 to 1881 on "black vomit" (the Spanish name for yellow fever) (Finlay, 1886).

The American anti-mosquito movement raised up the analysis concerning the influence of mosquito vectors in pathogen transmission and public health concern regarding life-threatening effects of mosquito vectors in the late 1800s. The taxonomists such as Frederic Skuse, Johan Fabricius, and Daniel Coquillett in the 1900s had identified approximately 160 types of mosquitoes at the species level (Christophers, 1960). During this period many people could have protested against them. However, nothing was done during the late 19th century, the scientific community opened a new chapter concerning the link between human beings and mosquitoes with special emphasis on the pathogen transmission by vector mosquitoes (Howard, 1921).

The establishment of the irrigation network and railways without keeping a proper drainage system during the 1840s in India has created several aquatic habitats for the mosquito vectors. The aforementioned condition has forced the British officers in India to pay extensive efforts to maintain proper drainage systems together with chemoprophylaxis using Quinine. In addition, the economic loss, heavy death followed by the life-threatening issues from vulnerable zones like Punjab to British officers has been recognized as additional prominent reasons to develop proper vector control approaches (India, 1909). Until 1871, no medical textbooks recognized any infections that could be spread by an insect. However, throughout the next 30 years, Harris Graham (dengue), Carlos Finlay (yellow fever), Roland Ross (malaria), and Patrick Manson (filariasis) identified and characterized the role of mosquito vectors in pathogen transmission. During the early twentieth century, the revolutionary campaigns of Malcolm Watson in the Federated Malay States, Oswaldo Cruz in Brazil, William Gorgas in Panama and Havana, verified the efficacy of mosquito control in falling mosquito-borne pathogens. At the same time, the demand for vector control programmes received impetus in human populations seeking relief from mosquito-borne diseases. The American anti-mosquito movement stimulated over three different eras during the twentieth century: i) the mechanical control era from 1900 to 1942, ii) the chemical control era from 1942 to 1972 and that iii) followed by integrated mosquito management era from 1972 to present.

The mechanical era illustrates the Gorgas's success in Panama and Havana. In 1901, a group of individuals from South Orange, New Jersey created a village improvement society to reduce the life-threatening effects of mosquitoes.

Surgeon-Major Sir Ronald Ross in 1881 joined the Indian Medical Services and in 1897, he established the life cycle of the malarial parasite in *Anopheles* mosquitoes. Later in 1902, he was knighted and received Nobel prize in medicine. This has unlocked new prospects in vector control research towards a novel direction, principally concentrating on the extermination of mosquitoes. John Smith, Professor of Entomology at Rutgers University, has published *Mosquitoes of New Jersey* which drew up a scientifically grounded campaign against vector mosquitoes.

William Herms, an Ohio entomologist-initiated California's (Penryn, California) first campaign in 1910 to prevent the proliferation of *Anopheline* mosquitoes. Throughout the next 20 years, the control of mosquito vectors based on draining and ditching with the limited use of oil was established in Illinois, Florida, and Utah (Smith, 1904). Stephens, James, and Christophers in the 1900s directed a comprehensive study in the military cantonments in Punjab with special inference to the epidemiological concerns of mosquitoes. Christophers has supervised the Punjab Malaria Surveys (1909–1911) and All-India Malaria Conferences (1900–1909) (http://www.nls.uk/indiapapers/index.html.). Capt. S. P. James wrote reports on the various aspects of malaria prevention and health care needs (James, 1906). Bently (1911) and Maj. Marjoribanks (1914) drew up the significance of drainage systems for effective mosquito-control programmes. Initial epidemics of dengue fever in India were reported and virologically verified in 1963-1964 on the Eastern Coast regions of India. It was then spread towards the north and southern provinces of India and reached at Delhi and Kanpur in 1967 and 1968 respectively. Simultaneously, the whole country has faced the threat from dengue

epidemics followed by the prevalence of all the dengue serotypes (Myers, Carey, Banerjee, Reuben, & Ramamurti, 1968).

Myers et al. in 1968 had conveyed the presence of dengue fever vector *Aedes aegypti* and DV-3 in patients at Vellore in 1966. In addition to this, four types of dengue viruses were characterized and isolated from mosquitoes and patients during the epidemic in 1968. Since 1968, the dengue epidemics have been drastically observed every 3-4 years; 1970, 1982, 1988, 1996, 2003, 2006, 2010, 2013, and 2015 in Delhi, the capital of India (Daudé, Mazumdar, & Solanki, 2017). The statistical analysis revealed that approximately 5,574 hospitalizations followed by eight deaths were reported during the 2013 outbreak. One of the significant reasons for the afore-mentioned drastic situation is the presence of several poor areas offering sufficient environmental conditions and habitats for the breeding of the dengue vector *Aedes aegypti*. In this regard, the World Health Organization-South East Asia (WHO-SEA) has positioned India in 'Category A.' The afore-mentioned concepts and the mosquito-borne epidemics put intense pressure on the scientific community to develop novel vector control strategies (Nagpal et al., 2016).

Jamison, Jha, Malhotra and Verguet (1900) reported that the transmission of mosquito-borne diseases is allied with three significant factors such as i) host susceptibility, ii) vector competence and iii) virulence of the infectious agent. In addition, they also reported that the development of insecticide resistance has been well studied in several arthropod disease vectors. One of the most significant ways to prevent mosquito-borne diseases is the prominent use of insecticides. In

this regard, several chemical compounds of synthetic origin have applied to aquatic habitats as effective larvicides. Adulticides were used for indoor residual sprays (IRS) and outdoor space applications (Jamison et al., 1900). The concepts of vector control had emerged in Malaya during 1901-1903 (Watson, 1921). In South Africa, the synthetic insecticide pyrethrum was used as an effective vector control agent during the early thirties.

However, the notable discovery of the use of DDT (1942) as a prominent insecticide by Paul Muller has reorganized the vector control field (Russell, West, Manwell, & Macdonald, 1963). Based on the past history of vector control in India, two types of eras have been distinguished; i) Pre-DDT era, and ii) Post-DDT era. Previous studies have verified that several vector control strategies have been commonly used during 1936 and oils, larvivorous fishes, and Paris greens were the major constituents of the afore-mentioned strategies. The proper drainage system may also help to exterminate the vector's population. As part of the antimalarial campaign in India during 1944, the DDT was chosen as an eminent insecticide for mosquito control (White, 1945). Hexachlorocyclohehane/ Benzenehexa chloride (HCH/BHC), another synthetic insecticide is used as an effective mosquito control agent in Assam, India during the 1950s.

In 1958, the National Malaria Eradication Programme (NMEP) has launched for the prevention of malaria and other vector-borne diseases (Rao, 1958). However, the frequent use of synthetic insecticides to eliminate vector-borne diseases using different strategies were not fully successful (Sharma, 1984). Therefore, various insecticides from pyrethroid group (1980) were applied in the public health

programme to evade the mosquito borne-diseases and its vectors. Mainly four groups of insecticides such as organophosphates, pyrethroids, organochlorine (DDT) and carbamates have been extensively used for mosquito control programmes. Initially, DDT was introduced against agricultural pests in 1934. However, it was banned due to the adverse effects from it in the environment in 1983. Based on the advice received from the World Health Organisation, the public health community has used DDT as a common insecticide for indoor residual spraying (IRS). One of the major pointers behind the use of DDT by WHO is the eradication of malaria (Stein, 1970).

The organophosphate insecticide Malathion also possessed significant toxicity against the *Anopheles culicifacies* population during 1969 (Rajagopal, 1977). At the end of the 20th century, several other insecticides such as deltamethrin, lamda cyhalothrin, and cyfluthrin have shown their potential to support the public health programme. Many other important approaches have also been used for vector control during the same period over India. Advanced technologies in computer hardware have sturdily strengthened the development of specific software for creating vector distribution maps using digital databases, remote sensing (RS) together with Global Positioning Systems (GPS) (Montoya, 2003).

Soper (1963) suggested that the larvicides can be applied to the natural aquatic environments or breeding sites of the vector mosquitoes and are known to reduce the pathogen transmission. Although, in some aspects, the direct implantation of chemical insecticides to the aquatic ecosystem is totally difficult, since it is problematic to locate in cryptic breeding sites (Killeen et al., 2006). Morrison,

Zielinski-Gutierrez, Scott and Rosenberg (2008) support the afore-mentioned concept as for the reason that the economic and anthropological resources are limited, and therefore, it is hard to attain a coverage level necessary to diminish the threat from arthropod-borne diseases.

Ponlawat, Scott and Harrington in 2005 reported that broadcasting of larvicides in wide-area has provided wide coverage, and either vehicle-mounted sprayers or aircraft can be used for the same. However, their study also mentioned certain community concerns, environmental restrictions, or regulations together with insecticide resistance to the active constituents of existing insecticides. They also discussed the drawbacks of the aforementioned concepts including the expensive nature of larvicide formulations (Li & Liu, 2014).

The previous studies on various dimensions by WHO indicated that the vector control interventions using chemical insecticides have resulted in considerable range of reductions in morbidity and mortality induced by mosquito-borne diseases. In contradiction to the advantageous outcomes provided by the synthetic insecticides, a new global systematic survey among the nations at risk for mosquito-borne infections drew consideration to life-threatening deficits in the ability to manage mosquito control insecticides. The lack of proper guidelines for pesticide registration and lack of training followed by gaps in pesticide store and appropriation practices are included as the foremost life-threatening deficits (Matthews et al., 2011).

The extensive use of various insecticides of chemical origin has elevated concerns over the emergence of resistance to them thereby causing adverse effects on

human health and the environment. Many studies conducted during the 1960s outlined by Mouchet in 1972 revealed that several *Aedes* sp. populations from India and Southeast Asia region were resistant to some of the commonly used insecticides such as fenthion, DDT and dieldrin. A review by Ranson et al. in 2010 later updated by Vontas et al. in 2012 analyzed the range of insecticide resistance in *Aedes* sp. all over the world (Ranson & Hemingway, 2005). The Stockholm Convention on Persistent Organic Pollutants in 2012 highlighted the need for replacements to the use of DDT and other insecticides in mosquito control, given its harmfulness, bioaccumulation and the potential for transboundary actions together with environmental persistence (Van Den Berg et al., 2012). That is, most of all the studies concerning insecticides and mosquito control have indicated that the frequent introduction of insecticides has instigated several arthropods including disease vectors to create mechanisms to withstand extensive exposure from treatments. Certainly, there is presently extensive resistance to all types of insecticides among several arthropod vectors of epidemiological significance, making vector control a challenging aspect in itself (Van Den Berg et al., 2012).

McCaffery and Nauen (2006) and Britch et al. (2010) suggested that routine and extended use of insecticides by ultra-low volume of aerosolized insecticides, permethrin, deltamethrin, and malathion against *Aedes aegypti* may develop insecticide resistance. According to Faucon et al. (2015), the possible mechanism concerning the resistance towards pyrethroid in *Aedes aegypti* is the target site mutations along with variations of enzyme activity. In addition to this, Dong (2007) mentioned that mutations occurred in the voltage-gated sodium channel

(VGSC) may be responsible for the impeding action of pyrethroids, thereby resulting in knockdown resistance (*kdr*). Previous studies conducted by Fernando et al. (2018) have supported the afore-mentioned concepts by evidencing the influence of S989P, V1016G, F1534C in pyrethroid resistance in the dengue fever vector *Aedes aegypti*. A further major alarm is the influence of human urbanization as well as the environmental circumstances in insecticide resistance demonstrated by (Nkya et al., 2014). Augmented susceptibility of *Aedes* mosquitoes to routinely used insecticides could be either directed by detoxification processes over enzymes or through target site modification. Overexpression of concerned genes/enzymes (Glutathione S-transferases (GSTs), Cytochrome P450s, Mono-oxigenases) has been exposed to confer resistance in resistant strains of *Aedes* mosquitoes all over the world (Vontas et al., 2012).

The Environmental Protection Act in 1969 has forced the public community to reduce the routine and improper use of insecticides in vector control programmes since many of the insecticides instigates environmental hazards, toxic effects to non-target organisms including human population, non-biodegradable nature, elevating insecticide resistance followed by the greater range of biological magnification over ecosystem (Bhatt & Khanal, 2009; Brown, 1986). This has impelled the scientific community to stare for alternative strategies stimulating the implementation of effective mosquito management programmes that focus on monitoring and surveillance and public education followed by source reduction and natural larval control. In this regard, the application of alternatives including natural control of mosquitoes has become the backbone of the vector control strategies in lieu of the synthetic insecticides (Ghosh et al., 2012). The

larvivorous fishes were predominantly used in peri-urban and urban areas for mosquito control during the early 20th century (Gratz & Pal, 1988). However, it doesn't provide the complete eradication of *Anopheles gambiae.* Additionally, the Toxorhynchites, nematode worms, dragonflies, bacteria, and cyclopoid copepods have been widely used for vector-borne disease eradication (Rozendaal, 1997). Basic sanitary measures, use of livestock and marsh alteration have also provided a significant contribution to the development of mosquito control interventions (Ault, 1994).

Till date, the bioactive elements of botanical origin from low concentration to higher concentration are prominently effective against *Aedes*, *Anopheles*, and *Culex* mosquito population. One of the best advantages of the insecticides of botanical origin is that it reduces the chance of insecticide resistance and even a layman can easily formulate inexpensive adulticides, ovicides or repellents at their home with low human toxicity (Isman & Grieneisen, 2014).

White (1973) reported that the floral biodiversity can be explored to enter the field of a sustainable method of vector control. Phy (1999) reported that the insecticidal potential of medicinal plants can be definitely used to develop a novel weapon in the synthetic insecticides resource and, in future, it may be recognized as an alternative natural product to combat vector-borne diseases.

The application of bioactive constituents from traditional medicinal plants to fight against vector-borne diseases has emerged since the 1920s, and the discovery of DDT, as well as other synthetic insecticides, made diminished use of phytochemical constituents in mosquito control strategies. However, after facing

many adverse effects by the routine use of synthetic insecticides, the scientific community has re-focussed on bioactive elements that are easily isolated from medicinal plants. From that moment onwards, the search for novel bioactive elements from the plant biodiversity and an effort to its commercial production has been started (Isman, 1997).

Shaalan, Canyon, Younes, Abdel-Wahab and Mansour (2005) reviewed different kinds of phytochemical elements (terpenoids, phenolics,steroids, and alkaloids) and their toxic potential towards the insects. They also mentioned various plant families that have many kinds of larval and repellent activities against mosquitoes of epidemiological importance in the current scenario. The families include; i) Solanaceae, ii) Cladophoraceae, iii) Miliaceae, iv) Asteraceae, v) Rutaceae, vi) Oocystaceae and vii) Labiatae. Additionally, they have reviewed some insecticides of botanical origin (Hellebore, d-limonene camphor, Pyrethrum, Nicotine, Turpentine, Derris, Anabasine, Quassia, and Azadirachtin) used in different nations during the early 20^{th} century.

Kishore, Mishra, Tiwari and Tripathi (2011) reviewed the larvicidal potential of phytochemical constituents against various mosquito species based on their chemical nature, presence of secondary metabolites like simple aromatics, alkenes, alkanes, alkynes, lactones, steroids, fatty acids, lignans, terpenes, isoflavonoids, alkaloids, and pterocarpans. Additionally, they also documented the characterization of several bioactive elements from various plants and discussed their toxicity against mosquitoes of epidemiological significance.

In 2012, Ghosh et al. listed some of the bioactive compounds isolated from various plants; and are Octacoane, Geranial, Germacrene D, Plumbagin, Pipernonaline, Marmesin, and Pachyrrhizine. They also discussed the larvicidal potential of the following medicinal plants such as *Jatropha* sp, *Ocimum sanctum*, *Citrus* sp, *Momordica charantia, Azadirechta indica, Piper* sp, *Curcuma domestica*, *Jatropha curcas* (Linnaeus, 1753), *Cestrum diurnum, Piper retrofractum, Citrullus vulgaris, Euphorbia* sp, *Annona* sp, *Euphorbia tirucalli*, *Annona squamosal, Solanum xanthocarpum, Solanum nigrum* and *Moringa oleifera*.

Similarly, Traboulsi et al. (2005), and Rahuman, Gopalakrishnan, Venkatesan and Geetha (2008a) reported the insecticidal activities of aromatic plants such as *Ferula hermonis, Pinus pinea, Eucalyptus spp, Citrus sinensis*, *Laurus nobilis*, *Momordica charantia*, *Coccinia indica*, *Citrullus colocynthis*, *Trichosanthes anguina*, *Cucumis sativus, Phyllanthus amarus, Pedilanthus tithymaloides, Euphorbia tirucalli, Euphorbia hirta,* and *Jatropha curcas*. Numerous investigations have exposed the significance of medicinal plants in mosquito vector control strategy. For example, Benelli (2015) reviewed the larvicidal potential of the following plants; *Acalypha indica, Acalypha alnifolia, Andrographis paniculata Ageratum houstonianum, Albizia lebbeck, Andrographis lineata, Annona senegalensis, Artemisia annua, Aristolochia bracteata, Asparagus racemosus, Basella rubra, Caesalpinia pulcherrima, Cananga odorata Calotropis procera, Cinnamomum zeylanicum, Citrullus colocynthis, Coccinia indica, Cassia occidentalis, Cucurbita maxima, Cymbopogon citratus, Cardiospermum halicabum, Coccolus hirsutus, Cassia*

fistula (Linnaeus, 1753), *Cleome viscosa, Cuminum cyminum, Curcuma longa, Cymbopogon proximus, Cyperus scariosus, Eclipta alba, Delonix elata, Eclipta prostrate* (Linnaeus, 1753), *Ervatamia coronaria, Erythrina indica, Euphorbia hirta, Gliciridia sepium, Ipomoea carica, Juniperus macropoda, Limonia acidissima, Lippia multiflora, Mentha spicata var. viridis, Moringa oleifera, Nigella sativa, Ocimum basilicum, Ocimum canum, Parthenium hysterophorus, Pemphis acidula, Pimpinella anisum, Pinus caribaea, Pinus tropicalis, Piper nigrum, Pithecellobium dulce, Polygonum hydropiper, Azadirachta indica, Pongamia glabra, Rosmarinus officinalis, Spilanthes mauritiana, Syzygium aromaticum, Solenostemma argel, Tagetes erecta* and *Zingiber officinalis.* Kumar, Wahab, Mishra, and Warikoo (2012b) and Oliveira et al. (2010) reported the lavicidal potential of following plants: *Abutilon indicum, Achyranthes aspera, Phyllanthus emblica, Cassia occidentalis, Allium sativum, Zingiber officinale, Momordica charantia, Lantana camara, Ricinus communis, Trachyspermum ammi, Putranjiva roxburghii, Chrysanthemum indicum, Myristica fragrans, Bauhinia tomentosa, Melaleuca bracteata, Coccoloba mollis, Eschweilera ovate, Guettarda grazielae, Merremia aegyptia, Ouratea nitida, Protium heptaphyllum, Rourea doniana, Spermacoce verticillata, Tovomita brevistaminea,* and *Triplaris americana.*

Various biochemical processes that happened in the plant has led to the discharge of an array of bioactive elements. The bioactive elements from botanicals have been touted as an effective alternative to chemical pesticides for mosquito control. Previous studies have already revealed the possible reason for the afore-mentioned natural strategy since the bioactive elements are known to play a significant role

in several metabolic processes associated with thermal tolerance, defense against herbivores and attracting pollinators (Nyasembe & Torto, 2014). But, it should not be expected that the bioactive elements are less susceptible to resistance as for the reason that they may possess similar modes of action against the mosquito vectors (Oladipupo, Callaghan, Holloway, & Gbaye, 2019). However, the development of novel and eco-attractive tools for preventing the proliferation of mosquitoes is of paramount prominence in order to confirm our future capacity to eradicate the vector-borne diseases transmitted by mosquitoes. Because of the vast diversity, presence of various potent phytochemical constituents, the medicinal plants are recognized as important sources of novel natural insecticides. Additionally, the natural complexity of insecticides of botanical origin can offer an additional benefit in the fight against mosquito-borne diseases: since the bioactive elements isolated from the plant extracts can act synergistically within the insect vector, the possibility of the persistence of individuals exhibiting resistance against any one of those compounds is impressively reduced (Siegwart et al., 2015).

It has been reported by previous studies that the prominent toxicity of most of all the plant species attributable to the occurrence of a wide variety of alkaloids, indol alkaloids, glycoalkaloids and pirrolizidin, are produced as a defense mechanism against predators, infectious agents, and insects (Ogunlesi, Okiei, Ofor, & Osibote, 2009). Cui, Tan, Ouyang, Jiang, and Pawliszyn (2009) reported the insecticidal activity of various alkaloids such as α-chaconine, α-solanine and α-tomatine with special reference to economic and medical importance. Nyasembe and Torto (2014) highlighted different types of plant volatile compounds from

many chemical classes, including aldehydes, ketones, phenols, terpenes, and alcohols against mosquitoes. In this regard, with the understanding of the importance of plant volatile compounds in vector control, there has been a growing concern in the chemistry and probable mode of action of these compounds.

This has resulted in the development of appropriate technologies for the isolation and exploration of bioactive compounds from medicinal plants (Harborne, 1998). The choice of the most effective technique for the isolation of bioactive elements is reliant on the type of plant species being studied. Several techniques including Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS), bioassay-guided chromatographic fractionation have been used for the collection and analysis of Phyto-compounds against mosquitoes (Tholl et al., 2006).

Kiprop, Kiprono, Rajab and Kosgei (2007) isolated limonoid calodendrolide from the plant *Calodendrum capense* Thunb with potent toxicity against *Aedes aegypti* larvae. They have also isolated pedonin and harrisonin from the Harrisonia abyssinica Oliv with larvicidal efficacy against the dengue fever vector *Aedes aegypti*. Silva et al. (2009) isolated and characterized isobrucein B and neosergeolide from the medicinal plant *Picrolemma sprucei* Hook against *Aedes aegypti*. Gu, Cheng, Huang, Chen and Chang (2009) reported the larvicidal potential of cubebol, epi-cubebol and ferruginol against *Aedes aegypti*. They have isolated the afore-mentioned compounds from *Cryptomeria japonica*. Similarly, Madhu, Shaukath, and Vijayan, (2010) studied the toxic effect of

sesquiterpenoid metabolites such as neoprocurcumenol and 9-oxoneoprocurcumenol isolated from *Curcuma aromatic* Salisb against *Culex quinquefasciatus*.

Rahuman et al. (2008b) isolated and characterized the tetracyclic triterpene derivative gluanol acetate from the herb *Ficus racemosa* L against *Aedes aegypti, Culex quinquefasciatus*, and *Anopheles stephensi.* Innocent et al. (2008) performed bio-assay guided fractionation of the root bark extract of the traditionally used medicinal plant *Lantana viburnoides subsp viburnoides var. kisi* against *Anopheles gambiae.* Their study also revealed the presence of lantadene-type triterpene camaric acid and lupine triterpene betulinic acid in the active fractions. Rahuman et al. (2008b) isolated saponin and β-sitosterol from *Achyranthes aspera* and *Abutilon indicum* respectively against *Aedes aegypti* larvae. Cheng, Huang, Chen, Kuo and Chang (2008) reported the potent larvicidal efficacy of the anthraquinone tectoquinone from *Cyptomeria japonica*. Sreelatha et al. (2010) performed the bioassay-guided fractionation of *Plumbago capensis* extracts and revealed the presence of two novel napthaquinone derivatives together with six known compounds against *Aedes aegypti* mosquitoes. A similar study conducted by Maniafu, Wilber, Ndiege, Wanjala and Akenga (2009) exposed the presence of 5-hydroxy-2-methyl-1,4-naphthoquinone and β-sitosterol against *Anopheles gambiae*. Garcez, Garcez, da Silva and Hamerski (2009) used bioassay-directed fractionation of *Ocotea velloziana*, and isolated aporphine alkaloid (+)-dicentrine against *Aedes aegypti*.

Innocent et al. (2008) reported the presence of furanonaphthaquinones regio-isomers and lantadene triterpenoid camaric acid from the bioactive fraction of *Lantana viburnoides*, against *Anopheles gambiae*. Pohlit, Rezende, Baldin, Lopes and de Andrade Neto (2011) described the isodillapiol, and n-propyloxy and n-butyloxy propan-2-yl ether as effective larvicides. In addition, they have reviewed the larvicidal potential of the following chemicals: Trans-anethole, Terpineol, β-Pinene, Borneol,α-Pinene, Borneol acetate, Linalool, Camphor, Pulegone, 1,8-Cineole, Citronellal, p-Cymene, Thymol, and Eugenol against *Aedes aegypti* (Pohlit et al., 2011).

Preethi, Raveen, Arivoli, Samuel and Madhanagopal (2014) investigated the larvicidal potential of the methanol, aqueous, and chloroform extracts of *Jasminum officinale, Jasminum grandiflorum*, and *Jasminum auriculatum* against the dengue vector *Aedes aegypti*. Muthee et al. (2011) documented the exploitation of several medicinal plants for the discovery of drugs. They have reviewed the medicinal potential of the following plant families: Anacardiaceae (*Rhus natalensis* and *Ozoroa insignis*), Amaranthaceae (*Sericocomposis hildebrandtii*), Apocynaceae (*Carissa edulis* and *Acokanthera schimperi*), Araliaceae (*Cussonia holstii*), Asclepiadaceae (*Mondia whytei*), Balanitaceae (*Balanites glabra*), Boraginaceae (*Cordia Africana* and *Kigelia africana*), Burseraceae (*Commiphora swynnertonii*), Capparidaceae (*Caparis tomentosa*), Cannelaceae (*Waburgia ugandensis*), Celastraceae (*Maytenus senegalensis*), Combretaceae (*Terminalia brownie* and *Combretum molle*), Compositae (*Psiadia punculata*), Commeliaceae (*Comelina africana*), Cucurbitaceae (*Zehneria scabra*), Ebenaceae (*Euclea divinorum*), Euphorbiaceae (*Ricinus communis*,

Euphorbia cuneata, *Croton megalocarpus* and *Euphorbia candelabrum*), Fabaceae (*Indigofera arrecta*, *Cajanus cajan*, *Acacia xanthophloea*, *Ormocarpum kirkii*, *Albizia anthelmintica* and *Erythrina abyssinica*) Flacourtiaceae (*Dovyalis abyssinica*), Liliaceae (*Aloe secundiflora*), *Leucas calustachys*), Loganiaceae (*Strychnos henningsii*), Labiatae (*Leucas calustachys*, *Leucas pododiskos* and *Iboza multiflora*), Meliaceae (*Azadirachta indica* and *Turraea mombassana*), Moraceae (*Ficus thonningi* and *Ficus sycomorus*), Musaceae (*Musa acuminata*), Myrsinaceae (*Rapanea melanophloes*), Olacaceae (*Ximenia americana*), Oleaceae (*Olea africana*), Oliniceae (*Olinia usambarensis*), Polygonaceae (*Rumex usambarensis*), Proteaceae (*Faurea saligna*), Rhamnaceae (*Rhamnus staddo* and *Rhamnus perinoides*), Rosaceae (*Prunus africana*), Rubiaceae (*Galium aprimnpoides*, *Hymenodictyon parvifolium* and *Vangueria tomentosa*), Rutaceae (*Calodendrum capense*, *Clausena anisata*, *Zanthoxylum usambarense* and *Teclea simplicifolia*), Salvadoraceae (*Salvadora persica)* (Linnaeus, 1753), Samydaceae (*Trimelia bakeri*), Santalaceae (*Osyris lanceolatat*), Sapindaceae (*Pappea capensis*), Simaroubaceae (*Harisonia abyssinica*), Solanaceae (*Solanum taitanse* Vatke and *Solanum incanum*), Sterculiaceae (*Dombeya rotundifolia*), Urticaceae (*Urtica dioica*), Useneaceae (*Usnea* spp.), Verbenaceae (*Clerodendrum myricoides* and *Lippia kituiensis*), and Vitaceae (*Cissus quandringularis* and *Rhoicissus tridentata*).

The major focus of their study is to offer a platform for studies whose aim is to develop plant-based products. This indicated that the plant-based insecticides are prominently drawing the attention of the research community due to its cost-effective, eco-attractive and bio-degradable nature. A recent study by Iqbal et al.

(2019) was directed to the biological synthesis of zinc oxide nanoparticles (ZnONPs) using the various extracts of the plant *Rhamnus virgate*, as a stabilizing agent. Pavela, Maggi, Iannarelli and Benelli (2019) reviewed the mosquitocidal activity of isolated bioactive compounds from plant extracts against vectors belonging to the genera *Culex, Anopheles*, and *Aedes* with special consideration of the probable mode of the mechanism of action. They have also discussed the mode of action of alkamides, triterpenes, anthraquinones, aliphatics, coumarins, acetogenonins, flavonoids, alkaloids, sesquiterpenes, sterols, sesquiterpenes, alkaloids, sesquiterpenes, alkaloids, aliphatics, flavonoids, sesquiterpenes and xanthones on mosquitoes from neurotoxic effects to enzymatic actions.

Benelli, Pavela, Drenaggi and Maggi (2019) reported the presence of volatile elements such as (*E*)-caryophyllene, myrcene , caryophyllene oxide, and β-pinene from *Acmella oleracea* against *Culex quinquefasciatus*. Simon-Oke and Akeju (2019) reported the toxic effects of *Citrus limonum, Citrus aurantifolia,* and *Citrus sinesis*. Sengar, Joshi, Prasad and Hemalatha (2015) has scientifically verified the anti-pyretic, analgesic and anti-inflammatory activities of *Jasminum sambac*. In 2014, Preethi et al. investigated the potential larvicidal efficacy of aqueous, methanol, and chloroform extracts of *Jasminum auriculatum*, *Jasminum grandiflorum*, and *Jasminum officinale* against *Aedes aegypti* at varying concentrations. Similarly, Pavela et al. (2019) reviewed the modes of action of bioactive elements from the medicinal plant *Jasminum nervosum*. Lallawmawma et al. (2015) suggested that the biosynthesized AuNPs and AgNPs (silver and gold nanoparticles) using the *Jasminum nervosum* extract could be an eco-attractive, safer natural insecticides and offered significant larvicidal potential against *Culex*

quinquefasciatus which could be employed for the prevention of various mosquito-borne diseases.

Koul, Kaur, Goomber and Wahab (2004) studied the mode of action and bioefficacy of rocaglamide isolated from *Aglaia elaeagnoidea.* Benelli et al. (2018) determined the significant mosquito nanolarvicides from the *Aglaia elaeagnoidea* with special reference to its ethnopharmacological concern and non-target mosquito natural predators including waterbugs, larvivorous fishes and backswimmers. Bacher, Hofer, Brader, Vajrodaya and Greger (1999) have isolated nine novel flavaglines, benzo [b] oxepines (thapoxepines), and cyclopenta [bc] benzopyrans (thapsakins) from the root extract of *Aglaia edulis* (Roxb, 1840) with insecticidal property. But, they did not mention the larvicidal property of the afore-said compounds against mosquitoes in the afore-mentioned study. Koul et al. (1997) screened the larval growth-inhibiting effects of *Aglaia elaeagnoidea*, *Aglaia roxburghiana,* and *Aglaia odorata* against *Helicoverpa armigera* and *Spodopteva litura*.

Dreyer et al. (2001) described the isolation of cyclopentatetrahydrobenzofurans of the rocaglamide from the plant *Aglaia oligophylla*. Anjana and Thoppil (2013) evaluated the antimitotic and cytotoxic potential of *Pogostemon auricularius* (Linnaeus, Hassk, 1843). Das et al. (2015) studied the synergistic mosquito-repellent activity of *Pogostemon heyneanus* against *Aedes albopictus*. Murugan, Mallavarapu, Padmashree, Rao and Livingstone (2010) evaluated the presence of acetophenone, patchouli alcohol, β-pinene, and (E)-nerolidol from *Pogostemon heyneanus* Benth using GC-MS. Hussaini, Agarwal, Roy, Prakash and Shoeb

(1988) have isolated three novel diterpenoid acids from *Pogostemon auricularis* (15-dien-18-oic acid and 7-hydroxy-and 7-acetoxycleistanth-13, 15-dien-18-oic acids and cleistanth-13). Hussaini et al. (1993) have isolated and characterized a novel Diterpenoid from *Pogostemon auricularis*.

As far as the review of literature could ascertain, the studies on the isolation of bioactive compounds from three traditionally used medicinal plants such as *Jasminum brevilobum* (A. DC. 1844), *Pogostemon auricularius*, and *Aglaia edulis* against *Aedes aegypti* with special reference to their probable mode of action, reactive oxygen species formation and insecticide resistance are not well established.

RELEVANCE OF THE STUDY

RELEVANCE OF THE STUDY

The plant-based products have been used against vector mosquitoes for generations in traditional approaches. The knowledge concerning the prominent use of plant-based products gained through phytochemical research is considered as a significant resource for the development of novel mosquito control agents. The importance of commercial mosquito controlling products containing plant-based ingredients has extended much more popularity among the consumers in the current scenario since most of the previous studies have reported the deleterious effects of long-established synthetic mosquito controlling products with special reference to insecticide resistance.

The phenomenon of adaptation in mosquito vectors towards new living conditions and the environment is one of the vital concepts in evolutionary biology. The development of insecticide resistance in the target organisms has been recognized as a significant part of natural selection. Studies on the various factors allied with the aforementioned perspectives have a prominent impact in the current scenario. Hence, changes in the detoxifying enzymes and the emergence of insecticide resistance in context with the natural products, natural selection, evolutionary perspectives, and DNA damage are core areas of interest of this investigation. The susceptibility status, qualitative and quantitative analysis of certain detoxifying enzymes from F_1-F_{10} generation of *Aedes aegypti* with special reference to insecticide resistance illustrates the role of natural selection in vector control perspectives. This investigation was intended to convey the need of

understanding the potential position and extensive value of plant-derived products and their prominent role in mosquito disease control with special reference to environmental sustainability, reactive oxygen species formation, natural selection, evolution and DNA damage.

GENERAL METHODOLOGY

GENERAL METHODOLOGY

Study area and plant material

The plant specimens were collected from Pothumoola, Thirunelly in Wayanad, Kerala, India based on the cultivational status, easily availability, medicinal potential, traditional uses, economic perspective, commercial probability and aromatic property together with conservational aspects. The collected plants were identified to species level with the help of taxonomists. The GPS point of all the plant specimens were collected. A herbarium specimen of the plant is kept in Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Josephs's College, Irinjalakuda, Kerala, India with reference number.

Crude and standard extraction

The collected plants were washed and grounded with mortar and pestle. The crude extract of the plant specimen was kept for 24 hours and the desired volume of the extract was taken after 24 hours for larval bioassay. The shade-dried powder of plant specimens that possess potent larvicidal efficacy among the 50 traditionally used medicinal plants were extracted using a Soxhlet extractor. Twenty grams of dried powder of plant specimen which contains the bioactive elements to be extracted were placed in a thimble. The thimble is usually made up of filter paper material that allows liquids and essential contents to pass through it. Each solvent (250 mL) was added to a round-bottomed flask, which is attached to a Soxhlet extractor and condenser on an isomantle. The organic solvents of increasing polarity such as petroleum ether, ethanol, acetone, and water as

separate solvents were heated at specific boiling temperatures for 6 to 12 hours and it initiates the movement of bioactive elements into the condenser. Once the level of the solvent containing bioactive elements reaches the siphon, it pours back into the round-bottomed flask (250 mL). The process was repeated continuously until all the constituents from the plant specimen were extracted into the organic solvent. The extracts obtained from the afore-mentioned steps were evaporated and used for further investigations including chromatographic analysis and larvicidal bioassay.

Experimental insect

The early fourth instar *Aedes aegypti* larvae were used for the accomplished experiments. The larvae used in this study were from the laboratory colony reared at Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Josephs's College, Irinjalakuda, Kerala, India. The *Aedes aegypti* eggs were placed in the plastic trays (28 L × 39 W× 14 D cm) and were transferred into another plastic tray (28 L × 39 W× 14 D cm) when accomplished with hatching. They were maintained and fed on dog biscuits and yeast in the ratio 3:1. The environmental conditions for rearing is as follows; 12 L:12 D photoperiod cycle, 27± 2°C temperature, followed by 55–60% relative humidity.

Larval bioassay

The graded series of extracts were prepared using methanol, petroleum ether and acetone as the solvent. Twenty-five healthy 4th instar larvae of *Aedes aegypti* were released into a 250 mL glass beaker containing 100 mL solution. Four replicates were set up for each concentration. *Aedes aegypti* larvae were counted as dead if

they failed to reach outward for respiration. The dead larvae in four replicates will be combined and expressed as the percentage of larval mortality for each concentration. In controls, the larvae were exposed to each solvent.

Chromatographic analysis

Repeated chromatographic separation was used for the isolation of bioactive elements from *Jasminum brevilobum*, *Aglaia edulis* and *Pogostemon auricularius*. The analytical grade acetone, hexane, and methanol were used for Column-Thin layer chromatography (TLC) analyses. Thin-layer chromatography for *Jasminumbrevilobum* acetone extract was done by using hexane and acetone as the mobile phase. *Aglaia edulis* acetone extract was subjected to TLC analysis using methanol (M), acetone (A) and hexane (H) as the mobile phases. The bioactive elements of *Pogostemon auricularius* extract were isolated using methanol, acetone, and hexane. The bioactive fractions of acetone extract of the three medicinal plants were separated using column chromatography. Silica gel for column chromatography (60-120 M) was packed in column (40 × 330 mm) (Merck KGaA, Darmstadt, Germany). Analytical grade methanol, acetone, and hexane were used as the mobile phases. Twenty grams of the acetone extract of *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius* were loaded on to the column packed with silica and mobile phase. All the isolated bioactive fractions have proceeded for the bioassay protocol provided by WHO. Sample names were provided for each bioactive fraction. The purified bioactive fractions were stored at -20 °C for further analysis.

Identification of purified bioactive compounds

The isolated bioactive elements such as CC7, EE5, and PP4 were subjected to GC-MS (MSQP2010 (Shimadzu, Kyoto, Japan). The peak areas of the isolated bioactive elements were matched with those on the National Institute of Standards and Technology library database (NIST 08-S, available at https://chemdata.nist.gov/). Fourier-transform infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy (Thermo Fisher, U.S.), a rapid technology to identify and elucidate the structure have been used in this study to fingerprint the isolated bioactive compounds from *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius*.

Susceptibility status

Bioactive elements, other chemicals, and selection experiments

The bioactive elements were isolated from *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius*. All the other chemicals used in this study were of HPLC grade. A susceptible colony for *Aedes aegypti* was maintained. The parental strain was colonized for 15 generations and selected as F_1. The bioassay was performed as per the standard protocol provided by WHO, 1981. Parallel control was maintained for all the executed experiments. The level of resistance was calculated using the method suggested by Marcombe, Chonephetsarath, Thammayong and Brey (2018) and Gopalan, Prakash, Bhattacharya, Anand and Rao (1996).

Qualitative enzyme assay- Poly Acrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) was used as qualitative analysis to compare the bioactive elements exposed and susceptible lines of *Aedes aegypti*. The glucose 6-phosphate dehydrogenase (G6PD), α-esterase, β-esterase, and cytochrome P450 were examined to get the differential isozyme profile.

Quantitative assay

The microplate assay was performed according to the method suggested by Hemingway and Karunaratne (1998). The esterase activity was examined using the Esterase assay. The influence of Cytochrome P450 in insecticide resistance is determined by means of monooxygenase assay (P-450). The effect of dehydrogenase activity was determined with the glucose 6-phosphate dehydrogenase assay (G6PD).

Toxicity towards microorganisms

The screening of antibacterial potential of the isolated bioactive elements were done against the bacterial population that reside inside the mosquito larval gut viz, *Klebsiella pneumonia* (MTCC 661), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160), *E. coli* (MTCC 443), and *Proteus mirabilis* (MTCC 442). The Minimum Inhibitory Concentration (MIC) was determined using the method given by Wiegand, Hilpert and Hancock (2008) and Bussmann et al. (2010). Triplicates were maintained for each executed experiments.

Phytochemicals induced free radical generation and DNA lysing potential

The lethal effects of three isolated compounds induced free radical formation were determined using the ammonium molybdate method (Prieto, Pineda, & Aguilar,

1999) with slight modifications. Twenty-fourth instar larvae of *Aedes aegypti* exposed with the isolated bioactive fractions were homogenized as separate samples. Controls were maintained for each experiment. The level of isolated compounds prompted free radicals formed in the target was determined using the Ultraviolet-visible spectroscopy (Thermo Fisher, U.S.).

Statistical analysis

All data were entered and systemized in Microsoft Excel 2010 and 2019 spreadsheet. The data were then exported into SPSS version 24.0.0 and R software version 3.2.3 for probit regression analysis (to get the LC_{50} and LC_{90} of crude extracts and isolated bioactive elements) and to create pictorial charts. The data on corrected mortality rates were calculated as the mean percentage ± standard deviation (SD) for each bio-assays. The graphs were plotted with GraphPad Prism 7.0 and the SPSS version 24.0.0 for Windows operating systems. The resistance ratio determination and other statistical analyses were also performed using the aforementioned software.

CHAPTER 1

Screening of Aromatic Plants against *Aedes aegypti*

1.1. INTRODUCTION

Aedes aegypti is a daytime, domestic foremost vector mosquito that preferably breeds in discarded plastic containers, stream pools, tree hole, automobile tires, mud pot, cemented tanks, plant pots, tin, coconut shell, bamboo and containers (De Lima Santos et al., 2012). It has extensive significance in tropics and subtropics worldwide since it is recognized as the vector of many infectious diseases including dengue fever, yellow fever and probably the zika fever (Anoopkumar et al., 2017a; Murrell, Wu, & Butler, 2011). In recent decades, the dengue viral infection has grown into an increasing global health threat with 390 million dengue infections annually (WHO, 2014). The lack of an effective vaccine against the dengue fever infection illustrates the importance of *Aedes* mosquito proliferation prevention in disease control; the only way to diminish the risk of dengue epidemics distribution.

The synthetic chemicals, principally the carbamates, pyrethroids, and organophosphates including DDT are used to prevent the rapid proliferation of larvae, pupae, and adult *Aedes aegytpi* mosquitoes. The afore-mentioned compounds are known to exhibit extensive environmental threat, due to severe confrontational effects on non-target organisms including the human population (Sánchez-Fortún & Barahona, 2005). The strategies to fight the mosquito-borne diseases mostly rely on the disruption of the transmission cycle either by pointing the breeding sites of mosquito larvae through insecticides spraying or by repelling/killing the adults using insecticides.

Table 1.1. Alternatives for DDT with active ingredients

No.	Active Ingredient	Product Type	WHO Class
1	*d*-allethrin (SP)	Mat, Coil	II
2	*d*-allethrin (Pynamine Fort) (SP)	Coil	II
3	*d-trans*-allethrin (SP)	Coil	II
4	Alpha Cypermethrin (SP)	WP, EC, SC	II
5	Bioallethrin (SP)	Aerosol	II
6	S-Bio-allethrin (SP)	Coil	II
7	Fenitrothion (OP)	EC	II
9	Imiprothin (SP)	Aerosol	III
10	Lambda Cyhalothrin (SP)	WP, EC	II
11	Malathion (OP)	EC	III
12	Permethrin (SP)	Aerosol, Powder, EC, WP, DP	II
13	Temephos (OP)	EC, G	U
14	Tetramethrin (SP)	RS, Aerosol	U
15	D-Tetramethrin (SP)	Aerosol	U
16	Transfluthrin (SP)	Coil	U
17	Chlorpyriphos (OP)	EC	II
18	Cypermethrin (SP)	EC	II
19	Deltamethrin (SP)	Chalk, EC, SC, WP, Flow, DP	II
20	Diazinon (OP)	EC	II
21	ETOC (Prallethrin) (SP)	Mat, Coil, Vaporizer	II
22	Fenthion (OP)	EC	II
23	Phenthoate (OP)	Liquid, EC	II
24	Pirimiphos Methyl (OP)	EC	III
25	Pynamine Fort (SP)	Liquid vaporizer	III
26	Prallethrin (SP)	Aerosol, Coil, Mat, Vaporizer	II
27	Propoxur (Car)	Aerosol	II
28	Sumione (SP)	Coil	II
29	Phenthoate (OP)	Liquid, EC	II
30	Pirimiphos Methyl (OP)	EC	III

*****WHO Class II specifies moderately hazardous (non-carcinogenic, non-teratogenic, etc.), WHO Class III specifies slightly hazardous and WHO Class U specifies unclassified ("SP- synthetic pyrethroid, OP-organophosphate, Car-carbamate.")

The frequent and routine use of chemical spraying has instigated alarm in the scientific community attributable to several investigations exposing the adverse effect on arthropod species, fish, wildlife and humans and several other non-target organisms (Desneux, Decourtye, & Delpuech, 2007). Moreover, the equally frightening adverse effects are the emergence of pesticide resistance (towards very low concentration) in insect populations all over the globe (de Azambuja Garcia et al., 2018).

An effective alternative to the afore-mentioned condition is the search for novel natural insecticides for mosquito control since they are easily available and biodegradable in nature. There are many defensive elements found in plants. The plant-based products contain various kinds of phytochemical constituents that exert toxic effects on the insect's population. The discovery of the antimalarial drug artemisinin (Fig. 1.1; Page No. 84) has verified this concept by altering the struggles to combat mosquito-borne diseases as a paradigm shift. Additionally, the phytochemical constituents from plants are also able to i) disrupt developmental processes including metamorphosis, ii) exert annoyance and disgusting effects and iii) promote morphological changes (Deletre et al., 2013).

Medicinal plants are recognized as the biochemical factories of the environment, synthesizing several phytochemical constituents including flavonoids, tannins, and alkaloids; most of them have pesticidal as well as therapeutic potential. The benefits of the natural insecticides constituted by blends of multiple bioactive phytochemical constituents are that they can act simultaneously on both behavioural as well as physiological processes (Pilaquinga et al., 2019). Hence,

prominent use of insecticides of botanical origin would offer a more effective and sustainable answer against the vector-borne diseases transmitted by *Aedes aegypti*. Succeeding this greener and harmless alternative approach, the medicinal plants have potential as a natural insecticide since it reduces the chance of emerging resistance (Oliveira et al., 2010; Pilaquinga et al., 2019).

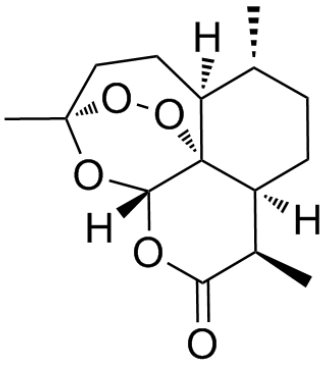


Fig. 1.1. Structure of Artemisinin

Several plant-based insecticides principally focus on the midgut of the vector mosquitoes in its larval stages, and some of them can prevent the distinct stages of development of an adult. The high efficacy and least expensive nature make the plant-based products an interesting prospect in integrated pest management (De Lima Santos et al., 2012). Therefore, in recent years, the use of biodegradable and eco-attractive insecticides of botanical origin has gained renewed attention for mosquito control. More than 2000 medicinal plants from the families Asteraceae, Oocystaceae, Miliaceae, Rutaceae, Cladophoraceae, Solanaceae and Labiatae have been known to synthesize metabolites and bioactive elements which have great significance in vector control programmes (Shaalan et al., 2005). Taken all together, the growing documentation of adverse impacts of synthetic insecticides with special reference to strict environmental regulation has brought about

renewed attention in the development of natural insecticides for mosquito control. Therefore, the present objective was intended to screen at least fifty locally available and traditionally important medicinal plants against *Aedes aegypti*, using organic solvents of increasing polarity.

1.2. MATERIALS AND METHODS

1.2.1. Study area and plant material

A total of 50 traditionally (Table 1.2; Page No. 95-107) used medicinal plants were collected on the basis of their medicinal potential, the possibility of commercialization, easy availability, and uncomplicated cultivation. It kept maximum attention that the fifty plant specimens were not endangered, and not threatened. The traditionally used medicinal plants were gathered from the neighbouring regions of Pothumoola, Thirunelly in Wayanad, that is part of the Western Ghats, Kerala, India and brought to CDRL in different polythene pouches from 18-11-16 to 04-04-17 (Fig. 1.2; Page No. 87). A herbarium was prepared and kept in Communicable Disease Research Laboratory, Department of Zoology, St. Joseph's College, Irinjalakuda, Kerala, India for future reference.

Different parts, mainly the bark and leaf, with the eminent fact of traditional use and non-toxicity to human population, were alienated from each plant and were carefully cleaned with water to remove dust or other waste particles stuck over their body. The plants selected for collection were observed sensibly with maximum care to discover any kind of infection or diseases and if any, such parts were removed and not used for further investigations. The collected plant specimens were kept at room temperature ($27 \pm 2°C$) for drying. The collected plants were identified to species level with the help of taxonomists. The GPS point of all the randomly collected traditionally used medicinal plants were taken

using the offline application software GPS info developed by Romuld Zdebskiy in Microsoft platform.

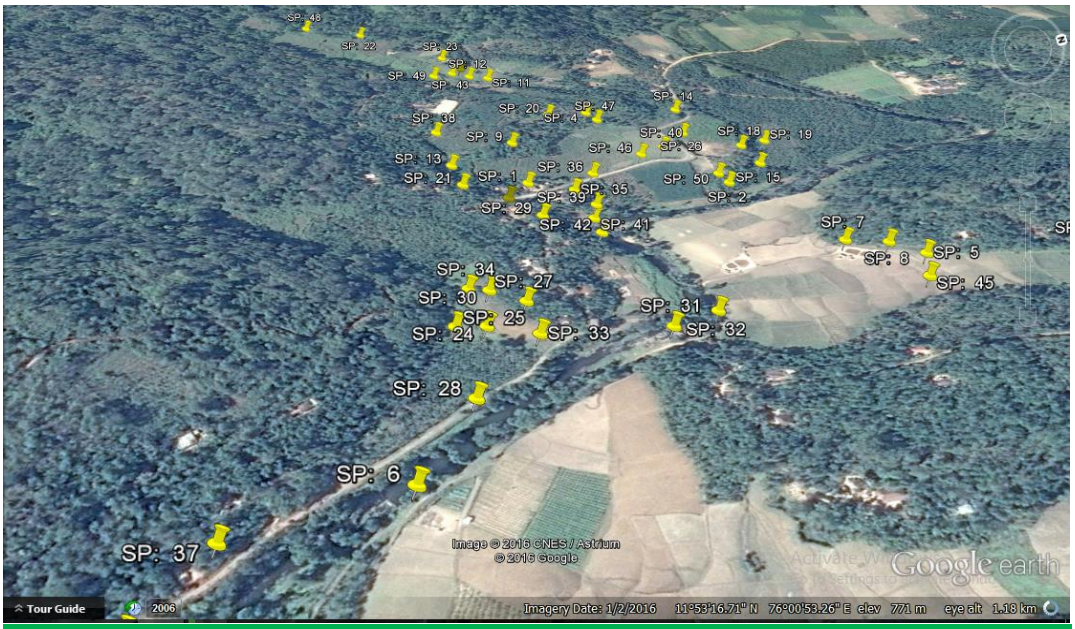


Fig. 1.2. Study site

Fig. 1.3. Shade dried plant specimens

1.2.2. Extraction

Soxhlet apparatus was used for extraction to get the non-polar bioactive elements. Twenty grams of shade dried (Fig. 1.3; Page No. 87) powder of plant specimens (sp:1 to sp:50) which contains the bioactive elements to be extracted was placed in

a thimble. The thimble is usually made up of filter paper material that allows liquids and essential contents to pass through it. The 250 mL of each solvent such as petroleum ether, methanol, acetone, and water was added to the round bottomed flask of the apparatus. The aforementioned organic solvents of increasing polarity were heated at specific boiling temperatures for 6 to 12 hours and this process initiates the movement of bioactive elements into the condenser. Once the level of the solvent comprising bioactive elements reaches the siphon, it pours back into the round bottomed flask. The process of extraction was repeated continuously until all the ingredients from the plant specimen are extracted into the organic solvents (Fig. 1.4; Page No. 89). The extracts obtained from the afore-mentioned step were concentrated using a rotary evaporator and kept at 4ºC for further studies such as bio-active guided fractionation and chromatographic analysis.

1.2.3. Experimental insect and larvicidal bio assay

Aedes aegypti maintained using standard rearing techniques at Communicable Disease Research Laboratory, Department of Zoology, St Joseph's College, Irinjalakuda were used for executed experiments. The larvicidal bioassays were performed as per WHO protocol. The petroleum ether, methanol, acetone and water extracts of fifty medicinal plants as listed in Table 1.2 (Page No. 95-107) were tested against *Aedes aegypti* to determine their larvicidal potential. Twenty-five early fourth instar larvae of *Aedes aegypti* were taken and put on the glass beaker containing 99 mL of distilled water and 1000 mg/L of each plant extract. Four replicates were maintained for all executed experiments. Control groups were also maintained using each solvent alone. After continuous exposure to 24

hours, the number of moribund and dead larvae were recorded as larval mortality. The mortality was determined by spotting the movement of *Aedes aegypti* larvae after 24 hours of continuous exposure. The death of larvae were confirmed when they showed no signs of movement. The larvae were recognized as moribund if they showed a little bit movement but did not exhibit any kind of swimming like movement. The plant specimens that failed to deliver a significant range of toxicity were no longer used for bio-assay guided chromatographic fractionation.

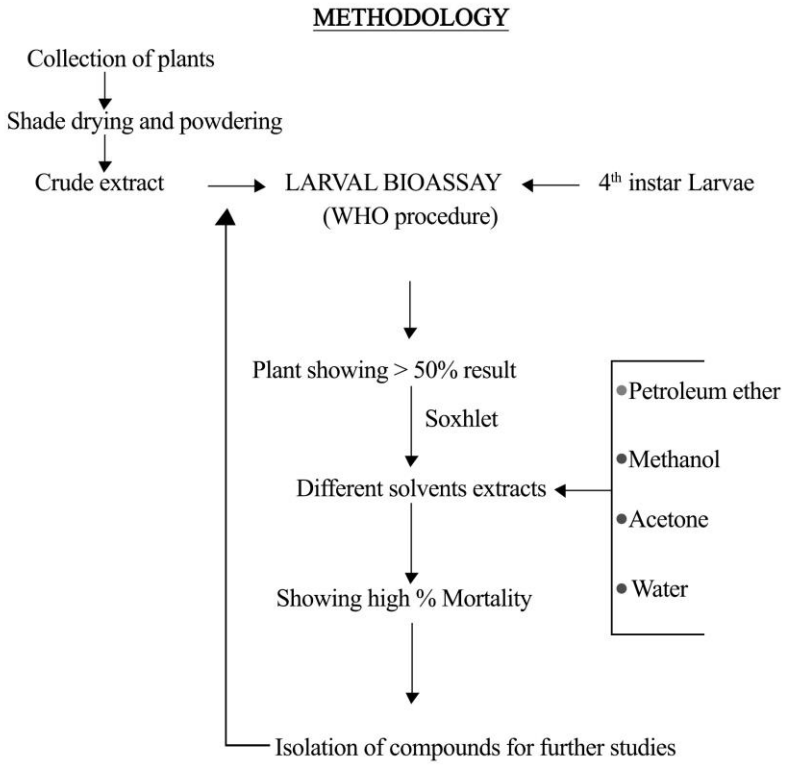


Fig. 1.4. Methodology involved in extraction and screening

1.2.4. Statistical analysis

All data were entered and systemized in Microsoft Excel 2010 and 2019 spreadsheet. The data were then exported into SPSS version 24.0.0 and R software version 3.2.3 to find out the percentage of toxicity exhibited (MEAN ±

SD) by the 50 medicinal plants against *Aedes aegypti*. The pictorial charts were also prepared using the afore-mentioned software. The data on corrected mortality rates were calculated as the mean percentage ± Standard Deviation (SD) for each bio-assay. The graphs were plotted with the SPSS version 24.0.0 for Windows Operating Systems.

1.3. RESULTS

In this objective, 1000 mg/l of the petroleum ether, methanol, acetone and water extracts of fifty traditionally used medicinal plants, collected from Pothumoola, Thirunelly in Wayanad, Kerala, India were tested against early fourth instar larvae of *Aedes aegypti*. The mean percentage of mortality recorded after 24 hours of continuous exposure have revealed that out of 50 plants (Figs. 1.5-1.10; Page No. 108-113.), 21 plants exhibit more than 41% of toxicity, 4 plants show 31-40% of toxicity, 8 plants shows 20-30% of mortality and 11 plants exhibited 10-19% of mortality.

The details of the medicinal plants collected, their scientific name, habitat, plant part used, date of collection, GPS (Latitude and Longitude), date of experiment, solvents used, followed by the percentage of mortality is presented in Table 1.2 (Page No. 95-107). The result revealed that the acetone extract made from the *Euphorbia thymifolia* (Linnaeus, 1753) (leaf) (Sp: 12) and *Catunaregam spinosa* (Thumb, 1978) (seeds) (Sp: 15) were the most potent extract exhibiting 96% and 92% respectively. The petroleum ether (90.66±2.31), methanol (92.00±0.0) and water (96.00±0.00) extracts of *Euphorbia thymifolia* also showed potent toxicity against the *Aedes aegypti* larvae. Similarly, the various extracts prepared from the *Catunaregam spinosa* (seeds) (Sp: 15) provided significant toxicity towards the *Aedes aegypti* population. The petroleum ether (92.00±4.00) and aqueous extracts (90.66±2.30) prepared using the seeds of *Catunaregam spinosa* have shown a prominent range of toxicity against *Aedes aegypti*. The methanol (90.66±2.30)

and acetone (78.66±2.31) extracts of the same have shown a relatively considerable range of toxicity against *Aedes aegypti*.

In addition, the acetone extract made from the *Jasminum brevilobum* (Sp: 13) and *Aglaia edulis* (Sp: 20) were considered as effective with 85% and 81% of toxicity respectively. It was also perceived that the methanol, water and acetone extracts made from *Cardiospermum halicacabum* (Sp: 32), *Persicaria hydropiper* (Sp: 46) and *Pogostemon auricularius* (Sp: 47) possessed the effective larvicidal potential of 73%, 76% and 76% respectively against *Aedes aegypti*. The other extracts made from the afore-mentioned medicinal plants (Sp: 13, Sp: 20, Sp: 32, Sp: 46 and Sp: 47) have also exhibited a great range of toxicity. The petroleum ether, water and acetone extracts prepared from *Cardiospermum halicacabum* (Sp: 32) showed 63% (63.33±4.16), 68% (68.00±4.00), 63% (63.33±4.16) of larvicidal potential respectively against *Aedes egypti* larvae. The petroleum ether, methanol and water extracts prepared from the *Persicaria hydropiper* leaf provided 54%, 65%, and 76% of larvicidal potential respectively.

The leaf extract of *Glycosmis pentaphylla* (Sp: 6) *Ageratum conyzoides* (Linnaeus, 1753) (Sp: 10) and *Plectranthus hadiensis* (Sp: 31) was found to be effective with a great range of toxicity from 60% to 63%. Among the four different types of extracts prepared from *Glycosmis pentaphylla* (Sp: 6), the acetone (62.66±2.31) extract is recognized as most effective than others. However, the other extracts of *Glycosmis pentaphylla* also showed a considerable range of toxicity (methanol: 36.00±6.93, petroleum ether: 52.00±0.0, water: 53.33±2.31). The water and methanol extracts prepared from the *Plectranthus hadiensis* leaf have exhibited 63

% (63.33±4.16) and 61 % (61.33±1.15) respectively. The acetone extract of the same has relatively showed a great range of toxicity (50.66±2.31). However, its petroleum ether extract is recognized as the least effective.

Likewise, the petroleum ether and water extract made from the *Mimosa pudica* (Linnaeus, 1753) (Sp: 5) and *Sphaernthus indicus* (Linnaeus, 1753) (Sp: 45) exhibited 54% (54.66±16.16) and 52% (52.00±4.00) of mortality respectively. The medicinal plants such as *Asclepias curassavica* (Linnaeus, 1753) (Sp:2), *Jatropha curcas* (Sp: 14), *Toddalia asiatica* (Sp: 22), *Scoparia dulcis* (Linnaeus, 1753) (Sp:30), and *Thottea siliquosa* (Sp:48) have exhibited a prominent range of larvicidal potential from the range of 50% to 60%. The petroleum ether and water extract prepared from the *Asclepias curassavica* exhibited similar results (50.66±2.31). Among the four extracts prepared from the *Jatropha curcas*, the methanol (53.33±2.31) extract is recognized as the most prominent one than others. Likewise, the water extract of *Scoparia dulcis* showed potential toxicity (52.66±5.03). The *Thottea siliquosa* methanol (51.33±1.15) extract also showed potent toxicity, however, the petroleum ether (43.33±5.03), acetone (43.33±5.03), and water (41.33±2.31) extracts prepared from the same have recognized as less active than the former one.

In addition, the other various extracts of the aforementioned medicinal plants exhibited potent toxicity towards the dengue fever vector *Aedes aegypti*. However, some of the other plant species are considered as least effective since they exhibit the larvicidal potential of below 10%. The medicinal plant *Tamilnadia utiginosa* (Retz, 1979) (Sp: 19) does not exhibited potent toxicity.

Based on the great percentage mortality exhibited by the medicinal plants, the conservational status, their easy availability, commercial probability and cultivation status, three plant specimens such as *Jasminum brevilobum* (Sp: 13), *Aglaia edulis* (Sp:20), and *Pogostemon auricularius* (Sp: 47) were selected for bioassay-guided chromatographic fraction. The petroleum ether extract made from *Jasminum brevilobum* (Sp: 13) showed 73% of mortality (77.33±2.31) whereas its methanol and water extract possessed 62% (62.66±2.31) and 74% (74.66±2.31) respectively. Similarly, the petroleum ether, methanol and water extracts made from the *Aglaia edulis* (Sp: 20) exhibited potent toxicity ranging from 62% to 69%. The *Pogostemon auricularius* (Sp: 47) petroleum ether extract exhibited the least larvicidal potential (59.33±1.15) than the methanol (63.33±9.86), and water (72.00±4.00), and acetone (76.00±3.46) extracts. The maximum toxicity provided by the three medicinal plants such as *Jasminum brevilobum* (Sp: 13), *Aglaia edulis* (Sp:20), and *Pogostemon auricularius* (Sp: 47) made them effective larvicides against *Aedes aegypti*; hence, these specimens have been used for further investigations.

Table 1.2. List of plant specimens collected and screened against *Aedes aegypti* fourth instar larvae

SP. No.	Local name	Scientific name	Herbarium-Reference Number	Family	Habitat	Plant part used	Date of collection	GPS		Date of expt.	Solvents used	Percentage of Mortality Mean ± SD
								Latitude	Longitude			
1.	Thoppipoov	*Asystasia gangetica* (Linnaeus, 1753)	Hb/CDRL/16-044	Acanthaceae	Paddy field	Leaf	01/04/16 02/04/16 10/06/16	11˚53ʹ21.48ʺN	76˚0ʹ52.03ʺE	18/11/16	Petroleum ether Methanol Acetone Water	14.66±2.31 18.66±2.31 13.33±2.31 12.00±0.0
2.	Kammal poov	*Asclepias curassavica*	Hb/CDRL/16-045	Apocynaceae	Paddy field	Leaf	05/04/16 31/04/16 10/06/16	11˚53ʹ28.46ʺN	76˚0ʹ53.45ʺE	19/11/16	Petroleum ether Methanol Acetone Water	50.66±2.31 44.00±0.0 46.66±2.31 50.66±2.31
3.	Janglimulli	*Blumea lacera* (Burm.f, 1834)	Hb/CDRL/16-046	Asteraceae	Paddy field	Leaf	06/04/16 25/04/16	11˚53ʹ35.63ʺN	76˚0ʹ1.83ʺE	21/11/16	Petroleum ether Methanol Acetone Water	40.66±1.15 42.66±231 48.00±0.0 41.33±2.31

4.	Vendak	*Lagerstroemiamicrocarpa* (Wight, 1839)	Hb/CDRL/16-047	Lythraceae	Land	Leaf	10/04/16 12/06/16	11˚53′25.45″ N	76˚0′46.37″E	24/11/16	Petroleum ether Methanol Acetone Water	36.00±0.0 36.00±0.0 33.33±2.31 38.66±2.31
5.	Tottavadi	*Mimosa pudica*	Hb/CDRL/16-048	Fabaceae	Paddy field	Leaf	11/04/16 12/06/16	11˚53′31.40″N	76˚0′0.58″E	19/10/16	Petroleum ether Methanol Acetone Water	54.66±16.16 42.00±0.0 48.00±0.0 50.66±2.31
6.	Paanal	*Glycosmis pentaphylla*	Hb/CDRL/16-049	Rutaceae	Land	Leaf	11/04/16 14/06/16	11˚53′15.41″N	76˚0′3.15″E	26/11/16	Petroleum ether Methanol Acetone Water	52.00±0.0 36.00±6.93 62.66±2.31 53.33±2.31
7.	White	*Heliotropium indicum*	Hb/CDRL/16-050	Boraginaceae	Paddy field	Leaf	16/04/16 14/06/16	11˚53′29.80″N	76˚0′58.82″E	29/11/16	Petroleum ether Methanol Acetone Water	32.00±4.00 29.33±2.31 53.33±2.31 21.33±2.31

8.	Appa	*Ageratum conyzoides*	Hb/CDRL/16-051	Asteraceae	Land	Leaf	16/04/16 28/04/16 18/06/16	11˚53'30.87" N	76˚0'59.49" E	06/12/16	Petroleum ether Methanol Acetone Water	21.33±2.31 25.33±4.62 25.33±2.31 26.66±2.31
9.	Kadalari	*Cyanthillium cinereum* (Linnaeus, 1753)	Hb/CDRL/16-052		Land	Leaf	17/04/16 18/06/16	11˚53'21.78"N	76˚0'48.84"E	09/12/17	Petroleum ether Methanol Acetone Water	12.00±0.0 12.00±0.0 10.66±2.31 10.66±2.31
10.	Nherinjil	*Acanthospermum hispidum* (DC, 1836)	Hb/CDRL/16-053		Land	Leaf	19/06/16	11°53'27.80"N	76° 047'80"E	11/12/17	Petroleum ether Methanol Acetone Water	12.00±0.00 12.00±2.31 10.66±4.62 12.00±0.00
11.	Muyalcheviyan	*Emilia sonchifolia* (Wight, 1834)	Hb/CDRL/16-054		Paddy field	Leaf	03/05/16 04/05/16 19/06/16	11˚53'22.50"N	76˚0'41.63"E	14/12/17	Petroleum ether Methanol Acetone Water	10.66±2.31 12.00±0.0 12.00±0.0 10.66±2.31

12.	Mookkilpodi cheruth	*Euphorbia thymifolia*	Hb/CDRL/16-055	Euphorbiaceae	Paddy field	Leaf	22/04/16 19/06/16	11˚53′21.74″N	76˚0′41.35″E	16/12/17	Petroleum ether Methanol Acetone Water	90.66±2.31 92.00±0.0 96.00±0.0 90.66±2.31
13.	Kaattumulla	*Jasminum brevilobum*	Hb/CDRL/16-056	Oleaceae	Land	Leaf	23/04/16 24/06/16	11°53'22.40″N	76° 0'52.10"E	10/01/17	Petroleum ether Methanol Acetone Water	77.33±2.31 62.66±2.31 85.33±4.62 74.66±2.31
14.	Kadalavanakk	*Jatropha curcas*	Hb/CDRL/16-057	Euphorbiaceae	Land	Leaf	04/05/16 05/05/16 24/06/16	11˚53′29.24″N	76˚0′46.55″E	11/01/17	Petroleum ether Methanol Acetone Water	49.33±4.62 53.33±2.31 44.00±4.00 49.33±2.31
15.	Kattukarakka	*Catunaregam spinosa*	Hb/CDRL/16-058	Rubiaceae	Land	seed	07/05/16 08/05/16 09/05/16	11˚53′30.25″N	76˚0′52.25″E	13/01/17	Petroleum ether Methanol Acetone Water	92.00±4.00 76.00±4.00 78.66±2.31 90.66±2.30

16.	Kanikkonna	*Cassia fistula*	Hb/CDRL/16-059	Fabaceae	Land	Bark	12/05/16 30/06/16	11˚53′20.53″N	76˚0′53.43″E	27/01/17	Petroleum ether Methanol Acetone Water	12.00±0.0 10.66±2.31 12.00±0.0 13.33±2.31
17.	Kanjunni	*Eclipta prostrata*	Hb/CDRL/16-060	Asteraceae	Land	Leaf	01/07/16	11˚53′21.62″N	76˚0′40.00″E	28/01/17	Petroleum ether Methanol Acetone Water	12.00±0.0 17.33±4.62 13.33±2.31 12.00±0.0
18.	Alatta	*Sida acuta* (Burm.f., 1768.)	Hb/CDRL/16-061	Malvaceae	Land	Leaf	02/07/16	11˚53′30.30″N	76˚0′50.55″E	30/01/17	Petroleum ether Methanol Acetone Water	26.00±2.00 21.33±2.31 12.00 14.66±4.12
19.	Kaattukarakka valuth	*Tamilnadia utiginosa*	Hb/CDRL/16-062	Rubiaceae	Land	Leaf	02/07/16	11˚53′31.37″N	76˚0′50.35″E	9/02/17	Petroleum ether Methanol Acetone Water	0 0 0 0

20.	Kalikutty	*Aglaia edulis*	Hb/CDRL/16-063	Meliaceae	Land	Leaf	04/07/16	11°53'22.60"N	76° 0'56.30"E	12/02/17	Petroleum ether Methanol Acetone Water	62.66±2.30 58.66±2.31 81.33±2.30 69.33±2.30
21.	Valapan	*Derris trifoliate* (Lour, 1793)	Hb/CDRL/16-064	Fabaceae	Land	Leaf	06/07/16	11˚53'19.23"N	76˚0'52.26"E	14/02/17	Petroleum ether Methanol Acetone Water	26.66±2.31 5.33±2.31 12.00 8.00
22.	Kattu Naranga	*Toddalia asiatica* (Linnaeus, Lam, 1797)	Hb/CDRL/16-065	Rutaceae	Land	Leaf	10/07/16	11˚53'17.80" N	76˚0'34.88"E	17/02/17	Petroleum ether Methanol Acetone Water	38.66±2.31 33.33±2.31 46.66±2.31 50.66±2.31
23.	Kattuchemb	*Piper methysticum* (Forst, 1786)	Hb/CDRL/16-066	Piperaceae	Land	Leaf	15/07/16 13/08/16	11˚53'21.09"N	76˚0'38.36"E	21/02/17	Petroleum ether Methanol Acetone Water	18.66±6.11 13.33±2.31 16.00±6.93 12.00±0.0

24.	Payachedy	*Verbena urticifolia* (Linnaeus, 1753)	Hb/CDRL/16-067	Verbenaceae	Land	Leaf	16/07/16	11˚53′18.14″N	76˚0′0.70″E	22/02/17	Petroleum ether Methanol Acetone Water	6.66±2.31 5.33±2.31 5.33±2.31 6.66±2.31
25.	Vellila	*Mussaenda frondosa* (Linnaeus, 1753)	Hb/CDRL/16-068	Rubiaceae	Land	Leaf	18/07/16 06/08/16	11˚53′17.31″N	76˚0′0.70″E	24/02/17	Petroleum ether Methanol Acetone Water	21.33±1.15 24.00±0.0 25.33±2.31 23.33±1.15
26.	Paimbala	*Hyptis saveolens* (Linnaeus, 1759)	Hb/CDRL/16-069	Lamiaceae	Land	Leaf	20/07/16 06/08/16	11˚53′28.60″N	76˚0′48.91″E	25/02/17	Petroleum ether Methanol Acetone Water	41.33±1.15 41.33±1.15 46.66±2.31 44.00±3.46
27.	Kilukki	*Crotalaria pallida* (Aiton, 1789)	Hb/CDRL/16-070	Fabaceae	Land	Leaf	24/07/16	11˚53′19.50″N	76˚0′59.61″E	27/02/17	Petroleum ether Methanol Acetone Water	8.00±2.31 4.00±0.00 4.00±0.00 8.00±1.15

28.	Ilatha	*Persicaria virginiana* (Linnaeus, 1753)	Hb/CDRL/16-071	Polygonaceae	Land	Leaf	15/05/16 28/07/16	11˚53′17.23″N	76˚0′3.62″E	01/03/17	Petroleum ether Methanol Acetone Water	12.00±0.0 12.00±0.0 21.00±2.31 14.66±4.62
29.	Avanakk	*Justicia adhatoda* (Linnaeus, 1753)	Hb/CDRL/16-072	Acanthaceae	Land	Leaf	01/08/16	11˚53′21.28″N	76˚0′54.89″E	02/03/17	Petroleum ether Methanol Acetone Water	12.00±0.0 14.66±4.62 12.00±0.0 12.00±0.0
30.	Kallurukki	*Scoparia dulcis*	Hb/CDRL/16-073	Plantaginaceae	Paddy field	Leaf	04/08/16	11˚53′18.62″N	76˚0′58.88″E	03/03/17	Petroleum ether Methanol Acetone Water	44.00±4.00 35.33±3.05 39.33±3.05 52.66±5.03
31.	Bhaya	*Plectranthus hadiensis* (Forssk, 1894)	Hb/CDRL/16-074	Lamiaceae	Land	Leaf	08/08/16	11˚53′22.95″N	76˚0′1.82″E	05/03/17	Petroleum ether Methanol Acetone Water	36.00±3.46 61.33±1.15 50.66±2.31 63.33±4.16

32.	Marunnevalli	*Cardiospermum halicacabum* (Linnaeus, 1753)	Hb/CDRL/16-075	Sapindaceae	Land	Leaf	16/05/16 10/08/16	11˚53′24.42″N	76˚0′1.40″E	06/03/17	Petroleum ether Methanol Acetone Water	63.33±4.16 73.33±2.31 63.33±4.16 68.00±4.00
33.	Kattupukayila	*Lobelia nicotianifolia* (Linnaeus, 1753)	Hb/CDRL/16-076	Campanulaceae	Land	Leaf	16/08/16 01/09/16	11˚53′19.44″N	76˚0′1.23″E	08/03/17	Petroleum ether Methanol Acetone Water	42.66±2.31 45.33±4.62 37.33±2.31 45.33±6.11
34.	Kandonekkuthy	*Bidens pilosa* (Linnaeus, 1753)	Hb/CDRL/16-077	Asteraceae	Land	Leaf	19/08/16 02/09/16	11˚53′18.05″N	76˚0′58.80″E	09/03/17	Petroleum ether Methanol Acetone Water	26.66±8.32 28.00±4.00 20.00±0.0 40.00±6.93
35.	Paranga	*Ficus hispida* (Linné, Carl von, 1782)	Hb/CDRL/16-078	Moraceae	Land	Leaf	20/08/16 03/09/16	11˚53′22.95″N	76˚0′52.93″E	10/03/17	Petroleum ether Methanol Acetone Water	40.00±0.0 21.33±2.31 41.33±2.31 14.66±4.12

36.	Chunda	*Solanum Torvum* (Sw, 1788)	Hb/CDRL/16-079	Solanaceae	Land	Leaf	28/08/16	11˚53′24.03′′N	76˚0′51.64′′E	11/03/17	Petroleum ether Methanol Acetone Water	6.00±0.6.11 4.33±4.00 5.33±0.00 6.00±1.15
37.	Chinungi	*Impatiens glandulifera* (Royle, 1835)	Hb/CDRL/16-080	Balsaminaceae	Land	Leaf	28/08/16	11˚53′11.48′′N	76˚0′7.06′′E	13/03/17	Petroleum ether Methanol Acetone Water	10.66±2.31 20.66±7.57 8.00±4.00 14.66±4.62
38.	Velippon	*Conyza Canadensis* (Linnaeus, 1753)	Hb/CDRL/16-081	Asteraceae	Paddy field	Leaf	06/09/16 07/09/16	11˚53′19.16′′N	76˚0′47.65′′E	15/03/17	Petroleum ether Methanol Acetone Water	8.00±0.0 9.33±2.31 9.33±2.31 8.00±0.0
39.	Malabar kino	*Pterocarpus marsupium* (Roxb, 1798)	Hb/CDRL/16-082	Fabaceae	Land	Bark	08/09/16 17/09/16 18/09/16	11˚53′23.35′′N	76˚0′54.10′′E	16/03/17	Petroleum ether Methanol Acetone Water	35.33±3.05 32.00±0.00 14.66±4.62 30.66±6.11

40.	Nerrori cheruth	*Stachytarpheta jamaicensis* (Linnaeus, 1753)	Hb/CDRL/16-083	Verbenaceae	Land	Leaf	09/09/16 18/09/16 19/09/16	11˚53ʹ27.36ʺ N	76˚0ʹ49.97ʺE	18/03/17	Petroleum ether Methanol Acetone Water	14.66±4.62 17.33±4.62 34.00±2.00 21.33±2.31
41.	Nanhuli	*Cassia alata* (Linnaeus, 1753)	Hb/CDRL/16-084	Fabaceae	Land	Leaf	15/09/16	11˚53ʹ22.93ʺ N	76˚0ʹ55.14ʺE	19/03/17	Petroleum ether Methanol Acetone Water	35.33±1.15 46.66±2.31 42.66±2.31 48.00±0.0
42.	chutakkarpuram	*Cinnamomum camphora* (Linnaeus, 1753)	Hb/CDRL/16-085	Lauraceae	Land	Leaf	16/09/16	11˚53ʹ22.84ʺ N	76˚0ʹ56.18ʺE	21/03/17	Petroleum ether Methanol Acetone Water	29.33±4.62 12.00±0.0 12.00±0.0 12.00±0.0
43.	Mookkilpodi Valuth	*Hypericum japonicum* (Thunb, 1784)	Hb/CDRL/16-086	Hypericaceae	Paddy field	Leaf	16/09/16	11˚53ʹ21.08ʺ N	76˚0ʹ40.86ʺE	22/03/17	Petroleum ether Methanol Acetone Water	90.66±2.31 84.66±1.15 88.00±0.0 96.00±0.0

44.	Menthonni	*Gloriosa superba* (Linnaeus, 1753)	Hb/CDRL/16-087	Colchicaceae	Land	Leaf	22/09/16 29/09/16	11˚53′9.98″N	76˚0′8.79″E	24/03/17	Petroleum ether Methanol Acetone Water	24.00±0.0 14.66±4.62 12.00±0 20.00±0.0
45.	Adakkamaniyan	*Sphaernthus indicus*	Hb/CDRL/16-088	Asteraceae	Paddy field	Seed	26/09/16	11˚53′30.67″N	76˚0′1.79″E	26/03/17	Petroleum ether Methanol Acetone Water	37.33±1.15 53.33±2.31 48.00±4.00 52.00±4.00
46.	Kovvanenji	*Persicaria hydropiper*	Hb/CDRL/16-089	Polygonaceae	Paddy field	Leaf	27/09/16 01/10/16	11˚53′26.33″N	76˚0′50.83″E	26/03/17	Petroleum ether Methanol Acetone Water	54.00±3.46 65.33±6.11 72.66±5.03 76.66±1.15
47.	Njandukanni	*Pogostemon auricularius* (Linnaeus, Hassk, 1843)	Hb/CDRL/16-090	Lamiaceae	Paddy field	Leaf	02/10/16	11°53'25.30"N	76° 0'47.30"E	28/03/17	Petroleum ether Methanol Acetone Water	59.33±1.15 63.33±9.86 76.00±3.46 72.00±4.00

48.	Kaipa	*Thottea siliquosa* (Lam, 1981)	Hb/CDRL/16-091	Aristolochiaceae	Land	Leaf	04/10/16	11˚53ʹ15.37ʺ N	76˚0ʹ33.64ʺE	01/04/17	Petroleum ether Methanol Acetone Water	43.33±5.03 51.33±1.15 42.66±2.31 41.33±2.31
49.	Cheroola	*Aerva lanata* (Linnaeus, 1758)	Hb/CDRL/16-092	Amaranthaceae	Land	Leaf	08/10/16 15/10/16	11˚53ʹ20.18ʺ N	76˚0ʹ41.48ʺE	02/04/17	Petroleum ether Methanol Acetone Water	39.33±4.16 41.33±2.31 35.33±1.15 44.00±0.0
50.	Tharakeera	*Spermacoce ocymoides* (Burm.f., 1768)	Hb/CDRL/16-093	Rubiaceae	Paddy field	Leaf	15/10/16 18/10/16	11˚53ʹ28.43ʺ N	76˚0ʹ52.63ʺE	04/04/17	Petroleum ether Methanol Acetone Water	12.00±0.0 14.66±4.62 13.33±2.31 9.33±2.31

*statistical significance ($P < 0.05$)

Fig. 1.5. Plant specimens collected and screened against *Aedes aegypti* fourth instar larvae (SP:1 to SP:9)

Fig. 1.6. Plant specimens collected and screened against *Aedes aegypti* fourth instar larvae (SP:10 to SP:18). Cont.

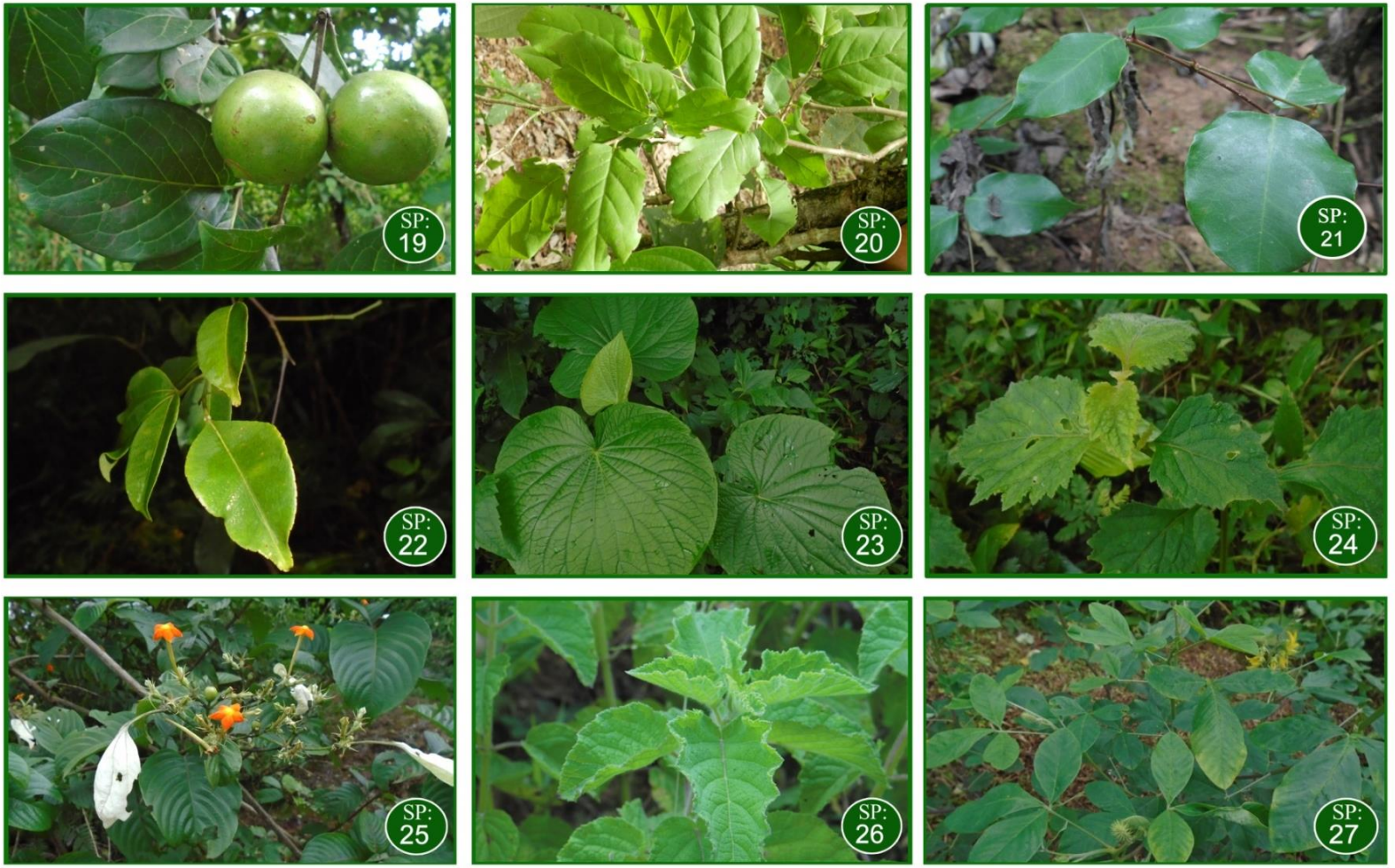


Fig. 1.7. Plant specimens collected, screened against *Aedes aegypti* fourth instar larvae (SP:19 to SP:27). Cont.

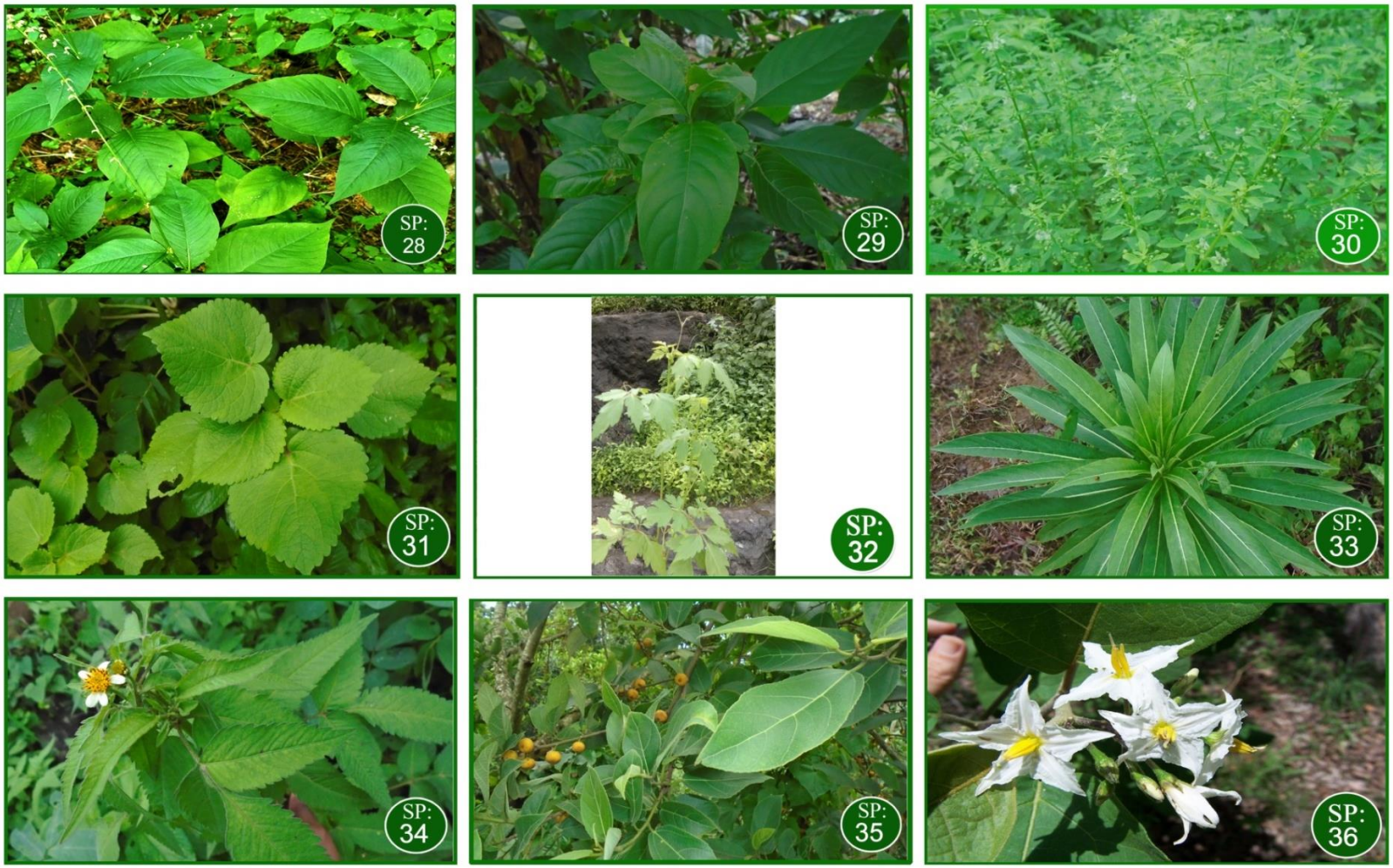


Fig. 1.8. Plant specimens collected and screened against *Aedes aegypti* fourth instar larvae (SP:28 to SP:36). Cont.

Fig. 1.9. Plant specimens collected and screened against *Aedes aegypti* fourth instar larvae (SP:37 to SP:45). Cont.

Fig. 1.10. Plant specimens collected and screened against *Aedes aegypti* fourth instar larvae (SP:46 to SP:50).

1.4. DISCUSSION

The findings of this investigation have exposed that the various phytochemical constituents from fifty traditionally used medicinal plants could be a pioneering application for the development of novel insecticides of botanical origin that could be considered as an alternative to synthetic insecticides in future. Diverse medicinal plants used in this study were as potent against *Aedes aegypti* as described in the previous study (Anoopkumar, Puthur, Rebello, & Aneesh, 2017b; Puthur, Anoopkumar, Rebello, & Aneesh, 2018). The use of traditionally used medicinal plants not only extensively augment the larvicidal potential against mosquitoes but also reduces the chance of insecticide resistance (Anoopkumar et al., 2017b). This study determined that most of all the traditional medicinal plants are effective to kill off fourth instar larvae of *Aedes aegypti*. The high larvicidal potential and low cost make medicinal plants an interesting concern for vector control programmes (Procópio et al., 2015). Therefore, this study can provide significant information on the larvicidal potential of traditionally used medicinal plants with special reference to their phytochemical constituents with the polar features.

As described in Table 1.2 (Page No. 95-107), the various extracts of the medicinal plant used in this study have exhibited effective larvicidal potential against *Aedes aegypti*. The extracts of medicinal plants prepared using organic solvents of increasing polarity have revealed that the same plant species has a different range of toxicity in different solvents. For instance, the acetone extract (1 mL) prepared

from the *Jasminum brevilobum* leaf exhibited 85% of larvicidal potential (85.33±4.62), whereas the petroleum ether (1 mL) (77.33±2.31), methanol (1 mL) (62.66±2.31) and water (1 mL) (74.66±2.31) extract prepared from the same plant has exhibited considerably less toxicity than acetone extract. One of the probable reasons for this condition is the critical difference in polarity (Preethi et al., 2014). The polarity index and type of solvents are already known to have a significant role in toxic effects (Do et al., 2014), therefore, the percentage of toxicity between the afore-mentioned extracts has greatly varied. The same pattern was observed in the traditionally used medicinal plants such as *Blumea lacera*, *Glycosmis pentaphylla*, *Heliotropium indicum* (Linnaeus, 1753), *Euphorbia thymifolia*, *Aglaia edulis*, *Hyptis saveolens*, *Persicaria virginiana*, *Stachytarpheta jamaicensis*, and *Pogostemon auricularius*. The aforementioned concepts are akin to early reported findings (Rafińska et al., 2019; Wakeel, Jan, Ullah, Shinwari, & Xu, 2019) who described the significance of polarity index in the quality and quantity of plant extracts and various biological activities influenced by secondary metabolites. This indicates that the various bioactive elements have different polarity index; can be extracted with a solvent encompassing specific polarity index. Additionally, among the fifty medicinal plants tested, 22 plants could be more potent than others. Among them, based on the diversity, easy availability, and non-toxic nature towards the human population, we found that the three traditionally used medicinal plants such as *Jasminum brevilobum* (Sp: 13), *Aglaia edulis* (Sp: 20), and *Pogostemon auricularius* (Sp: 47) can be used as effective larvicides against *Aedes aegypti*.

The results from this study were similar to a study concerning the larvicidal potential of *Jasminum* sp. (Oleaceae) against the dengue fever vector *Aedes aegypti* by (Preethi et al., 2014). They verified the larvicidal potential of the flower extracts of *Jasminum grandiflorum, Jasminum officinale,* and *Jasminum auriculatum*. Previous studies have mentioned that the preliminary screening of various extracts is a good means of assessing the mosquitocidal potential (Tennyson, Ravindran, & Arivoli, 2012). Studies on the larvicidal potential of *Jasminum grandiflorum, Jasminum officinale,* and *Jasminum auriculatum* on mosquitoes were performed with effective results, specifying the occurrence of larvicidal elements in these plants. Previous studies have reported the toxic effects of various phytochemical constituents such as Hexadecanoic acid, Eicosanoic acid, Linolenic acid, Linoleoyl chloride, (2E, 6E)-farnesyl benzoate, Hexatriacontane, Benzyl linoleate, Squalene, Heptacosane, Hentriacontane, Phenylethanolamine, Cinnamic acid, and 3,7,11-trimethyl-3-hydroxy-6,10-dodecadien-1-yl acetate isolated from *Jasminum fluminense*. The aforementioned compounds act as potent toxicants against both adult and larval stages of mosquito vectors, while some of them act as repellents or growth inhibitors (Arivoli et al., 2018). They also suggest that the phytochemical compounds gained from *Jasminum fluminense* can serve as effective tools for the management of vector mosquitoes. Uniyal et al. (2016) reported that the compound Jasmine is the predominant phytochemical constituent in the *Jasminum grandiflorum* (Oleaceae). Moreover, the results of this study corroborate with previous reports of *Aglaia odorata* leaf extracts yielded bioactive elements against *Aedes aegypti* (Nugroho et al., 1999). They have also reported the presence of four new insecticidal

derivatives. The effective larvicidal potential of various extracts of *Aglaia edulis* against *Aedes aegypti*, as verified in the present study, could provide a route to the development of natural insecticides against *Aedes aegypti*. The observations from the present study is in agreement with previous reports of larvicidal and insecticidal potential of other *Aglaia* sp. including *Aglaia elaeagnoidea*, *Aglaia odorata*, *Aglaia malabarica*, *Aglaia elliptifolia* and *Aglaia rubiginosa* (Benelli et al., 2018; Komalamisra, Trongtokit, Rongsriyam, & Apiwathnasorn, 2005; Koul, Singh, Singh, & Multani, 2005; Tawatsin et al., 2006). The aforementioned findings uphold the results gained in this study that, the *Aglaia edulis* act a potent larvicide against *Aedes aegypti* population. The reported results of this study concerning the larvicidal potential of *Aglaia edulis* are far by far prominent than those studied by others in *Aedes* sp. The non-toxicity to human beings, biodegradable nature, effective larvicidal potential and therapeutic benefits exhibited by *Aglaia edulis* are recognized as additional benefits to vector control.

Regarding the toxicity of plant extracts against *Aedes* sp, several studies pointing on the exploration of phytochemical constituents based larvicidal potential indicated that *Pogostemon cablin*, *Pogostemon heyneanus*, and *Pogostemon parviflorus* are effective (Gokulakrishnan, Kuppusamy, Shanmugam, Appavu, & Kaliyamoorthi, 2013). In the present study, for the first time, the larvicidal potential of various extracts prepared from the *Pogostemon auricularius* leaf against *Aedes aegypti* was reported. As suggested by Gokulakrishnan et al. (2013), the use of bioactive elements (β-Eudesmol, Elemol, Carotol, and Patchoulol) from *Pogostemon cablin*, *Daucus carota*, and *Amyris balsamifera* could be an alternative tool for vector control management. Therefore, the

medicinal plant *Pogostemon auricularius* can be used for further investigation with special reference to the isolation of their phytochemical constituents with mosquitocidal toxicity.

This study also showed that the various extracts prepared from *Euphorbia thymifolia, Catunaregam spinosa, Cardiospermum halicacabum, Persicaria hydropiper, Glycosmis pentaphylla, Ageratum conyzoides,* and *Plectranthus hadiensis* have potential larvicidal toxicity against *Aedes aegypti*. Previous studies have mentioned the prominent larvicidal potential of medicinal plant species from the family Euphorbiaceae against *Aedes aegypti*. They include *Euphorbia tirucalli, Euphorbia hirta, Jatropha curcas, Pedilanthus tithymaloides,* and *Phyllanthus amarus* (Rahuman et al., 2008a). They discussed that all the extracts prepared by them have shown great larvicidal efficacy. However, the maximum larvicidal potential was found in the petroleum ether extracts made from *Euphorbia tirucalli, Euphorbia hirta, Jatropha curcas, Pedilanthus tithymaloides,* and *Phyllanthus amarus*. However, in the present study, it was observed that the acetone extract prepared from the *Euphorbia thymifolia* have potent toxicity. The family Euphorbiaceae is principally known by the presence of a huge number of phytochemical constituents including polyfunctional diterpenoids. In addition to this, several toxic and nonirritant polyfunctional macrocyclic diterpenoids have also been separated from the medicinal plants of Euphorbiaceae (Rahuman et al., 2008a). They also reported the pharmacological, antifeedant, repellent, anthelmintic effects of *Euphorbia hirta* L. which is distributed in the Western Ghats of India (Tamil Nadu region). Another medicinal plant, *Euphorbia tirucalli*, is reported with larvicidal potential by Yadav,

Srivastava, Chandra and Singh (2002). The present study strongly agrees with Panneerselvam, Murugan, Kovendan, Kumar and Subramaniam (2013) since they reported that several Euphorbiaceae species are being used for traditional medicines and various biological activities.

The fruits of *Catunaregam spinosa* are well known for their immunomodulatory and antidysenteric properties. The plant is primarily used as a traditional medicine against various diseases including jaundice, emetic, asthma and gonorrhoea. Earlier phytochemical studies have opened the way for the isolation and characterization of various phytochemical constituents including iridoid glucosides, lignans, coumarin glucosides and triterpenoid saponins (Gao, Lu, Tao, Zhang, & Wang, 2011). They isolated four new compounds from the stem bark of *Catunaregam spinosa*. One of the probable reasons for the potent larvicidal efficacy of *Catunaregam spinosa* seed extract obtained in this study is might be the presence of similar phytochemical constituents obtained from its bark. Suryawanshi, Patil, Borase, Narkhede and Patil (2015) reported the insecticidal properties of plants from Apocynaceae and Rubiaceae family since these plants are well known for the cyclotides expression. The various phytochemical constituents isolated from the chloroform, hexane, benzene, methanol and ethyl acetate extracts of *Cardiospermum halicacabum* showed strong repellent activity against *Culex quinquefasciatus* (Govindarajan & Sivakumar, 2012). Additionally, they suggested that the afore-mentioned extracts provided strong protection to the test person without inducing any allergic reaction.

Our previous study (Anoopkumar et al., 2017b) verified that the leaf extracts of *Persicaria hydropiper* and *Plectranthus hadiensis* showed remarkable larvicidal efficacy against *Aedes aegypti* fourth instar larvae. This study also reported the larvicidal potential of various extracts prepared from the traditionally used medicinal plants such as *Hydrocotyle javanica* (Apiaceae), *Acanthospermum hispidum*, *Triumfetta rhomboidea*, *Sphaernthus indicus*, *Toddelia asiatica*, *Deris trifoliata* and *Drymaria cordata*. The members of the families of Asteraceae, Caryophyllaceae, Rutaceae, Lamiacea, Malvaceae, Polygonaceae, and Fabaceae possess various kinds of inhibiting activity against mosquitoes (Anoopkumar et al., 2017b; Arivoli, Tennyson, & Martin, 2011; Pavela et al., 2019). Various secondary metabolites including tannin, flavonoids, alkaloids and saponins in the plants enhance the demand of traditionally used medicinal plants for vector control approaches (Mimica-Dukic & Bozin, 2008).

Concerning the larvicidal potential of *Blumea lacera*, *Heliotropium indicum*, *Hyptis saveolens*, and *Stachytarpheta jamaicensis*; comparison of the results from this study to those found in the previous reports yielded the following findings: a) the various extracts of *Blumea* sp. (*Blumea martiniana*, *Blumea perrottetiana*, *Blumea balsamifera*, *Blumea mollis* and *Blumea brevipes*) are reported with various biological activities including insecticidal toxicity (Zhu & Tian, 2011); b) the *Heliotropium* sp. mediated silver nanoparticles showed significant larvicidal efficacy against various mosquito species such as *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Santhosh, Ragavendran, & Natarajan, 2015); c) the genera *Hyptis* and *Stachytarpheta* are known to constitute species with prominent insecticidal efficacy (Conti et al., 2012; Mgbemena, Ebe,

Nnadozie, & Ekeanyanwu, 2015); d) (Anoopkumar et al., 2017b) reported the larvicidal potential of *Persicaria hydropiper* against *Aedes aegypti* and (Hossain & Khalequzzaman, 2018) described the oviposition deterrent activity of the same against *Bactrocera cucurbitae*. The variations observed in the toxicity may be attributable to the different methods of extraction, selection of organic solvents, difference in polarity index or different types of phytochemical constituents present in the plants.

Moreover, as stated in the results, this study supports the view that the different kinds of plant families have significant biological properties. Keeping in attention the context of their larvicidal efficacy, a total of 50 plant families were screened against the fourth instar larvae of *Aedes aegypti*. The major plant families that are included in the present study are; Acanthaceae, Apocynaceae, Asteraceae, Lythraceae, Fabaceae, Rutaceae, Boraginaceae, Euphorbiaceae, Oleaceae, Malvaceae, Rubiaceae, Meliaceae, Piperaceae, Verbenaceae, Lamiaceae, Polygonaceae, Plantaginaceae, Sapindaceae, Campanulaceae, Moraceae, Solanaceae, Balsaminaceae, Lauraceae, Hypericaceae, Colchicaceae, Aristolochiaceae, and Amaranthaceae. Islam et al. (2012) reported that a large number of plants have been reported with mosquito repellent activity, anti-plasmodial activity and larvicidal activities from Asteraceae and Acanthaceae family. The results from the present investigation are comparable with the previous report published by (Suryawanshi et al., 2015). They evaluated the insecticidal properties of the flower and leaf extracts of plants from the families Apocynaceae and Rubiaceae. Another study undertaken by Samidurai (2012) assessed the ovicidal and larvicidal potential of acetone, benzene and methanol

solvent extracts prepared from the medicinal plants of Lythraceae family. Govindarajan, Rajeswary and Amsath (2013) discussed the various biological activities of medicinal plants from the families Fabaceae and Rutaceae. Johnson, Maharajan and Janakiraman (2015) reported the medicinal potential of various plants from the families Boraginaceae and Euphorbiaceae. In addition to this, the scientific literature revealed that the silver and gold nanoparticles synthesized from Oleaceae plant possessed significant toxicity against *Culex quinquefasciatus*. Kumar, Warikoo and Wahab (2010) explained the structural deformations induced by the medicinal plants from the families Meliaceae, Piperaceae, and Verbenaceae in *Aedes aegypti*. Jacobson (1975a, b), and Sharma and Samant (2014) reported the various biological activities of medicinal plants from the families Lamiaceae, Polygonaceae, Plantaginaceae, Sapindaceae, and Campanulaceae. A review made by Kaushik, Sharma, Thomas, Sharma and Bansal (2019) reported the larvicidal properties of many indigenous plants from the families Solanaceae, Balsaminaceae and Amaranthaceae against *Aedes aegypti*, a dengue fever vector. The findings of this study can be used to enlighten the possible mode of action of bioactive elements over the target insect *Aedes aegypti*.

The results from the present study also suggest that the different plant species exhibit different magnitude of toxicity. The magnitude of the toxic effects induced by the medicinal plants has differed among various solvents. Consistent with the insecticidal properties, most of the plant species used in this study can be useful for mosquito control. The screening of traditionally used medicinal plants against *Aedes aegypti*, as described in this study, could enlighten the development

of natural insecticides over the synthetic insecticides. Based on the previous studies these plant species can be either used for spatial spray treatment or indoor residual spraying (IRS) or direct spraying to the larval habitat. In this regard, with the exceptions of a few research articles, one of the major problems found in the scientific community is the lack of reported statistics concerning the practical assumptions in control of mosquitoes. Therefore, the practical perspectives of the use of the plant species in this study are needed to be performed in future studies to accomplish the afore-mentioned concept. Further isolation, identification, and mode of action of bioactive elements from medicinal plants have acted as a backbone for the development of next-generation insecticides of botanical origin against mosquito vectors.

1.5. SUMMARY

The importance of healthcare services in the diagnosis and treatment of arboviral diseases has increased in the current scenario since the threat of morbidity and mortality is increasing day by day. The increased population rate, urbanization and international travel build a suitable environment for arboviral disease vectors, especially the *Aedes aegypti*. *Aedes aegypti*, is primarily recognized as one of the prominent vectors of various arboviral diseases including dengue fever, Mayaro, chikungunya, yellow fever, and zika fever. The epidemics of dengue are endemic in more than 100 nations in the Eastern Mediterranean, America, Africa, and Southeast Asia. Every year, approximately 50 million dengue infections happen together with 18,000 deaths. The WHO (World Health Organization) fact sheet in 2008 verified that 80% of the people from African and Asian follow traditional medicinal plants for their health care needs. So far, vaccinations and precise medication are not existing commercially against dengue fever. The only effective method used to diminish the rate of dengue transmission is by the control of *Aedes aegypti*. For the past years, the control measures for *Aedes aegypti* were followed on the routine and indiscriminate use of chemical insecticides including organophosphates, organochlorines, pyrethroids and carbamates. The blind use of them have given rise to the development of insecticide resistance and thereby reinforced the link between environment, health and phytochemical constituents. The use of medicinal plants and their products for vector control are growing globally as they don't possess toxic effects on humans. Bearing in mind the fact

that the increased documentation of health and environmental impacts induced by synthetic insecticides, the present study focusses on the development of natural insecticides for mosquito control.

The larvicidal efficacy of acetone, methanol, petroleum ether and water extracts prepared from fifty traditionally used medicinal plants was carried out against *Aedes aegypti*, a dengue fever vector. Most of all the extracts showed their potency to impose mortality in *Aedes aegypti* fourth instar larvae. Among the fifty traditionally used medicinal plants, 21 plants exhibit more than 41% of toxicity, 4 plants show 31-40 of toxicity, 8 plants show 20-30% of mortality. Only 11 plants exhibited 10-19% larvicidal potential. The medicinal plants with potent larvicidal efficacy are; *Euphorbia thymifolia, Catunaregam spinosa, Cardiospermum halicacabum, Persicaria hydropiper, Ageratum conyzoides, Glycosmis pentaphylla, Plectranthus hadiensis, Asclepias curassavica, Mimosa pudica, Toddalia asiatica, Scoparia dulcis, Jasminum brevilobum, Aglaia edulis,* and *Pogostemon auricularius.* The various extracts of the afore-mentioned medicinal plant exhibit a different range of toxicity in different extracts. One of the probable reasons for the aforementioned condition is the polarity index of the phytochemical constituents present in the extract. Based on the effective toxicity, easy availability, cultivation status and commercial probability along with conservational aspects, the three traditionally used medicinal plants such as *Jasminum brevilobum* (Sp: 13), *Aglaia edulis* (Sp:20), and *Pogostemon auricularius* (Sp: 47) were selected for the bioassay-guided chromatographic fraction. The acetone extract of these three plants was chosen for further analysis. It could also be perceived from the previous studies that some bioactive elements

in the plants act as general toxicants. The initial screening is a better way of evaluating the significant larvicidal activity of medicinal plants. The findings gathered from this study have revealed that the phytochemical constituents of various extracts could be a ground-breaking notion for the development of natural insecticides.

CHAPTER II

Identification of Bioactive Compounds

2.1. INTRODUCTION

Globally, insect vectors are the most life-threatening group, as they are responsible for the dissemination of several severe diseases to humans, in which, mosquitoes are considered as important groups that cause a number of life-threatening diseases. They are dengue fever, yellow fever, malaria, West Nile virus, Ross River fever, Japanese encephalitis, filariasis, chikungunya and Western equine encephalitis. Severe dengue infection was recognized as a principal cause of death among humans in Africa, Latin American, Eastern Mediterranean and Asian countries. Four types of dengue virus serotypes (Flaviviridae family) are responsible for infection (DENV1, DENV2, DENV3, and DENV4). Moreover, a new fifth serotype (DENV-5) has reported in 2013 (Anoopkumar et al., 2019; Mustafa et al., 2015). The afore-mentioned viruses were entering into the human body through the bites of infected *Aedes aegypti* mosquitoes. Nowadays, the major approach to prevent or control the dengue transmission is to combat the *Aedes aegypti* mosquitoes. This can be achieved through i) Mosquito breeding prevention, ii) Personal protection and iii) vector control. In addition, there exists ongoing research in the scientific community in search of new innovative approaches that will assist global efforts to prevent the dengue transmission, as well as other arthropod-borne diseases (WHO, 2014).

For the control of *Aedes aegypti*, the communities and societies prominently used the synthetic insecticides such as Dichloro Diphenyl Trichloroethane's (DDT), organophosphates, carbamates, and organochlorines. Furthermore, synthetic

insecticides are expensive and they develop environmental issues besides inducing toxic effects on non-target organisms. Therefore, the scientific community has been seeking for an alternative strategy for mosquito control. Researches that are more intensive have been recently performed to prevent mosquito proliferation in an integrated way (Ragavendran et al., 2019; Singh, Dhiman, & Mittal, 2006).

The various phytochemical constituents in medicinal plants have been well reported for developing an environmentally established vector control programme. Because of their low toxicity to mammals, wide-spectrum activity and low environmental pollution, the phytochemical constituents in plants are regarded as alternatives to synthetic insecticides. A large number of such plants have been used for mosquito control ever since ancient times. Previous studies have reported the usefulness of plant-based products as effective mosquito agents and repellents devoid of inducing toxic effects to humans. At present, more than 2000 plants have been well-known to produce bioactive elements of value in mosquito control. But, the fact is that only a few plants have been used for practical assumptions in mosquito control programmes (Kumar et al., 2012b).

Researchers have verified that the bioactive elements isolated from the crude extracts of various plants possess potent toxicity against *Aedes aegypti* mosquito vectors. The most favourable botanical groups that show potent toxicity against mosquitoes are Acanthaceae, Apocynaceae, Asteraceae, Lythraceae, Fabaceae, Rutaceae, Boraginaceae, Euphorbiaceae, Oleaceae, Malvaceae, Rubiaceae, Meliaceae, Piperaceae, Verbenaceae, Lamiaceae, Polygonaceae, Plantaginaceae, Sapindaceae, Campanulaceae, Moraceae, Solanaceae, Balsaminaceae, Lauraceae,

Hypericaceae, Colchicaceae, Aristolochiaceae, and Amaranthaceae. Some of the afore-mentioned families are regarded as aromatic plants. One of the earliest studies concerning the use of medicinal plants against mosquito vectors is reputed to be Campbell and co-workers (1933), who discovered that the various phytochemical constituents like methylanabasine, nicotine, lupinine, and anabasine isolated from *Anabasis aphylla*, exhibited larvicidal efficacy against mosquitoes (Amer & Mehlhorn, 2006a). These kinds of phytochemical constituents are those formed through a complex metabolic pathway. They do not directly link with primary biochemical events that promote growth and reproduction. The distribution and presence of secondary metabolites in medicinal plants can be linked with a defensive purpose against pathogenic organisms, pests, herbivores, and insects. The major classes of secondary metabolites include alkaloids, phenolics and terpenoids (Amer & Mehlhorn, 2006a).

Taking the whole ideas stated above into consideration, it is clear that the isolation of phytochemical constituents from medicinal plants is a complex process. Extraction is the initial step and it is obligatory to extract the specific bioactive elements from traditional medicinal plants for further characterization. Various methods are available for extraction and are; heating under reflux, soxhlet extraction, and sonification. Owing to the fact that the various extracts of medicinal plants constitute phytochemical constituents with different polarity index, their isolation, and separation still continues to be a challenge. It is true that the availability of modern techniques such as Thin Layer Chromatography (TLC), column chromatography, Gas Chromatography-Mass Spectrometry (GC-

MS), Fourier-transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopy (NMR) make bioactive element isolation easier than earlier (Sasidharan et al., 2011). A study by Talontsi et al. (2011) applied Bioactivity-guided chromatographic separation for the isolation of alkaloids (wuchuyuamide I, rutaecarpine and evodiamine) with potent larvicidal efficacy. Similarly, previous studies have used the afore-mentioned techniques for the isolation of phenolics and terpenoids with a significant range of toxicity against *Aedes aegypti* mosquitoes. This indicates that the importance of studies concerning the medicinal plants and their active phytochemical constituents against mosquitoes is increasing day by day all around the world. In the course of mass screening of traditional medicinal plants in the previous objective, the three plants such as *Jasminum brevilobum* (Sp: 13), *Aglaia edulis* (Sp:20), and *Pogostemon auricularius* (Sp: 47) was found to show prominent larvicidal efficacy against the *Aedes aegypti* fourth instar larvae.

The genus *Jasminum* L constitutes about 200 plant species over the world; in India, the genus is characterized by 47 species. Previous studies have reported the toxic effects of various *Jasminum* species (*Jasminum officinale*, *Jasminumgrandiflorum Jasminum auriculatum*, *Jasminum nervosum*) against mosquito vectors (Preethi et al., 2014). However, studies on the above-mentioned aspects in *Jasminum brevilobum* with special reference to the isolation of bioactive elements from its acetone extracts are scanty in the current scenario.

The genus *Aglaia* Lour is considered as the largest group comprising about 115 species. For the past few years, the *Aglaia* Lour genus has gained aggregating

scientific focus on account of various bioactivity potential (Muellner, Samuel, Chase, Pannell, & Greger, 2005). The medicinal and larvicidal potential of *Aglaia odorata* Lour, *Aglaia elaeagnoidea*, *Aglaia rubiginosa*, *Aglaia elliptifolia*, *Aglaia malabarica* have been reported in previous studies. Previous studies in *Aglaia edulis* have revealed the presence of nine novel flavaglines with insecticidal property. However, the larvicidal property of the isolated compounds have not been described so far. This indicates that the larvicidal potential of *Aglaia edulis* with special reference to the bioactive guided fractionation using chromatographic approaches are not well established in previous studies.

The genus *Pogostemon* Desf. is represented by 340 species in India. The mosquito repellent, pupicidal efficacies of various *Pogostemon* sp. were evaluated previously by (Das et al., 2015) and (Gokulakrishnan et al., 2013). Similarly, the larvicidal potential of *Pogostemon cablin* against *Culex pipiens pallens* was verified by Park and Park (2012). Most of all the studies concerning the larvicidal potential and pharmacological benefits are focussed on the *Pogostemon heyneanus*. Murugan et al. (2010) verified the presence of patchouli alcohol and other important phytochemical constituents from the medicinal plant *Pogostemon heyneanus.* However, the larvicidal potential, as well as the isolation of responsible bioactive elements from the medicinal plant *Pogostemon auricularius*, are not well established in previous studies.

Among the forecasting approaches to mosquito control, the chemical method is of great position, but the deleterious effects of such synthetic products may result in severe health effects including respiratory problems in human beings. Moreover,

such synthetic approaches inevitably harm environmental sustainability thereby augmenting the rapid emergence of insecticide resistance (Hamid et al., 2017). One of the prominent ways to prevent the afore-said problems concerning environmental sustainability and human health is searching for natural products. In contrast with the disadvantages of synthetic insecticides, natural insecticides are promising alternatives. There exist many added advantages for natural insecticides and are, a) eco-friendly, b) generate a low level of resistance, c) quick decomposition, d) comparatively less toxicity to nontarget organisms and e) cheap.

In view of the above-mentioned concepts, the three medicinal plants screened from the previous objective have been chosen for bioactive guided fractionation. Altogether, the present study intends to represent a modern strategy integrating the traditional knowledge on medicinal plants to the mosquito larval control, with negligible harmfulness against non-target organisms. The assessment of bioactive element isolation for larvicidal efficacy may help in the development of novel strategies that prevent the proliferation of *Aedes* mosquito vectors.

2.2. MATERIALS AND METHODS

2.2.1. Plant collection and extraction

Fresh leaves of *Aglaia edulis* (11°53'22.60"N; 76° 0'56.30"E), *Pogostemon auricularius* (11°53'25.30"N; 76° 0'47.30"E) and *Jasminum brevilobum* (11°53'22.40"N; 76° 0'52.10"E) were collected from different areas of Thirunelly, Wayanad (a part of Western Ghats), Kerala, India. The shade dried leaves of the afore-mentioned plants were powdered using an electric grinder. As per the results from the previous objective, various extracts of three plants were prepared using Soxhlet extraction. Acetone, methanol, petroleum ether and water were used as solvents for Soxhlet extraction. Twenty grams of fine powder prepared from *Pogostemon auricularius, Jasminum brevilobum,* and *Aglaia edulis* which contains the bioactive elements to be extracted were placed in a separate thimble. The 250 mL of each solvent such as petroleum ether, methanol, acetone and water was added to the round bottomed flask of the apparatus. The solvents were heated at specific boiling temperature for 6 to 12 hours. The heating initiates the movement of phytochemical constituents into the condenser. Once the level of the solvent containing bioactive elements reaches the siphon, it pours back into the round bottomed flask. The process of extraction for each plant is repeated continuously until the colour of the solvent becomes transparent. The acetone extracts prepared from the *Pogostemon auricularius, Jasminum brevilobum* and *Aglaia edulis* were then concentrated using rotary evaporator and kept at 4ºC for future investigative studies.

2.2.2. Experimental insects

Early fourth instar larvae of *Aedes aegypti* were used for the executed experiments. The larvae were reared at Communicable Disease Research Laboratory, St. Josephs's College, Irinjalakuda, Kerala, India. The eggs of *Aedes aegypti* were placed in trays containing dechlorinated water and the eggs were hatched within 2-3 days. The first instar larvae of *Aedes aegypti* were then transferred into another tray and they were provided fed upon dog biscuits and yeast in the ratio 3:1. The pupae formed were then transferred into another plastic bowl, which is placed inside a cloth cage. After the adult emergence, the female mosquitoes are supplied with blood meals for egg maturation. The following environmental conditions were executed during the rearing process; 27±2°C temperature, 55-60% relative humidity followed by 12 L:12 D photoperiod cycle.

2.2.3. Larval bioassay

The larvicidal efficacy of isolated bioactive fractions and compounds were followed by the method recommended by WHO. All the experiments were executed against *Aedes aegypti* fourth instar larvae. The control group was maintained for each experiment. After 24 hours of continuous exposure, the larval mortality was recorded.

2.2.4. Fractionation and isolation of bioactive compounds from the three plants

The purified phytochemical constituents were obtained through repeated chromatographic purification (Column chromatography and Thin layer chromatography). The analytical grade acetone, methanol, and hexane were used for Thin-layer chromatography (TLC) analyses. The Silica gel having a mesh size

of 70–30 and TLC plate was obtained from Merck Co. (Germany). The acetone extract prepared from *Pogostemon auricularius, Jasminum brevilobum* and *Aglaia edulis* were used for thin-layer chromatographic analysis.

2.2.4.1. Fractionation and isolation of bioactive compounds from *Pogostemon auricularius*

Thin-layer chromatography for *Pogostemon auricularius* acetone extract yielded five subfractions such as FRC TL1P, FRC TL2P, FRC TL3P, FRC TL4P, and FRC TL5P. The mixture of acetone, hexane and methanol in the ratio 3:11:5 is used as the mobile phase in Thin layer chromatography. All the isolated fractions are then subjected to larval bioassay against *Aedes aegypti*. The most effective and potent bioactive fraction (FRC TL3P) was then again separated from the acetone extract by Thin layer chromatography until enough quantity of the subfraction gained. The FRC TL3P subfraction was then subjected to Column chromatography (methanol, hexane, and acetone in the ratio 4.5:4) and yielded 18 fractions and they were provided with sample names from P1 to P18. These fractions were then subjected to larval bioassay. The bioactive fraction P8 was again separated using Column chromatography with the same mobile phase used in the previous step. After getting a sufficient amount, the P8 fraction was subjected to Thin-layer chromatography (acetone, hexane and methanol in the ratio 2:7:6). This yielded 5 subfractions and they were provided with sample names from P8A, P8B, P8C, P8D and P8E, from which, the bioactive fraction P8D was subjected to Column chromatography (hexane and acetone in the ratio 8:3). About, fourteen subfractions were isolated from P8D fraction and they were labelled with sample names from PP1-PP14. The subfractions from PP1 to PP14

were then subjected for larval bioassay. The bioactive fraction PP4 was again isolated since it shows potent activity against *Aedes aegypti* fourth instar larvae. The isolated bioactive fraction PP4 was then stored at -20°C for further analysis.

2.2.4.2. Fractionation and isolation of bioactive compounds from *Jasminum brevilobum*

Thin layer chromatography for *Jasminum brevilobum* acetone extract yielded seven subfractions such as FRC TL1J, FRC TL2J, FRC TL3J, FRC TL4J, FRC TL5J, FRC TL6J, and FRC TL7J. The experiment was again performed to get a sufficient quantity of each afore-mentioned fractions using hexane and acetone as the mobile phase (11:5). The larvicidal potential of the afore-mentioned fractions was performed against *Aedes aegypti* fourth instar larvae. The subfraction FRC TL2J possessed significant larvicidal potential. Therefore, no consideration has been paid to other subfractions during further studies. Additionally, the separation of "FRC TL2J" was repeated to get enough quantity for further studies. The FRC TL2J sub-fraction was then proceeded for chromatographic analysis. The subfraction FRC TL2J isolated from acetone extract of *Jasminum brevilobum* was then loaded on to the column packed with silica and mobile phase. The acetone and hexane in the 5:11 ratio were used as the mobile phases in Column chromatography. This yield twelve subfractions and they were named from C1 to C12. All the isolated subtractions were subjected for larvicidal bioassay, in which, the subfraction C7 showed significant toxicity against *Aedes aegypti* fourth instar larvae. All the remaining fractions did not exhibit potent toxicity. Therefore, the column chromatography was again performed until a sufficient quantity of bioactive fraction C7 obtained. The bioactive fraction C7 isolated

from FRC TL2J was again separated using TLC and Column chromatography with Hexane (90 mL): Acetone (40 mL) as a mobile phase. The TLC analysis yield four subfractions such as C7A, C7B, C7C, and C7D. The bioactive guided fractionation using the afore-mentioned fractions such as C7A, C7B, C7C, and C7D revealed that the subfraction C7D is potent. The column chromatographic analysis of C7D yielded seven subfractions and they were provided with the sample names as CC1, CC2, CC3, CC4, CC5, CC6 and CC7. The bioactive guided fractionation using the afore-mentioned extracts (CC1-CC7) indicated that the CC7 is more effective than others, therefore, the column chromatographic separation of the same (CC7) was again repeated to get sufficient quantity of bioactive element.

2.2.4.3. Fractionation and isolation of bioactive compounds from *Aglaia edulis*

Thin-layer chromatography for *Aglaia edulis* acetone extract yielded seven subfractions such as FRC TL1E, FRC TL2E, FRC TL3E, FRC TL4E, FRC TL5E, FRC TL6E, and FRC TL7E. The mixture of methanol and hexane in the ratio 6:1.5 is taken as the mobile phase for TLC. These isolated bioactive fractions were then subjected to larval bioassay. The FRC TL2E is again separated since it shows potent toxicity against *Aedes aegypti*. The column chromatography of the bioactive fraction FRC TL2E yielded nineteen fractions and they were provided with sample names from E1-E19. The mobile phase used for column chromatography is as follows; methanol, hexane, and acetone in the ratio 6:1:5. After the larval bioassay, the bioactive fraction E11 was subjected to Thin layer chromatography and yield 6 subfractions named as E11A, E11B, E11C, E11D,

E11E, and E11F. The mixture of methanol and hexane in the ratio 7: 1.5 is used as the mobile phase in TLC. They were subjected for larval bioassay and the bioactive fraction E11D is then again separated to get enough amount of the same. The bioactive fraction E11D then separated using column chromatography and the ratio of the mobile phase is as follows; Methanol (70 mL): Hexane (15 mL): Acetone (1 mL). Five fractions such as EE1, EE2, EE3, EE4 and EE5 were separated from E11D. These isolated fractions were subjected to larval bioassay and the effective fraction (EE5) was stored at -20°C for further investigations.

2.2.5. Identification of purified bioactive compounds

The isolated bioactive fractions such as CC7, PP4, and EE5 were subjected to GC-MS (Shimadzu, Kyoto, Japan). Each bioactive elements were injected separately for GC-MS analysis. The following conditions were maintained during the analysis; 30 meter × 0.25 mm, film thickness 0.25 μm DB-5MS column, the initial temperature is 50˚C (2 min) simultaneously maintained a flow rate of 20˚C/min to 130˚C, 12˚C/min to a 180˚C. The final temperature was maintained to 280˚C at 3˚C per min for 15 min. The carrier gas (helium) was used at mL/min flow rate. Optimum temperature was maintained for injection port and ion source. Electron impact mode was selected during the process for 58 min (70 ev). For the confirmation of analytes, selective ion mode (SIM) was used (Abirami and Rajendran 2012). The peak area of the respective samples was matched with those on the National Institute of Standards and Technology library database. For the structural characterization, Fourier-transform infrared spectroscopy (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy were used. The FTIR was verified to be an effective tool for the characterization of bioactive elements from

plants (Eberhardt, Li, Shupe, & Hse, 2007). The unknown bioactive elements (CC7, PP4 and EE5) were identified by comparing their spectrum with the known library.

2.2.6. Statistical analysis

The data concerning the larvae mortality were subjected to probit regression analysis to determine the LC_{50} and LC_{90} values using the statistical package of social science (SPSS) version 24.0.0. for the Windows platform.

2.3. RESULTS

2.3.1. Larval bioassay

In this study, the larvicidal efficacy of bioactive fractions and bioactive elements present in the three traditionally used medicinal plants such as *Pogostemon auricularius, Jasminum brevilobum* and *Aglaia edulis* are evaluated against fourth instar larvae of *Aedes aegypti*, one of the prominent vectors of dengue fever. The larval mortality rates were evaluated after 24 hours of continuous exposure to varying concentrations of FRC TL 2J, C7, C7D, CC7, FRC TL 3P, P8, P8D, PP4, FRC TL2E, E11, E11D and EE5. The result of each executed experiment was noted as the lethal concentrations assessed to kill 50 % of the experimental insects (LC_{50}). The LC_{50} values for each bioactive fractions including bioactive compounds, along with LC_{90}, were given in Table 2.1 (Page No. 144).

As reported in our published article entitled "Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases", all the evaluated fractions and compounds had prominent larvicidal efficacy varying between 1.724 mg/L and 43.688 mg/L (Fig. 2.1; Page No. 145). Among the 12 bioactive fractions including 3 compounds, EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) showed the most effective larvicidal efficacy against *Aedes aegypti* fourth instar larvae with LC_{50} value of 1.724 (0.834-2.479) mg/l after 24 hours of continuous exposure. The next effective bioactive elements identified were CC7 (2.213 mg/L (1.292-3.032) (Jatamansone ((7R)-4a,8a-

dimethyl-7-propan-2-yl-3,4,5,6,7,8-hexahydro-2H-naphthalen-1-one) and PP4 3.356 mg/L (2.331-4.518) (3-hydroxy-2,2,4-trimethylpentyl isobutyrate). In addition to this, remaining bioactive fractions such as FRC TL 2J, C7, C7D, FRC TL 3P, P8, P8D, FRC TL2E, E11, and E11D also showed remarkable larvicidal potential. The larvae in control groups displayed normal physical appearance without any damage in the body. The rate of larval mortality was found to be directly proportional to the concentrations of isolated bioactive fractions and compounds. The larvicidal efficacy of isolated bioactive elements and fractions against the laboratory maintained *Aedes aegypti* larvae were found to be dosage-dependent with prominent mortality observed for *Aglaia edulis*.

2.3.2. Phytochemistry

The bioassay-guided chromatographic fractionation of the *Aglaia edulis*, *Pogostemon auricularius*, and *Jasminum brevilobum* acetone extracts yielded twelve bioactive fractions. The acetone extracts of the afore-mentioned plants have also yielded several other fractions too, however, due to the lack of larvicidal potential against *Aedes aegeypti* the below mentioned bioactive fractions have only opted for further characterization and experiments. The fractions that are unable to produce prominent larvicidal potential have been discarded immediately during the study. The bioactive fractions such as FRC TL 2J, C7, C7D, and CC7 were isolated from *Jasminum brevilobum*. Similarly, the chromatographic separation of acetone extracts prepared from *Pogostemon auricularius* yielded four fractions such as FRC TL 3P, P8, P8D and PP4. The bioassay-guided chromatographic fractionation of acetone extracts prepared using *Aglaia edulis* produced the following bioactive fractions; FRC TL2E, E11, E11D and EE5.

Based on the GC-MS, FTIR, and NMR, three compounds were isolated from *Jasminum brevilobum* (CC7), *Aglaia edulis* (EE5) and *Pogostemon auricularius* (PP4).The isolated compounds are i) Jatamansone (CC7- isolated from *Jasminum brevilobum*), ii) 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4 – isolated from *Pogostemon auricularius*) and iii) 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (Eb5 – isolated from *Aglaia edulis*). The isolated compounds were compared and identified using the database of the GC-MS library provided by NIST. The GC-MS and FTIR spectrum of the isolated bioactive fractions were shown in the Figs. 2.2-2.4 (Page No. 148, 149, 151). The structure, retention time, area, area %, molecular weight (g/mol) of the isolated bioactive elements such as CC7, EE5, and PP4 is provided in Table 2.5 (Page No. 152).

The spectroscopic data (FTIR and NMR) acquired from the present study were consistent with those found in the databases, thus confirming the identity of three isolated compounds. Based on the results obtained, the following three compounds were identified from *Jasminum brevilobum*, *Pogostemon auricularius*, and *Aglaia edulis*; 1) Jatamansone (CC7; $C_{15}H_{26}O$), 2) 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4; $C_{12}H_{24}O_3$), 3) 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (EE5; $C_{16}H_{30}O_4$) (Table 2.5; Page No. 152).

Table 2.1. Larvicidal efficacy of isolated compounds and fractions from *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius*

Sl. No.	Plant	Bioactive fraction	LC_{50} (Fiducial limit) (In mg/L)	LC_{90} (Fiducial limit) (In mg/L)	Chi-Square	df^b
1	*Jasminum brevilobum*	FRC TL 2J	43.688 (36.003-51.306)	97.735 (79.025-139.511)	2.652	3
2		C7	24.645 (19.696-30.140)	70.273 (51.329-131.480)	4.438	3
3		C7D	9.499 (6.872-11.868)	31.226 (22.465-62.403)	4.813	3
4		CC7	2.213 (1.292-3.032)	13.549 (8.445-39.290)	1.101	4
5	*Pogostemon auricularius*	FRC TL 3P	54.793 (45.389-64.209	122.560 (98.624-178.421)	2.918	3
6		P8	37.163 (30.883-43.799)	85.481 (67.705-128.922)	1.865	3
7		P8D	14.807 (10.863-18.883)	51.808 (37.218-93.188)	5.235	3
8		PP4	3.356 (2.331-4.518)	19.667 (11.460-68.386)	1.597	4
9	*Aglaia edulis*	FRC TL2E	23.645 (15.928-29.953)	76.953 (57.022-141.596)	2.418	3
10		E11	8.836 (4.532-12.692)	51.231 (32.994-131.421)	3.211	3
11		E11D	6.546 (4.411-8.308)	22.131 (16.109- 3.155)	2.887	3
12		EE5	1.724 (0.834-2.479)	11.775 (7.335-35.706)	4.760	4

*statistical significance ($P < 0.05$)

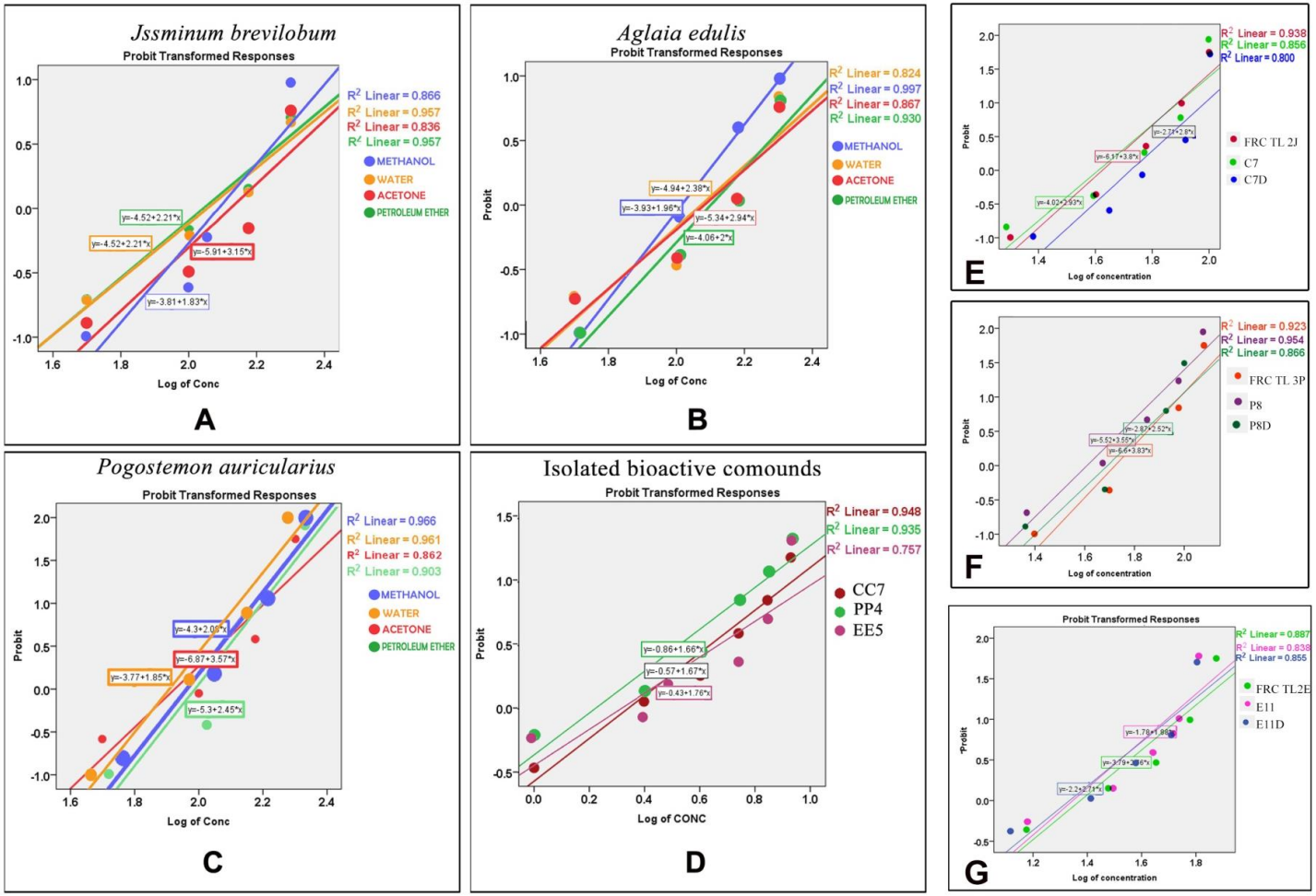


Fig. 2.1. Probit regression analysis – Larvicidal potential of (A) *Jasminum brevilobum*, (B) *Aglaia edulis*, (C) *Pogostemon auricularius*, (D) Isolated bioactive compounds, (E) Isolated bio active fractions of *Jasminum brevilobum*, (F) Isolated bio active fractions of *Aglaia edulis*, (G) Isolated bioactive fractions of *Pogostemon auricularius*.

The data obtained from ^{1}H-NMR spectrum of CC7 revealed the following data; ^{1}H NMR: δ 0.88-0.92 (9H, 0.90 (d, *J* = 6.8 Hz), 0.91 (s), 0.90 (d, *J* = 6.8 Hz)), 1.18 (3H, s), 1.36 (1H, ddd, *J* = 13.3, 10.3, 2.8 Hz), 1.31-1.51 (2H, 1.38 (ddd, *J* = 12.9, 10.2, 2.5 Hz), 1.44 (ddt, *J* = 13.0, 10.3, 2.9 Hz), 1.45-1.70 (6H, 1.58 (ddd, *J* = 12.9, 3.1, 2.5 Hz), 1.58 (ddt, *J* = 13.0, 2.8, 2.7 Hz), 1.64 (ddd, *J* = 13.3, 2.9, 2.7 Hz), 1.50 (dd, *J* = 13.2, 2.8 Hz), 1.59 (dddt, *J* = 4.1, 2.9, 2.8, 2.7 Hz), 1.50 (septd, *J* = 6.8, 4.1 Hz)), 1.71-1.96 (3H, 1.82 (ddddd, *J* = 13.0, 10.2, 10.2, 3.5, 2.5 Hz), 1.92 (dd, *J* = 13.2, 2.7 Hz), 1.81 (ddddd, *J* = 13.0, 3.4, 3.1, 2.5, 2.2 Hz)), 2.27-2.44 (2H, 2.32 (ddd, *J* = 14.4, 3.5, 2.2 Hz), 2.36 (ddd, *J* = 14.4, 10.2, 3.4 Hz)). The bioactive fraction CC7 was identified as Jatamansone, based on the comparison of ^{1}H NMR spectral data (Fig. 2.2; Page No. 148) with that reported in the literature (Table 2.2; Page No. 147).

The data gathered from the ^{1}H-NMR analysis of the bioactive fraction EE5, isolated from *Aglaia edulis* revealed the following profile; ^{1}H NMR: δ 0.85-0.89 (6H, 0.87 (d, *J* = 6.7 Hz), 0.87 (d, *J* = 6.7 Hz)), 0.90-0.91 (6H, 0.91 (s), 0.91 (s)), 1.09-1.14 (12H, 1.12 (d, *J* = 7.0 Hz), 1.12 (d, *J* = 7.0 Hz), 1.12 (d, *J* = 7.0 Hz), 1.12 (d, *J* = 7.0 Hz)), 1.83 (1H, dsept, *J* = 7.2, 6.7 Hz), 2.38-2.47 (2H, 2.42 (sept, *J* = 7.0 Hz), 2.43 (sept, *J* = 7.0 Hz), 4.43-4.48 (3H, 4.44 (s), 4.45 (d, *J* = 7.2 Hz), 4.44 (s). The bioactive fraction EE5 was identified as 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, based on the comparison of ^{1}H NMR spectral data with that reported in the literature. The data obtained from the ^{1}H NMR analysis of the EE5 bioactive fraction has been shown in the Table 2.3 (Page No. 147) and Fig. 2.3 (Page No. 149).

Table 2.2. The spectral data of the bioactive fraction CC7, isolated from *Jasminum brevilobum*

Assignment	Delta	Nb H	Mult	J (Hz)
24	2.321	1	ddd	14.42 3.49 2.22
23	2.36	1	ddd	14.42 10.17 3.42
36,37,38	0.902	3	D	6.80
39, 40, 41	0.902	3	D	6.80
27	1.58	1	ddd	12.90 3.10 2.52
28	1.378	1	ddd	12.90 10.24 2.50
35	1.498	1	septd	6.80 4.11
16	1.588	1	dddt	4.11 2.89 2.81 2.72
26	1.816	1	ddddd	13.04 10.24 10.17 3.49 2.52
25	1.815	1	ddddd	13.04 3.42 3.10 2.50 2.22
19	1.916	1	Dd	13.16 2.74
20	1.498	1	Dd	13.16 2.81
22	1.361	1	ddd	13.26 10.26 2.81
17	1.435	1	ddt	13.01 10.26 2.87
18	1.52	1	ddt	13.01 2.81 2.72

Table 2.3. The spectral data of bioactive fraction EE5 isolated from *Aglaia edulis*

Assignment	Delta	Nb H	Mult	J (Hz)
20	4.454	1	D	7.21
21, 22, 23	1.117	3	D	7.03
25, 26, 27	1.117	3	D	7.03
24	2.419	1	Sept	7.03
44, 45, 46	1.121	3	D	7.03
47, 48, 49	1.121	3	D	7.03
43	2.433	1	Sept	7.03
37, 38, 39	0.872	3	D	6.73
40, 41, 42	0.872	3	D	6.73
36	1.828	1	Dsept	7.21 6.73

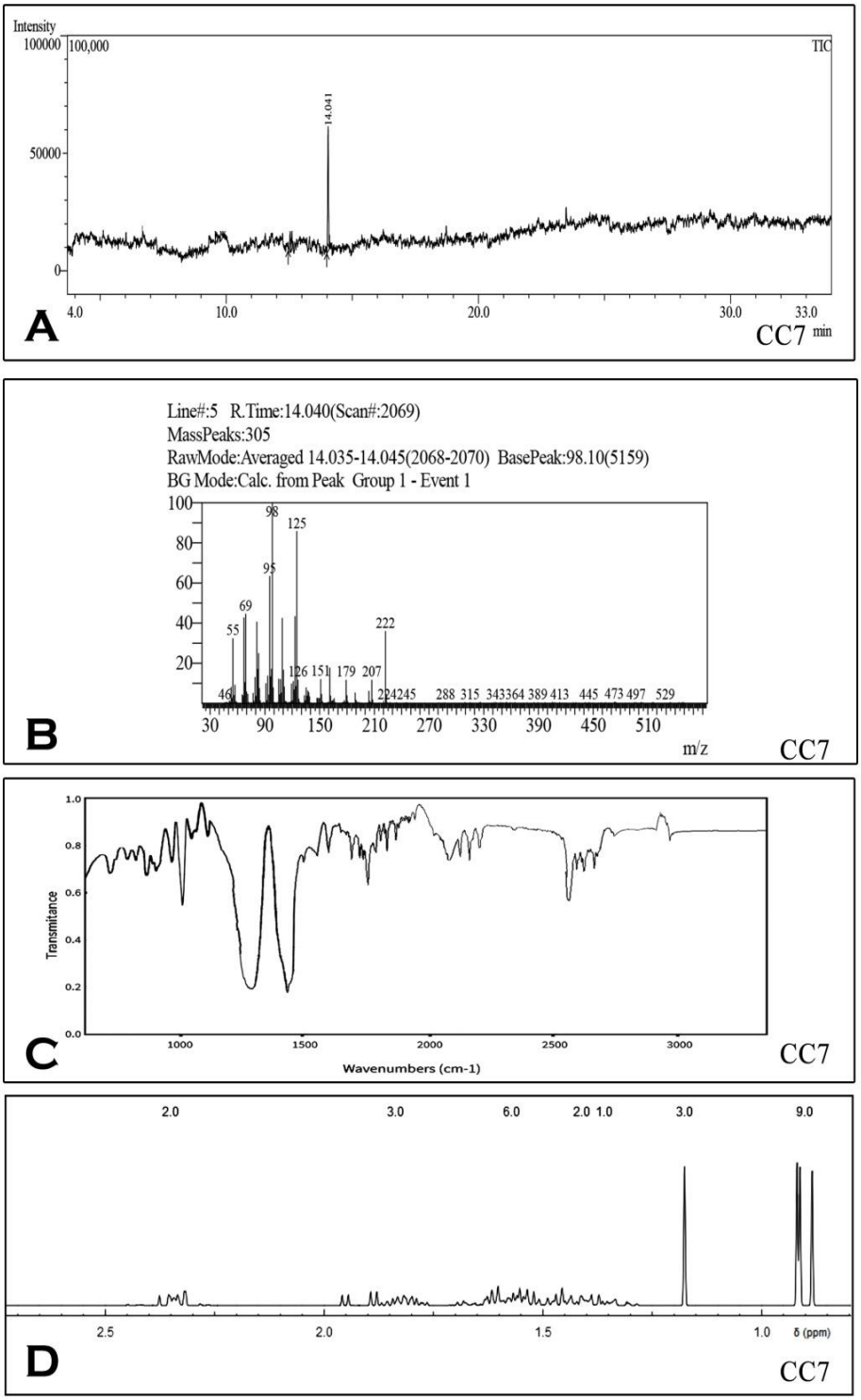


Fig. 2.2. Identification of purified bioactive compounds using GC-MS, FTIR and NMR. (A-B) Gas chromatography-mass spectrometer chromatogram and mass spectra of the isolated compound from *Jasminum brevilobum*; (C) Infra-red absorption spectra of of the isolated bioactive fraction CC7; (D) NMR spectra of the isolated bioactive fraction CC7.

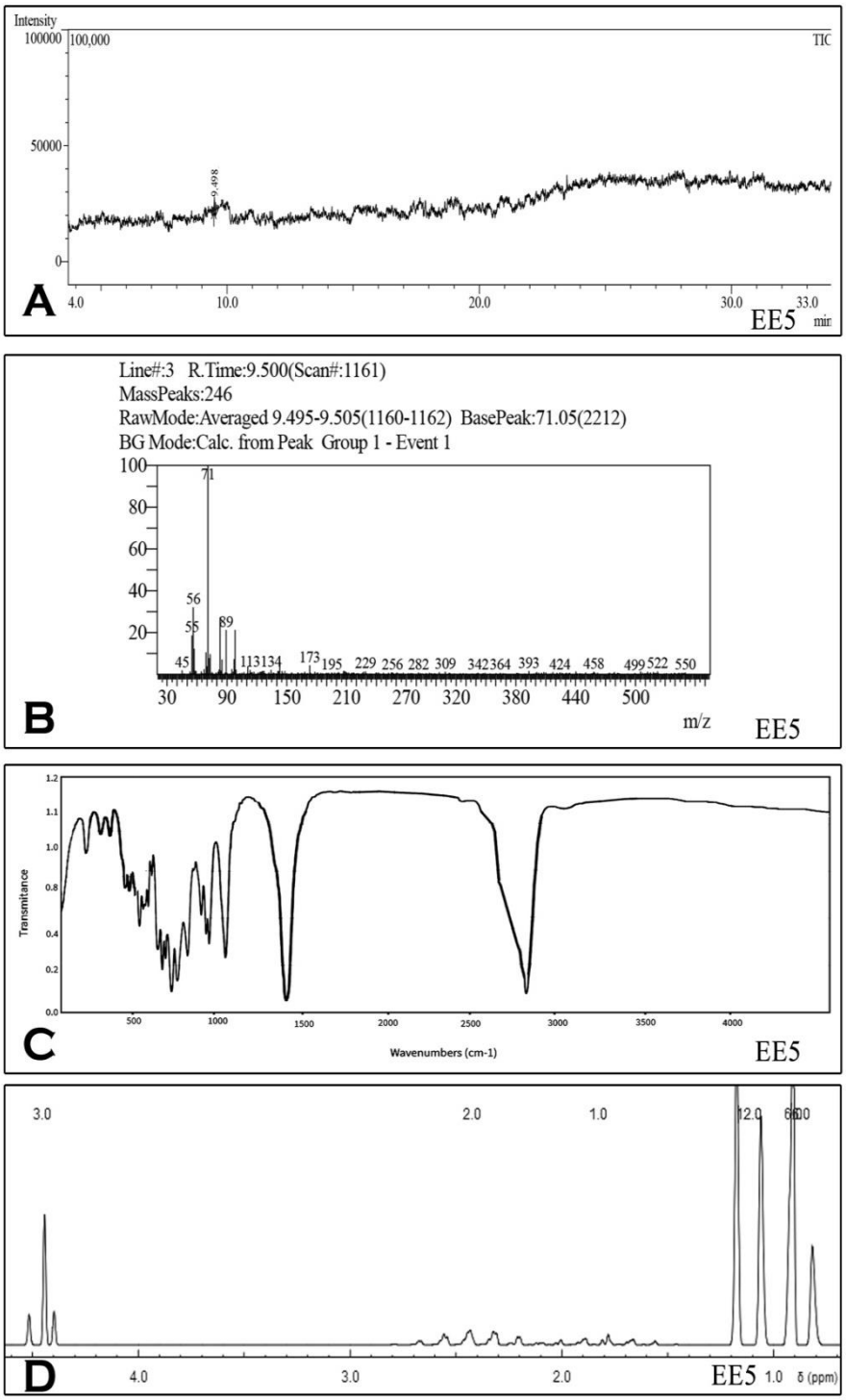


Fig. 2.3. Identification of purified bioactive compounds using GC-MS, FTIR and NMR. (A-B) Gas chromatography-mass spectrometer chromatogram and mass spectra of the isolated compound from *Aglaia edulis*; (C) Infra-red absorption spectra of of the isolated bioactive fraction EE5; (D) NMR spectra of the isolated bioactive fraction EE5.

The spectral data of the bioactive fraction PP4, isolated from *Pogostemon auricularius* has been depicted in the Table 2.4 (Page No. 150) and Fig. 2.4 (Page No. 151). The ^{1}H-NMR profile of PP4 revealed the following data; ^{1}H NMR: δ 0.87 (9H, s), 1.02 (3H, d, *J* = 6.8 Hz), 1.09-1.14 (6H, 1.11 (d, *J* = 7.0 Hz), 1.11 (d, *J* = 7.0 Hz)), 2.10 (1H, hd, *J* = 6.8, 2.7 Hz), 2.40 (1H, sept, *J* = 7.0 Hz), 3.34 (1H, d, *J* = 2.7 Hz), 4.32-4.37 (2H, 4.34 (d, *J* = 6.8 Hz), 4.34 (d, *J* = 6.8 Hz)). The bioactive fraction PP4 was identified as 3-hydroxy-2,2,4-trimethylpentyl isobutyrate, based on the comparison of ^{1}H NMR spectral data with that reported in the literature. The spectral data of bioactive fraction PP4 isolated from *Pogostemon auricularius* is shown in Table 2.4 (Page No. 150) and Fig. 2.4 (Page No. 151).

Table 2.4. The spectral data of bioactive fraction PP4 isolated from *Pogostemon auricularius*

Assignment	Delta	Nb H	Mult	J (Hz)
26	3.337	1	D	2.75
24	4.344	1	D	6.78
23	4.344	1	D	6.78
20, 21, 22	1.115	3	d	7.03
16, 7, 18	1.115	3	d	7.03
19	2.403	1	sept	7.03
27, 28, 29	1.023	3	d	6.79
25	2.104	1	hd	6.78 2.75

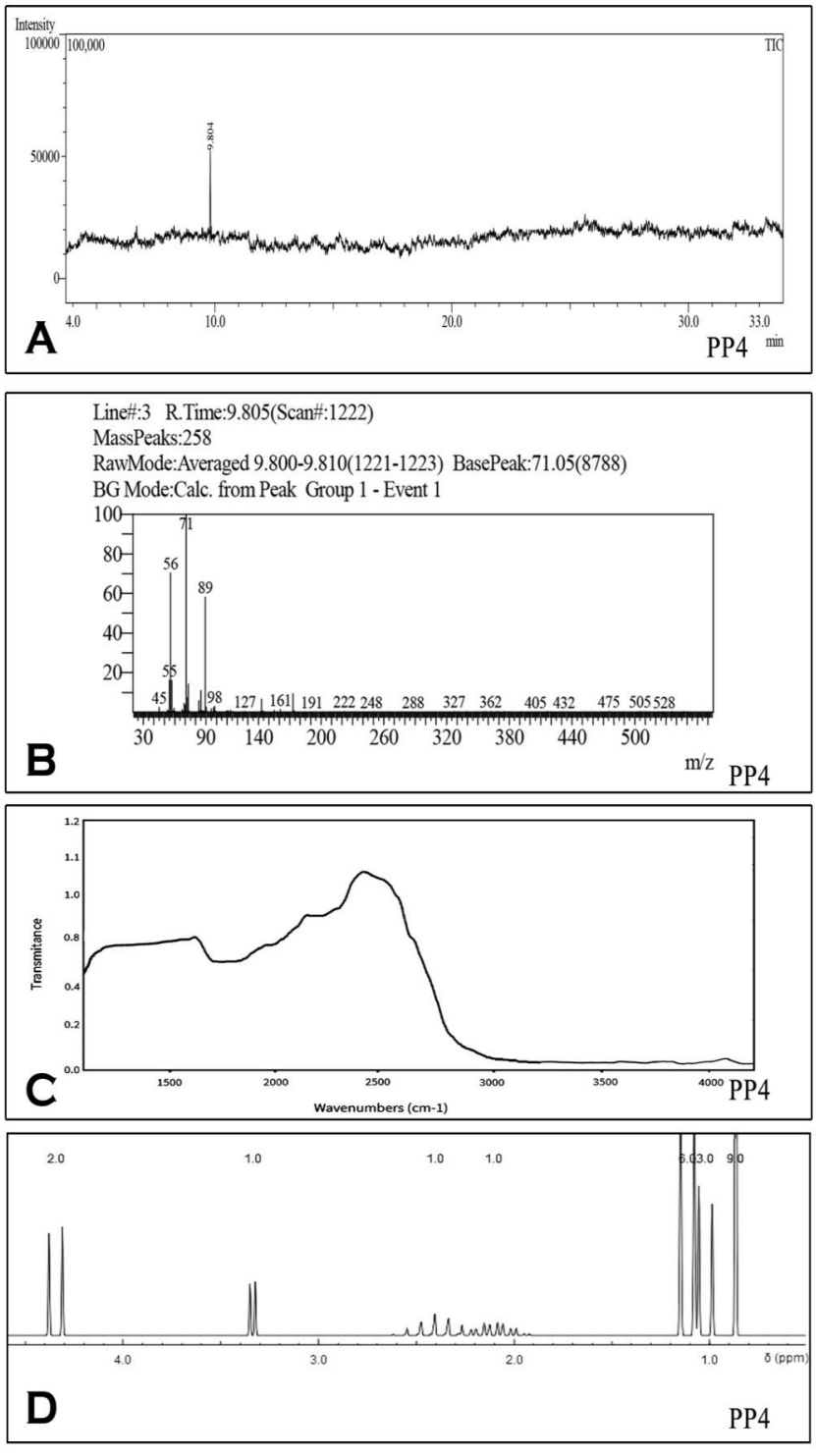
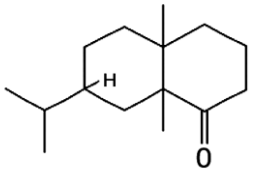
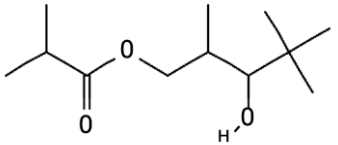
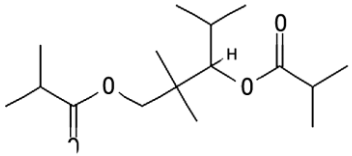


Fig. 2.4. Identification of purified bioactive compounds using GC-MS, FTIR and NMR. (A-B) Gas chromatography-mass spectrometer (GC-MS) chromatogram and mass spectra of the isolated compound from *Pogostemon auricularius*; (C) Infra-red absorption spectra of of the isolated bioactive fraction PP4; (D) NMR spectra of the isolated bioactive fraction PP4.

Table 2.5. Bioactive fractions and compounds isolated from three medicinal plants

Sl. No	Retention time	Area	Area%	Name of the compound	Molecular weight (g/mol)	Structure	Plant
1	14.041	123577	23.30	Jatamansone	222.37	 CC7	*Jasminum brevilobum*
2	9.804	53442	46.28	3-hydroxy-2,2,4-trimethylpentyl isobutyrate	216.32	 PP4	*Pogostemon auricularius*
3	9.498	12271	9.56	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	286.41	 EE5	*Aglaia edulis*

2.4. DISCUSSION

The findings of this study have revealed that the three bioactive elements such as Jatamansone ((7R)-4a,8a-dimethyl-7-propan-2-yl-3,4,5,6,7,8-hexahydro-2H-napht halen-1-one) (CC7); 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4); and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (EE5) isolated from three plants could be an innovative notion for the development of a natural insecticide that would be explored as an alternative to synthetic insecticides in future. In addition to this, the compound isolated from (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) *Aglaia edulis* could be more effective than the remaining two compounds. The Jatamansone is a sesquiterpenoid with three uninterrupted isoprene units and it exhibited pharmacological benefits. Likewise, the 3-hydroxy-2,2,4-trimethylpentyl isobutyrate isolated from *Pogostemon auricularius* was previously reported as an effective antibacterial agent which prevents the growth of *Clostridium perfringens.* This has confirmed the toxicity of afore-mentioned compounds against microorganisms. Incorporated with previous discoveries, for the first time, the present study has reported the toxic effects of 3-hydroxy-2,2,4-trimethylpentyl isobutyrate against *Aedes aegypti* fourth instar larvae. In addition to this, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate isolated from *Aglaia edulis* exhibited a prominent range of toxicity against *Aedes aegypti*. Previous studies have validated that a higher concentration of 2,2,4-Trimethyl-1,3-pentanediol can offer a considerable range of toxicity (Astill, Terhaar, & Fassett,

1972). However, no previous research has studied the toxic nature of 2,2,4-Trimethyl-1,3-pentanediol against *Aedes aegypti*.

Despite many studies focussed on the larvicidal efficacy of plant extracts against *Aedes aegypti*, it is found that almost all the investigations were focussed on the preliminary studies; none piloted investigations on the larvicidal potential of isolated bioactive elements from *Pogostemon auricularius*, *Jasminum brevilobum*, and *Aglaia edulis* with special reference to the free radical formation. This highlights the uniqueness and distinctness of the present investigation.

A number of previous reports have been carried out on the larvicidal efficacy of bioactive compounds from various medicinal plants. However, studies concerning the larvicidal potential of bioactive elements isolated from the medicinal plants *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius* are scanty in the current research. The medicinal and pharmacological benefits of various extracts prepared from these three plants were rarely studied in previous investigations. For example, the extracts prepared from *Aglaia* spp. (*Aglaia edulis*, *Aglaia odorata*, and *Aglaia elaeagnoidea*) are known to produce high pharmacodynamic activity against *Entamoeba histolytica* (Tasanor et al., 2007). The presence of various phytochemical constituents with potent cytotoxicity from *Aglaia edulis* was previously reported (Kim et al., 2006). The major compounds with potent cytotoxicity reported by them are i)19,20-dehydroedulirin A (7), isoedulirin A, ii) edulirin A 10-O-acetate, iii) 3‘-methoxyaglaroxin A 1-O-acetate and iv) aglaroxin A 1-O-acetate. Similarly, the two novel compounds (eximiamide A and eximiamide B) were isolated from the

bark of *Aglaia eximia* with potent cytotoxcicty. The afore-mentioned studies have verified two significant concerns; i) the *Aglaia edulis* contain various bioactive elements, ii) studies concerning the larvicidal potential of isolated bioactive compounds from *Aglaia edulis* are scanty in the previous studies.

In addition to this, the phytochemical analysis of *Jasminum* spp. for their pharmacological benefits has reported previously. The presence of secondary metabolites such as flavonoids, glycosides, tannins, alkaloids, steroids, terpenoids, anthocyanins, coumarins, phlobatinins, saponins and leucoanthcyanins in *Jasminum* spp. have verified the pharmacological benefits. The antioxidant, antimicrobial, antifertility, and insecticidal activities of them have already been reported. One of the major common species, which is known to produce the afore-mentioned effects, is the *Jasminum officinale*. The various extracts prepared from the *Jasminum officinale, Jasminum grandiflorum* and *Jasminum auriculatum* are also able to produce mosquito larvicidal activity against filarial vector *Culex quinquefasciatus*. However, studies concerning the larvicidal potential of various bioactive elements isolated from *Jasminum brevilobum* are rare in the current scenario, which makes this study unique.

The therapeutic potential of *Pogostemon* spp. have already been reported in previous studies since it is considered as an important element in fragrance industries. In addition to this, the traditional use of *Pogostemon auricularis* in India, China, Bangladesh and Malaysia for stomachache, hysteria, discomfort, headaches, abdominal pain, colds, nausea, snake bites, diarrhea, fever, insect bites, and vomiting has been previously reported. They also analyzed the presence of

various phytochemical constituents including phenolic, tannin, alkaloids, flavonoids, glycosides, phytosterols, saponins, fats, cardiac glycosides, fixed oils in the leaf of *Pogostemon auricularis* to confirm their pharmacological benefits (Kamaleswari & Nandagopalan, 2017). However, studies concerning the potential use of isolated bioactive compounds from *Pogostemon auricularis* for mosquito control are scanty in the current research. These afore-mentioned aspects have made this study unique. This has indicated that an important fragment of the flora consists of various aromatic and medicinal plants that are prominently used as the raw materials for fragrance, pharmaceutical, and drug industries. In spite of much development that happened in the drug research, plants and their phytochemical constituents are still recognized to be the important sources of insecticides. One of the basic reasons for the prominent use of plant-based products as an alternative to synthetic insecticides is that, it provides novel modes of action against mosquitoes. The plant-based products also reduce the chance of developing cross-resistance in target species (Liu & Liu, 2014). Therefore, studies based on the phytochemical constituents with special emphasis on insect control have gained much more significance than other studies.

Over the last decade, significant efforts have been developed to propose phytochemical constituents as effective alternatives to synthetic insecticides, due to their high biodegradability, effectiveness, and low toxicity towards non-target organisms. The lack of novel insecticides, the emergence of insecticide resistance, high cost, alarm for environmental sustainability, and extensive biological magnification have been recognized as foremost factors that forced the public community to use bio-derived products (Brown, 1986). In addition to this,

the Environmental Protection Act in 1969 has outlined a number of limitations and regulations regarding the frequent use of insecticides. The afore-mentioned facts have prompted the scientific community to search for alternative strategies to focus on the eco-attractive natural insecticides. The exploration of floral biodiversity can be considered as one of the most efficient approaches in natural insecticide development for mosquito control (Ghosh et al., 2012).

The plant-based insecticides constitute various phytochemical constituents, which act in a concerted manner on both physiological and behavioural processes. As a result, there is a very slight chance of emerging insecticide resistance in the target mosquitoes. Therefore, identifying bio-active elements that are effective against *Aedes aegypti* mosquitoes, as well as being suitable to ecological circumstances, is exigent for continued effective mosquito control management (Ghosh et al., 2012). This has indicated that the results from the present study provide a major milestone in the quest for innovative vector control strategies based on bioactive elements.

The previous studies have discussed the mosquitocidal activity of plants. However, most of the studies focussed on the basic research were based on the various extracts prepared from the plants. The following plants were previously reported with potent larvicidal efficacy against various mosquito vectors; *Abrus precatorius*, *Abutilon indicum*, *Acacia caesia*, *Acacia pennata*, *Acacia nilotica*, *Acalypha alnifolia*, *Acalypha indica*, *Acanthophora muscoides*, *Acer campestre*, *Acer cissifolium* (Siebold & Zucc.), *Acer negundo*, *Acer platanoides*, *Acer pseudoplatanus*, *Achillea millefolium*, *Achyranthes aspera*, *Adansonia digitata*,

Adenanthera pavonina, Adhatoda vasica Nees, *Adiantum raddianum, Aegle marmelos, Ageratina adenophora, Ageratum conyzoides, Aglaia elaeagnoidea, Albizia polyantha, Allium sativum, Aloe fibrosa, Aloe ngongensis, Aloe turkanensis, Aloe vera, Alstonia scholaris, Alternanthera sessilis, Anacardium occidentale, Andrographis paniculata, Andrographis lineata, Angelica archangelica, Anisomeles indica, Anisomeles malabarica, Annona glabra, Annona reticulata, Annona senegalensis, Annona squamosa, Anthemis tinctoria, Apium graveolens, Aristolochia bracteata, Aristolochia indica, Artemisia abrotanum, Artemisia afra, Artemisia annua, Artemisia campestris, Artemisia nilagirica, Asparagus racemosus, Asteranthe asterias, Asteranthe lutea, Astrodaucus persicus, Atalantia monophylla, Azadirachta indica, Balanites aegyptiaca, Balsamita major, Barleria cristata, Basella rubra, Biophytum sensitivum, Boenninghausenia albiflora, Bombax malabaricum, Borago officinalis, Bougainvillea glabra, Bryonia dioica, Bryopsis pennata, Bupleurum exaltatum, Caesalpinia pulcherrima, Callistemon rigidus, Callistemon viminalis, Calotropis procera, Campanula longistyla, Canna indica, Capsicum annuum, Cardiospermum halicacabum, Carica papaya, Carissa carandas,Cassia angustifolia, Cassia auriculata, Cassia mimosoides, Cassia obtusifolia, Cassia occidentalis, Cassia roxburghii, Catharanthus roseus, Cedrus deodara, Ceiba pentandra, Centella asiatica, Cestrum diurnum, Cestrum nocturnum, Chloroxylon swietenia, Chomelia asiatica, Chrysanthemum cinerariifolium, Chrysanthemum indicum, Cichorium intybus, Citrullus colocynthis, Citrus aurantifolia, Citrus sinensis, Citrus grandis, Clausena anisata, Clausena dentata, Clausena lansium, Cleistanthus collinus, Cleome rupicola, Cleome viscosa,*

Clerodendrum chinense, *Clerodendrum inerme*, *Clerodendrum phlomidis*, *Clitoria ternatea*, *Coccinia indica*, *Coccoloba mollis*, *Cocculus hirsutus*, *Coldenia procumbens*, *Coleus amboinicus*, *Coleus aromaticus*, *Commiphora berryi*, *Convolvulus cantabrica*, *Cosmos bipinnatus*, *Cotula cinerea*, *Couroupita guianensis*, *Croton bonplandianum*, *Croton macrostachyus*, *Croton sylvaticus*, *Cryptomeria japonica*, *Cucumis sativus*, *Cunninghamia konishii*, *Curcuma amada*, *Curcuma xanthorrhiza*, *Curcuma heyneana*, *Curcuma mangga*, *Cymbopogon citratus*, *Cynodon dactylon*, *Dalbergia sissoo*, *Delonix elata*, *Derris sp.*, *Derris heterophylla*, *Derris urucu*, *Dicranopteris linearis*, *Dictamnus albus*, *Dolichos biflorus*, *Dracocephalum moldavica*, *Dregea volubilis*, *Echinacea purpurea*, *Eclipta paniculata*, *Eichhornia crassipes*, *Ervatamia coronaria*, *Erythrina indica*, *Eugenia jambolana*, *Euodia ridleyi*, *Eupatorium odoratum*, *Euphorbia hirta*, *Euphorbia rothiana*, *Evodia rutaecarpa*, *Feronia elephantum* Corrêa, *Ferula lancerottensis*, *Ficus benghalensis*, *Ficus microcarpa*, *Ficus racemosa*, *Ficus religiosa*, *Foeniculum vulgare*, *Fumaria indica*, *Galatella villosa*, *Garcinia mangostana*, *Gardenia carinata*, *Gilia capitata*, *Glebionis coronaria*, *Gloriosa superba*, *Gluta renghas*, *Gmelina asiatica*, *Gossypium herbaceum*, *Gossypium hirsutum*, *Guarea kunthiana*, *Guettarda grazielae*, *Gymnema sylvestre*, *Habenaria plantaginea*, *Halodule uninervis*, *Halophila ovalis*, *Hedyotis puberula*, *Hemidesmus indicus*, *Hibiscus rosa-sinensis*, *Hiptage benghalensis*, *Holarrhena antidysenterica*,*Holostemma ada-kodien*, *Hoslundia opposita*, *Hugonia mystax*, *Humulus japonicus*, *Hymenodictyon orixense*, *Hydrocotyle javanica*, *Hypericum perforatum*, *Hypericum polyanthemum*, *Hyptis suaveolens*, *Hyssopus officinalis*, *Ichnocarpus frutescens*, *Imperatoria*

ostruthium, Indonesiella echioides, Inula britannica., Inula helenium, Inula racemosa, Ipomoea cairica, Ipomoea carnea, Jacobaea maritima, Jasminum nervosum, Jatropha curcas, Justicia procumbens, Kaempferia angustifolia, Kaempferia rotunda, Knema attenuata, Laburnum anagyroides, Lansium domesticum, Lantana camara, Laurencia papillosa, Laurus nobilis, Matricaria maritima, Medicago romanica, Melanochyla fasciculiflora, Millettia ferruginea, Mentha arvensis, Merremia aegyptia, Merremia emarginata, Microdictyon pseudohapteron, Mimosa pudica, Mimusops elengi, Momordica charantia, Nelumbo nucifera, Nerium oleander, Nicandra physalodes, Nicotiana tabacum, Nyctanthes arbortistis, Ocimum basilicum, Ocimum canum, Ocimum sanctum, Ocotea velloziana, Origanum vulgare, Ormocarpum cochinchinense, Ormosia arborea, Orthosiphon thymiflorus, Otanthus maritimus, Padina australis, Parthenium hysterophorus, Passiflora foetida, Pavonia zeylanica, Pedalium murex, Pedilanthus tithymaloides, Pelargonium graveolens, Pereskia bleo, Pergularia daemia, Persea americana, Pergularia extensa, Phyllanthus emblica, Physalis alkekengi, Piper aduncum, Piper longum, Piper nigrum, Piper ribesioides, Piper sarmentosum, Pithecellobium dulce, Plectranthus amboinicus, Plumbago zeylanica, Polyalthia tanganyikensis, Prosopis juliflora, Psychotria nilgiriensis, Pteridium aquilinum, Quassia africana, Quercus infectoria, Quisqualis indica, Reseda odorata, Rhinacanthus nasutus, Rhizophora apiculata, Rhizophora mucronata, Ricinus communis, Rourea doniana, Rubia tinctorum, Rubus ellipticus, Ruellia tuberosa, Salicornia fruticosa, Salvadora persica, Salvia farinacea, Salvia officinalis, Salvia verbenaca, Salvia verticillata, Salvia viridis, Sargassum binderi, Sargassum wightii, Sapindus emarginatus,

Saponaria officinalis, *Satureja hortensis*, *Schisandra chinensis*, *Scindapsus officinalis*, *Scrophularia nodosa*, *Senecio laetus*, *Senna didymobotrya*, *Senna occidentalis*, *Sesamum indicum*, *Sesbania grandiflora*, *Seseli tortuosum*, *Sida acuta Burm*, *Sideritis euxina*, *Solanum nigrum*, *Solanum torvum*, *Solanum variabile*, *Solanum villosum*, *Solanum violaceum*, *Solidago Canadensis*, *Sonchus arvensis*, *Sonneratia alba*, *Sonneratia caseolaris*, *Spermacoce hispida*, *Spermacoce latifolia*, *Spermacoce verticillata*, *Sphaeranthus indicus*, *Spilanthes acmella*,*Spilanthes acmella*, *Spilanthes mauritiana*, *Spilanthes paniculata*, *Spondias mombin*, *Stachys byzantina*, *Stachys cretica*, *Suaeda maritima*, *Syringodium isoetifolium*, *Syzygium aromaticum*, *Tabebuia avellanedae*, *Tagetes minuta*, *Tagetes patula*, *Tanacetum vulgare*, *Tephrosia purpurea*, *Terminalia chebula*, *Terminalia fagifolia*, *Tessmannia densiflora*, *Tessmannia martiniana*, *Teucrium chamaedrys*, *Teucrium hircanicum*, *Thespesia populnea*, *Thevetia peruviana*, *Thymus serphyllum*, *Thymus vulgaris*, *Tinospora cordifolia*, *Tithonia diversifolia*, *Trachyspermum ammi*, *Trema orientalis*, *Tribulus terrestris*, *Trichosanthes anguina*, *Tridax procumbens*, *Triplaris americana*, *Tropaeolum majus*, *Turnera ulmifolia*, *Ulva lactuca*, *Umbilicaria aprina*, *Uvaria faulknerae*, *Uvaria kirkii*, *Uvaria leptocladon*, *Uvaria lungonyana*, *Uvaria scheffleri*, *Uvariodendron pycnophyllum*, *Uvariodendron usambarense*, *Valeriana hardwickii*, *Ventilago maderaspatana*, *Vernonia ammophila*, *Vernonia cinerea*, *Verbascum pinnatifidum*, *Verbena officinalis*, *Vetiveria zizanoides*, *Vitex cymosa*, *Vitex negundo*, V*itex payos*, *Vitex trifolia*, *Withania somnifera*, *Wrightia tinctoria*, *Xanthium strumarium*, *Zanthoxylum* spp., *Zingiber officinale*, *Zingiber zerumbet*, and *Zornia diphylla* (Pavela et al., 2019).

In addition to this, the following studies analyzed the various extraction process, screening methodologies, botanical ovicides, synergistic effects of plant extracts, growth impeding phytochemicals and effects on non-target organisms with special reference to mosquito control; (Roark, 1947) and (Sukumar, Perich, & Boobar, 1991). The authors listed the larvicidal efficacy of various herbal products from trees, herbs, edible crops, marine plants and ornamental plants. They also mentioned the mode of action of bio-derived products towards the different life stages of various mosquito vectors as a reference point for future investigations.

The larvicidal potential of isolated bioactive elements against mosquitoes can vary depending on the plant part used plant species, age of the plant, the solvent used for extraction and the vector species.

Sukumar et al. (1991) have verified the variation in the larvicidal potential of phytochemical constituents on target mosquito vectors. The results from this study also point to the afore-mentioned concept since the three plant species such as *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius* showed different range of larvicidal efficacy against *Aedes aegypti*. Based on the previous studies we can assume that the probable reasons for the varied larvicidal efficacy against *Aedes aegypti*is might be due to i) The method of extraction, ii) the solvent used for extraction, iii) the polarity index, iv) the different range of toxicity induced by the various phytochemicals present in the plant and v) the plant part used. However, previous studies have reported that the geographical origin of the plant is having a significant role in determining its larvicidal potential (Ghosh et al. 2012). They have listed the following plants with diverse larvicidal potential

with special emphasis on their geographical origin; *Jatropha* sp, *Momordica charantia, Azadirechta indica, Curcuma domestica, Jatropha curcas, Cestrum diurnum, Tridax procumbens, Euphorbia* sp, *Euphorbia tirucalli, Azadirechta indica, Annona squamosal, Moringa oleifera, Ocimum sanctum, Cestrum diurnum, Solanum xanthocarpum,* and *Annona squamosal*. They also reported that the *Solenostemma argel* showed the lowest LC_{50} value against *Culex pipiens*; whereas, many other plants used in their study (*Cryptotaenia paniculata, Centella asiatica, Nyctanthes arbotristis,* and *Atlantia monophylla*) has shown promising larvicidal efficacy against same vector species. Considering these, previous studies suggest that the extracts would be fractioned in order to find out the particular bioactive elements responsible for toxicity. Therefore, the present study has fractioned the acetone extracts of *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius* to isolate the following bioactive elements; i) Jatamansone, ii) 3-hydroxy-2,2,4-trimethylpentyl isobutyrate, iii) 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate. Bioactive guided chromatographic fractionation, GC-MS, FTIR, and NMR has been used for the isolation and characterisation of bioactive elements from the three medicinal plants such as *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius*. The GC-MS analysis of the bioactive fractions EE5, CC7, and PP4 revealed the presence of three afore-mentioned compounds with a strong match to the National Institute of Standards and Technology (NIST) library. The GC-MS retention time, area, area%, molecular weight (g/mol) together with their mass spectrum (Table 2.5; Page No. 152) clearly confirmed their characterization.

As stated formerly, it is very common in the previous investigations to see the larvicidal efficacy of plant extracts against mosquitoes without a bio-assay guided fractionation. Often the activity of plant extracts is reported only and this study is left to assume, on which bioactive elements are responsible for the toxicity of the plant as a whole. There exists a comprehensive analysis of individual bioactive elements for a few of the phytochemical constituents isolated in the present study. For example, the Jatamansone was previously isolated from various medicinal plants and found to exhibit pharmacologological benefits. The 3-hydroxy-2,2,4-trimethylpentyl isobutyrate isolated from *Pogostemon auricularius* was previously reported from a few medicinal plants, and it was shown to exhibit antibacterial activity against *Clostridium perfringens*. 3-hydroxy-2,2,4-trimethylpentyl isobutyrate was reported to be toxic to several organisms. Moreover, the toxic nature of the afore-mentioned compounds against *Aedes aegypti* is not well established in previous reports. However, the toxic nature of other phytochemical constituents against mosquito vectors, especially against *Aedes aegypti* is popularly studied. For example, previous studies have reported that neoprocurcumenol and 9-oxoneoprocurcumenol isolated from *Curcuma aromatic* could be introduced as one of the most effective candidates to prevent the reappearance of mosquito vectors (Madhu et al., 2010).

The previous studies have reported the larvicidal efficacy of various bioactive compounds such as visnagin, khellin (furanochromones), visnadin (pyranocoumarins), squamocin G (acetogenins), artemisinin, dihydroartemisinin (sesquiterpene lactones), atalantin (limonoids), β-sitosterol (phytosterols), limonin (limonoids), cleomdiolic acid (secodammarane triterpenes), pectolinaringenin

(flavones), emodin (anthraquinones), α-mangostin (prenylated xanthones), nuciferine, neferine (alkaloids), persin (acetogenins), 1,2,4- trihydroxynona decane (alkane derivatives), piperine, piperlongumine (alkaloid amides), scinamide A, scinamide B, solasodine (alkaloids), spilanthol (alkylamides), thiotagetin A, thiotagetin B (thiophenes), deguelin, ephrosin, and rotenone (rotenoids) (Pavela et al., 2019).

A study on the larvicidal potential of squamocin G isolated from *Annona squamosal*reported a lower LC_{50} value of 0.01 against *Aedes aegypti*. In addition to this, artemisinin (isolated from *Artemisia annua*), emodin (isolated from *Coccoloba mollis*), α-mangostin (isolated from *Garcinia mangostana*) was known to exhibit least LC_{50} values ranging from 1.5 to 2.2. According to a study by Godara et al. (2018), the piperine and piperlongumine isolated from *Piper longum* and *Piper nigrum* have significant larvicidal potential on (LC_{50} 3 to 4.6) *Culex pipiens, Aedes aegypti*, and *Aedes togoi* mosquitoes. In corroboration with their significant results, the present study has verified the similar range (LC_{50} 1.724 to 3.356) of toxicity against *Aedes aegypti*. The results from the present investigation are similar to previous reports who determined the larvicidal activity of deguelin (LC_{50} = 1.6), tephrosin (LC_{50} = 1.4), and rotenone against *Aedes aegypti* mosquito vectors (LC_{50} = 1.6). Furthermore, according to the previous studies on the larvicidal potential of the afore-mentioned bioactive compounds, the authors not only focus on the larvicidal efficacy of compounds but also targeted on the mode of action of those compounds for effective vector control.

The advantage of examining the modes of action of phytocompounds on the target organism is that it will offer great support for effective mosquito control strategies. For example, the squamocin G has often been investigated for its larvicidal efficacy with special reference to the inhibition of NADH ubiquinone oxydoreductase, midgut damage, reduction of protein level, neuronal toxicity as modes of action (Chen et al., 2012). Meanwhile, artemisinin, emodin, piperine, and deguelin were reported with ROS generation, post ingestive damage, nicotinic acetylcholine activated cation-selective channel activity, and inhibition of NADH as modes of action in the target vector mosquitoes. Next, α-mangostin was reported for blocking of sterol carrying proteins in the *Aedes aegypti* and *Anopheles gambiae* mosquito vectors. Limonin and khellin were reported in previous studies for their inhibition action (inhibition of glutathione Stransferase) in the target mosquito vector *Aedes aegypti*. These afore-mentioned studies have indicated that some of the compounds have been allied with the GABAergic system (Kadir, Zakaria, Kechil, & Azirun, 1989).Considering all these afore-mentioned aspects, the output obtained from this study proved to be efficient to that of some commercially available insecticides. However, practical assumptions need to be studied in further investigations.

The studies concerning the use of plant-based products are moving forward as the users mandate means of escaping from arthropod bites since the natural insecticides are safe, biodegradable and eco attractive. Many studies were beheld at augmenting the formulations of bioactive elements from traditionally used medicinal plants through the bioassay-guided chromatographic fractionation and advanced technologies. The local production of natural insecticide using

bioactive elements from plants would definitely remove the considerable expense of importation in developing countries, like in the Indian scenario. The field of natural insecticide development from the traditional medicinal plants is extensively fertile due to the presence of a plethora of phytochemical constituents seen in plants as defenses against various insects. In the past few years, certain plant-derived compounds have been verified to be safe and efficacious to compete with synthetic insecticides in the field of vector control. However, the number of bioactive compounds discovered against mosquito vectors is scanty in the past few years since most of the studies have been a focus on the plant extracts as a preliminary investigation. In order to overcome such situations, the present study has used bio-assay guided chromatographic fractionation, FTIR, NMR, GC-MS to isolate and characterize efficient bioactive elements from three traditionally used medicinal plants such as *Pogostemon auricularius*, *Jasminum brevilobum*, and *Aglaia edulis*. This study highlights the use of the isolated bio-active elements such as Jatamansone, 3-hydroxy-2,2,4-trimethylpentyl isobutyrate, and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate; those can be exploited for mosquito control strategies in the future.

The output from this investigation can be exploited to diminish the chance of financial burden in developing countries augmented by the importing of synthetic insecticides. The local production of natural insecticide using the afore-mentioned compounds would definitely reduce the considerable expense of importation in developing countries, like those in the Indian scenario. The findings of this investigation underpin that these three bioactive compounds can be considered for mosquito control approaches thereby diminishing the chance of

financial burden in developing countries augmented by the importation of synthetic insecticides for mosquito control. In the subsequent chapters, the susceptibility status, influence of ROS production, the role of microbial consortia in vector control with special emphasis on the isolated bioactive elements will be discussed.

2.5. SUMMARY

Mosquitoes are recognized as a major group of arthropods transmitting severe diseases to humans. Unfortunately, the prevention of mosquito-borne diseases is becoming challenging in the current scenario since there are no specific vaccines available for most of the severe diseases including dengue. Approximately 700,000 people have died every year due to the threat allied with etiologic diseases transmitted by mosquito vectors. Therefore, mosquito control is still a significant form of disease eradication. Furthermore, the insecticide resistance and other environmental hurdles induced by the synthetic insecticides have lowered the efficacy of synthetic insecticide-based strategies in developing countries. Such drastic situations call for the use of insecticides prepared from medicinal plants as an effective alternative measure. The present study describes the isolation and characterization of bioactive elements from medicinal plants, highlights the importance of isolated compounds in preventing the rapid spread of dengue, and discusses the current status of phytochemical compounds-based studies which may lead to the development of natural insecticides for mosquito control.

The findings of this study have revealed that the three bioactive elements isolated from *Aglaia edulis, Jasminum brevilobum,* and *Pogostemon auricularius* could be considered as an innovative notion for the development of natural insecticide against *Aedes aegypti* in future. Among the three isolated compounds, "2,2,4-Trimethyl-1,3-pentanediol diisobutyrate" isolated from *Aglaia edulis* is considered

as the most efficient bioactive element that shows prominent larvicidal efficacy against *Aedes aegypti* with lower LC_{50} value of 1.724 (0.834-2.479).

Further more, the "Jatamansone" isolated from *Jasminum brevilobum* (LC_{50} - 2.213 (1.292 - 3.032) and "3-hydroxy-2,2,4-trimethylpentyl isobutyrate" isolated from *Pogostemon auricularius* (LC_{50} - 3.356 (2.331 - 4.518) also exhibited a prominent range of toxicity against *Aedes aegypti*. In addition to this, the present investigation also reported the larvicidal potential of various bioactive fractions isolated during the time of bio-assay guided chromatographic fractionation. The output gathered from this investigation suggests that the use of plant-based products for vector control would enhance the various mosquito control strategies thereby diminishing the risk to humans followed by reduction in the financial burden and environmental threats. However, further studies concerning the susceptibility status of the afore-mentioned bioactive elements should be performed to find out the chance of developing insecticide resistance in target mosquitoes.

CHAPTER III

Susceptibility Status of 4th Instar Larvae of *Aedes aegypti* towards the Plant Isolates

3.1. INTRODUCTION

The genus *Aedes* is considered one of the most common groups of mosquitoes usually found in the tropics and subtropics all over the world. Generally, the mosquitoes belong to this genus are known for their invasive nature and can transmit a wide range of infectious agents to humans. In which, *Aedes albopictus* and *Aedes aegypti* are illustrated as the primary vectors of major arboviral diseases that already induced drastic effects in public health (Hartjes, 2011).

Aedes aegypti is the major vector that transmits the dengue viruses to humans. Unplanned urbanization, rapid population growth, and poor water management systems have promoted the rapid proliferation of *Aedes aegypti* mosquitoes. Dengue fever, a rapid spreading arboviral disease is prevalent in more than 100 nations with 390 million infections every year. The countries that are in the Asia Pacific zones including India were reported with the heaviest burden of infection for the past years. The statistical analysis from the "National Vector Borne Disease Control Programme (NVBDCP), Ministry of Health & Family Welfare, Government of India" indicated that about 136422 of dengue cases followed by 132 deaths was observed from the various regions of India in 2019 (NVBDCP, 2020). However, it was observed that the death rate has reduced (from 172 to 132) from the previous year (2018). Although, the incidence of dengue infection has dramatically grown up in recent decades worldwide (1800 million people at risk). Due to the dengue endemicity that happened in Indian provinces, the World

Health Organization-South East Asia (WHO-SEA) has given the 'Category A' position for India (Nagpal et al., 2016).

This has indicated that the dengue infection is known to instigate economic challenges as well as public health problems that have been tried to suppress by the use of several vector control strategies. So, wide spectrum strategies, that are cost-effective and eco-attractive are proposed in dengue-infected regions. Based on the previous studies, it was observed that mainly three kinds of vector control strategies have been used. They are i) Physical Control, ii) Biological Control, and iii) Chemical Control (Rather et al., 2017).

The physical control strategies include GIS mapping, education of prevention methods, focused surveillance, community-based vector control programmes, characterization of oviposition sites. Advanced technologies like GIS mapping has been considered as an efficient tool in locating dengue epidemics since it can locate the dengue foci to treat the infected individuals with preventive measures (Gandhi, Chapla, Reddya Naik, & Gujju Gandhi, 2017). It has been previously reported that the success of vector control approaches depends upon the strategies involved, knowledge, the behaviour of the people and education. The education offers a base for an individual to deal with various preventive measures and the vector habitats. Madeira, Macharelli, Pedras and Delfino (2002) analyzed that sharing of information concerning the distribution of dengue fever conveys awareness among peoples to control dengue by the destruction of mosquito vector habitats. In addition to this, community-based control programmes are established to educate the community regarding the various preventive measures for the

extermination of mosquito habitats. One of the major advantages of employing community involvement is that it can possibly integrate several techniques for maximum control of the mosquito vector population. Moreover, the determination of the oviposition pattern of *Aedes aegypti* has been playing a significant role in the reduction of population density of *Aedes aegypti* mosquitoes.

The paratransgenesis, one of the prominent techniques used for the control of *Aedes aegypti* mosquitoes, is based on the applications of genetically modified symbiotic bacteria. The genetically modified microorganisms have instigated deleterious effects in the sexual cycle of target organisms thereby diminishing the host competence in the target organism (Wilke & Marrelli, 2015). The use of crustacean and larvivorous fishes illustrate an eco-attractive, cost-effective and innovative approach in controlling *Aedes aegypti* mosquito population.

The afore-mentioned tasks and strategies often necessitate the participation of governmental and non-governmental sectors, sustained financial support, sufficient numbers of trained experts and personnel, and well-established mosquito control strategies. Despite epochs of systematized vector control struggles, the various mosquito-borne diseases such as lymphatic filariasis, malaria, Japanese encephalitis including dengue remain real threats in several regions all over the world (Chareonviriyaphap et al., 2013).

However, the use of various chemical compounds, insecticide-treated bed nets, and indoor residual sprays (IRS) reduced the density of infection rates to humans. Therefore, during the past epochs, scaling up of indoor residual spraying and long-

lasting insecticidal nets using synthetic insecticides have been a focal element in vector control strategies. Mainly four groups of synthetic compounds have been used for the afore-mentioned strategies, and are i) organophosphates, ii) pyrethroids, iii) organochlorine (DDT) and iv) carbamates. Among them, DDT was popularly known for its toxic effects against various insect vectors, especially against mosquitoes. The use of synthetic insecticides against mosquitoes has begun a steady drop in the later decades since it instigated adverse impacts on the environment that forced the public community to ban its use for indoor residual spraying (Chareonviriyaphap et al., 2013).

Moreover, the unmanaged and unrestrained use of synthetic insecticides has brought about the emergence of insecticide resistance in the target insect. For instance, the extensive use of synthetic insecticides like DDT has resulted in the emergence of insecticide resistance in various mosquito vector populations of *Anopheles culicifacies*, *Anopheles philippinensis*, *Anopheles aconitus*, *Anopheles nivipes*, *Aedes aegypti*, and *Culex quinquefasciatus*. However, the insecticide resistance has also been allied with all types of compounds including insect growth regulators and microbial-based agents. The ability of an insect to resist the toxic effects of an insecticide by means of mutations and adaptive natural selection is considered as the insecticide resistance (Hemingway, Hawkes, McCarroll, & Ranson, 2004).

Mainly, four mechanisms are concerned with the insecticide resistance and they are, i) reduced penetration, ii) behavioural modification, iii) metabolic detoxification and iv) target-site insensitivity. The reduced penetration is a slow-

down mechanism that involves the modification of the cuticle composition through structural cuticular proteins or epicuticular lipids. This allows the detoxification enzymes to act more time thereby developing phenotypes that are more resistant indicating the prominent role of metabolic detoxification and target-site insensitivity (Hemingway & Karunaratne, 1998).

The target-site insensitivity inhibits the bindings of synthetic insecticides in the target site while metabolic detoxification has linked with the detoxification of various enzymes such as glutathione S-transferases (GSTs), cytochromeP450 monooxygenases, and esterases. The insecticide resistance instigated by deltamethrin or permethrin, malathion, and aerosolized insecticides against the various mosquitoes has previously been studied. The authors also mentioned that the probable mechanisms responsible for the resistance against the afore-mentioned insecticides are due to the target site mutations and enzyme activity. Previous investigations also discussed the role of urban pollutants in the emergence of insecticide resistance. The insecticide resistance at high levels in mosquito vectors including *Aedes aegypti* can seriously obstruct the mosquito control programmes (Rose, 2001).

This has indicated that during the outbreak of arboviral diseases, the emergence of insecticide resistance in mosquito vectors are considered as severe risks to public health. Previous studies have reported that the bio-derived products are safer than that of synthetic insecticides since the synthetic insecticides are known to expose insecticide resistance after its frequent use for vector control. However, Berling (2009) reported the possible side effects of the bio-pesticides with special

inference on insecticide resistance. The author also described that at least 27 insects have developed prominent insecticide resistance towards the commonly used bio-pesticide over the world. Therefore, for the long-term aim of bio-control, the prevention of insecticide resistance is vital. Studies based on the afore-mentioned concepts in *Aedes aegypti* with special reference to the bioactive elements isolated from three medicinal plants such as *Aglaia edulis, Jasminum brevilobum*, and *Pogostemon auricularius* have not been previously investigated and this makes the present study unique. Pursuing the investigation concerning the influence of insecticide resistance, instigated against natural insecticides will provide specific attention to reducing the rate of mosquito-borne diseases in endemic regions.

3.2. MATERIALS AND METHODS

3.2.1. Isolated bioactive elements

The three bioactive elements such as Jatamansone (CC7); 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4); and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (EE5) were isolated from *Jasminum brevilobum*, *Aglaia edulis* and *Pogostemon auricularius* respectively.

3.2.2. Mosquito colony

Maintained as explained in general methodology (Page No. 75).

3.2.2a. Selection experiments

A susceptible laboratory colonized *Aedes aegypti* was placed under selection pressure with three bioactive elements isolated from the medicinal plants such as from *Jasminum brevilobum*, *Aglaia edulis* and *Pogostemon auricularius*. The following three compounds were exposed on the fourth instar larvae of *Aedes aegypti* for 10 consecutive generations. The parental strain (susceptible) colonized for 15 consecutive generations is designated as the F_1 generation. The afore-mentioned parental strain is known to free from isolated bioactive elements. The larvae from this parental line were chosen for bioassay to get LC_{50} at each generation. The WHO standard method (WHO, 1981) was employed for bioassay experiments. The required quantity of isolated bioactive elements such as Jatamansone; 3-hydroxy-2,2,4-trimethylpentyl isobutyrate; and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate was mixed thoroughly with 249 mL of dechlorinated water in glass beakers (500 mL). Ascending series of six concentrations of each

of the afore-mentioned compounds were exposed to the *Aedes aegypti* larvae. The determination of doses for each compound was completely based on the preliminary experiments. Parallel control groups were also maintained for all the executed experiments. Twenty-five fourth instar larvae were used in each experiment. After, 24 hours of continuous exposure at 55-60% relative humidity, 27±2°C temperature, and 12 L:12 D, the mortality rate was recorded.

The bioactive element exposed larvae were employed for selection experiments in every generation from F_1 to F_{10}. The larvae that were survived during the previous experiments in each generation were washed with tap water and set aside for rearing. The bioactive elements exposed larvae that survived (LC60 level) during the experiment produced F_2 generation progeny. The afore-mentioned process was consequently repeated up to 10 generations (F_{10}), thus excluding the susceptible larvae every time. The level of resistance that emerged by the isolated bioactive compounds against *Aedes aegypti* larvae in the succeeding generation (F_1-F_{10}) was calculated by the following formula prescribed by Gopalan et al. (1996).

$$Resistance\ (Fold\ increased) = \frac{\text{LC50 or LC90 Values of the resistant larvae}}{\text{LC50 or LC90 Values of the susceptible larvae}}$$

Probit regression analysis was used to get the LC_{50} and LC_{90} of the respective compounds against *Aedes aegypti* larvae. If the control mortality is ranged between 5 to 20%, Abbott's formula (Abbott, 1925) was used to correct the mortality data. Based on the WHO criteria, if the value of RR_{50} is more than 10, the strain is considered as resistant. If the RR_{50} is between 2 to 10, it was

considered as moderately resistant. The strain is recognized as susceptible when the RR_{50} is less than two.

3.2.3. Definition of key terms

The present study used the terms “CC7” (Jatamansone), “EE5” (2,2,4- Trimethyl-1,3- pentanediol diisobutyrate), and “PP4” (3-hydroxy- 2,2,4- trimethylpentyl isobutyrate) for respective isolated compounds.

3.3. RESULTS

Aedes aegypti fourth instar larvae were placed under isolated bioactive elements selection pressure. Each generation from F_1 to F_{10} was examined for its susceptibility. The Table 3.1-3.6 (Page No. 186-191) shows the level of mortality induced by the isolated bioactive elements against various generations of *Aedes aegypti* larvae. The LC_{50} and LC_{90} values along with the degree of freedom, Chi-square, and Resistance Ratio (RR) for respective generations of *Aedes aegypti* are given in Table 3.4-3.6 (Page No. 189-191). Table 3.1 (Page No. 186) represents the toxicity of isolated bioactive element CC7 against 10 generations of *Aedes aegypti* with special reference to the susceptibility status of *Aedes aegypti* fourth instar larvae. Table 3.2 (Page No. 187) summarizes the toxicity of isolated bioactive element EE5 against 10 generations of *Aedes aegypti*. The susceptibility status of *Aedes aegypti* against EE5 in the larval stage is presented in Table 3.5 (Page No. 190).

As stated in Table 3.1 (Page No. 186), in the F_1 generation of *Aedes aegypti*, the CC7 in the concentration of 0.75, 2.25, 3.75, 5.25, 6.75, and 8.25 ppm produced mortality of 24, 36, 48, 64, 72, and 96 percent respectively. The F_2 generation of CC7 treated *Aedes aegypti* mosquito larvae produced maximum mortality of 70.66% of mortality at 4.3 ppm. Similarly, the F_3 and F_4 generations of CC7 treated *Aedes aegypti* showed the highest degree of mortality at 4.6 and 5 ppm respectively. Moreover, the F_5 and F_6 generation showed potent toxicity against the CC7 treated *Aedes aegypti* fourth instar larvae. Likewise, in the F_7 generation,

the doses of 5.3, 5.4, 5.5, 5.6, 5.7, and 5.8 ppm produced 9.33, 17.33, 26.66, 42.66, 61.33, and 78.66 percent toxicity. In the F_8 generation, the highest larvicidal activity has been observed at a concentration of 6.1 ppm of CC7. Further concentrations of CC7 in subsequent generations such as F_9, and F_{10} produced potent larvicidal efficacy against *Aedes aegypti* fourth instar larvae.

The data on the toxicity of isolated bioactive element EE5 shows the highest degree of mortality in every generation at different concentrations. The EE5 exposed *Aedes aegypti* population exhibited 96% of mortality at 6.5 ppm in the F_1 generation. The susceptibility tests performed out using the EE5 exposed *Aedes aegypti* revealed that the larval mortality in the F_2 generation ranged from 34.66 (4.4 ppm) -78.66 (5.4 ppm) while that in the F_3 generation ranged from 30.66 (4.8 ppm) -77.33 (5.8 ppm) (Table 3.2; Page No. 187). The larval bioassay performed against the F_4 generation confirmed potent toxic effects ranging from 34.66 (5.3 ppm) to 82.66 (6.3 ppm) percentages. It is imperative to note that the subsequent generations of *Aedes aegypti* (F_5 and F_6) are also of the highest mortality rates. In F_7 generation the EE5 in the concentration of 6.87, 7.2, 7.4, 7.6, and 7.8 ppm produced mortality of 20, 36, 44, 60, 72, and 84 percent respectively (Table 3.2; Page No. 187). Tests of F_8 progeny *Aedes aegypti* fourth instar larvae showed prominent larvicidal potential (20, 28, 40, 52, 68, and 80 %) after continuous exposure to the various concentrations (7.3, 7.5, 7.7, 7.9, 8.1, and 8.3 ppm) of EE5. The remaining two generations (F_9 and F_{10}) tested with the bioactive element EE5 showed significant mortality range from 28 to 88%.

The data on the susceptibility status of *Aedes aegypti* have been shown in Table 3.6 (Page No. 191). It states the highest degree of mortality rate against the isolated bioactive element PP4. The susceptibility values of *Aedes aegypti* against the isolated bioactive element PP4 are shown in Table 3.6 (Page No. 191), while the mortality rate is presented as percentage listed in Table 3.3 (Page No. 188). The bioassay revealed that the F_1 generation of *Aedes aegypti* has exhibited a prominent range of toxicity against the isolated bioactive element PP4 as reported in Table 3.3 (Page No. 188). In the F_2 generation, the mortality rate was comparatively lower (66%) at a concentration of 3.3 ppm, but the mortality rate was increased in the subsequent experiments performed in F_3, F_4, and F_5 generation. Further concentrations of 3.1, 3.4, 3.7, 4, 4.1, and 4.3 ppm produced 21.33, 33.33, 45.33, 54.66, 62.66, and 68 percent mortality respectively in F_3 generation. The maximum mortality in F_4 was observed at a concentration of 5.5 ppm. In F_5, the concentrations of 5.1, 5.3, 5.8, 6.2, 6.5, and 6.8 ppm resulted 32, 42.66, 54.66, 62.66, 70.66, and 76 percent mortality respectively. A similar pattern was perceived in the F_6 and F_7 generation since both of this showed the highest degree of mortality ranged from 28 to 84%. However, it was important to note that, the concentration of the isolated bioactive element PP4 required to destroy the *Aedes aegypti* has increased from 1 to 8.7 ppm. Further, concentrations of 7.1, 7.4, 7.7, 8, 8.3, and 8.6 ppm produced 34.66, 46.66, 54.66, 65.33,76, and 82.66 percent mortality respectively in F_8 generation. Mortality ranging from 17 to 78 percent and 24 to 80 percentages after continuous exposure to the PP4 was observed in F_9 and F_{10} respectively.

Figs. 3.1-3.3 (Page No. 189-191) revealed the probit regression analysis of the isolated bioactive elements against 10 successive generations of *Aedes aegypti* fourth instar larvae. Fig. 3.4 (Page No. 192) represents the resistance ratio of the 10-successive generations of *Aedes aegypti*, which illustrates the ratios of the bioactive elements tolerance increase. The determination of resistance ratio was based on the comparative susceptibility of the F_1 generation (LC_{50} - 2.771 ppm, LC_{90} -14.971 ppm for CC7; LC_{50} - 4.071 ppm, LC_{90} -6.383 ppm for EE5; LC_{50} - 1.952 ppm, LC_{90} -6.068 ppm for PP4). Under continuous bioactive element selection, both the LC_{50} and LC_{90} values increased from F_1 to F_{10} generation. The LC_{50} of CC7 exhibited a gradual increase from 2.771 ppm in the F1 to 6.375 in F_{10} (RR - 2.30) (Table 3.4; Page No. 189). Based on the WHO criteria, the afore-mentioned strain (CC7 exposed) was recorded as moderately resistant since the RR_{50} is found to be ranged between 2 and 10. If the RR_{50} is over, the strain will be considered as resistant. The lower range of RR_{50} like fewer than two is considered as susceptible. The LC_{50} of EE5 showed a prominent increase from 4.071 ppm in the $F_1$to 8.760 ppm in F_{10} (RR-2.15) (Table 3.5; Page No. 190). This indicates that the EE5 exposed strain was recognized as moderately resistant. In the case of PP4 exposed strains, the LC_{50} values ranged from 1.952 ppm to 9.302 ppm, indicating a gradual increase in the LC_{50} values from F_1 generation to F_{10} generation. The resistance ratio for the same was found to be 4.76, revealing its moderate range of resistance. Among the three strains (CC7, EE5, PP4 exposed *Aedes aegypti*), the resistance ratio was found to be relatively high in (RR- 4.76) PP4 exposed strain followed by the CC7 (RR- 2.30) exposed strain (Table 3.6; Page No. 191). An increase in the resistance ratio based on the LC_{50} values was

found to be significant in studied generations ($p<0.05$). The regression equation for respective generations exposed with CC7, EE5 and PP4 was given in Figs. 3.1-3.3 (Page No. 189-191). While following the WHO criteria, it was observed that all the strains in the present study are moderately resistant, indicating the significance of bioactive elements from plants in mosquito vector control.

Table 3.1. Percentage mortality of *Aedes aegypti* exposed to CC7 at different concentrations

F_1		F_2		F_3		F_4		F_5	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
0.75	24±1.00	3.8	21±0.57	4.1	16±1.00	4.5	20±1.00	4.8	16±0.00
2.25	36±1.00	3.8	37±0.57	4.2	29±0.57	4.6	35±0.57	4.9	25±0.57
3.75	48±1.52	4	47±0.57	4.3	43±0.57	4.7	49±0.57	5	44±1.00
5.25	64±1.00	4.1	55±0.57	4.4	51±1.00	4.8	53±0.57	5.1	49±1.00
6.75	72±1.00	4.2	63±0.57	4.5	57±0.57	4.9	61±0.57	5.2	69±1.00
8.25	96±0.57	4.3	71±0.57	4.6	65±0.57	5	73±0.57	5.3	75±1.00
Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00
F_6		F_7		F_8		F_9		F_{10}	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
5.1	12±0.00	5.3	9±0.57	5.6	15±0.57	5.8	17±1.15	6.1	24±0.00
5.2	20±0.00	5.4	17±0.57	5.7	23±0.57	5.9	27±1.52	6.2	29±0.57
5.3	40±1.00	5.5	27±0.57	5.8	33±1.52	6	43±0.57	6.3	36±0.00
5.4	55±0.57	5.6	43±2.08	5.9	55±0.57	6.1	51±1.15	6.4	49±0.57
5.5	69±0.57	5.7	61±0.57	6	67±0.57	6.2	64±0.00	6.5	57±0.57
5.6	77±0.57	5.8	79±0.57	6.1	85±0.57	6.3	81±0.57	6.6	85±0.57
Control	0±0.00	Control	0±0.00	Control	1±0.57	Control	0±0.00	Control	0±0.00

*statistical significance ($P < 0.05$)

Table 3.2. Percentage mortality of *Aedes aegypti* exposed to EE5 at different concentrations

F_1		F_2		F_3		F_4		F_5	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
3.5	32±0.00	4.4	35±0.57	4.8	31±0.57	5.3	35±2.51	5.7	24±1.00
4	52±1.00	4.6	55±0.57	5	48±1.00	5.5	51±0.57	5.9	39±0.57
4.5	60±1.00	4.8	59±0.57	5.2	58±0.57	5.7	59±0.57	6.1	49±0.57
5	72±1.00	5	68±0.00	5.4	63±0.57	5.9	68±0.00	6.3	56±0.00
6	80±0.00	5.2	75±0.57	5.6	71±0.57	6.1	75±0.57	6.5	64±0.00
6.5	96±0.57	5.4	79±0.57	5.8	77±0.57	6.3	83±0.57	6.7	73±0.57
Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	1±1.15
F_6		F_7		F_8		F_9		F_{10}	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
6.3	24±1.15	6.8	20±0.57	7.3	20±1.00	7.9	28±1.00	8.4	24±0.57
6.5	44±1.00	7	36±1.00	7.5	28±1.00	8.1	36±0.00	8.6	36±0.00
6.7	52±0.57	7.2	44±1.00	7.7	40±1.15	8.3	48±0.00	8.8	52±0.57
6.9	60±0.57	7.4	60±1.00	7.9	52±0.57	8.5	60±1.00	9	60±0.00
7.1	80±1.52	7.6	72±0.57	8.1	68±0.57	8.7	76±0.57	9	72±0.57
7.3	84±0.00	7.8	84±0.57	8.3	80±0.57	8.9	80±0.00	9.2	88±1.15
Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00

*statistical significance ($P < 0.05$)

Table 3.3. Percentage mortality of *Aedes aegypti* exposed to PP4 at different concentrations

F_1		F_2		F_3		F_4		F_5	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
1	21±0.57	1.9	19±1.15	3.1	21±0.57	4	29±0.57	5.1	32±0.00
1.3	36±0.00	2.2	27±0.57	3.4	33±0.57	4.3	37±0.57	5.3	43±0.57
1.6	40±0.00	2.5	35±0.57	3.7	45±0.57	4.6	45±0.57	5.8	55±0.57
1.9	45±0.00	2.8	48±0.00	4	54±0.57	4.9	52±1.00	6.2	63±0.57
2.2	53±0.00	3.1	59±0.57	4.1	63±0.57	5.2	63±0.57	6.5	71±0.57
2.3	61±0.00	3.3	67±1.15	4.3	68±0.00	5.5	68±0.00	6.8	76±0.00
Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	1±0.57
F_6		F_7		F_8		F_9		F_{10}	
Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD	Concentration (In ppm)	Mortality Mean ±SD
6	44±1.00	6.2	28±1.00	7.1	35±2.51	7.8	17±0.57	8.5	24±0.00
6.3	51±0.57	6.5	48±1.00	7.4	47±0.57	8.1	28±0.00	8.8	33±0.57
6.6	61±0.57	6.8	59±0.57	7.7	55±0.57	8.4	43±0.57	9.2	43±1.15
6.9	71±1.15	7.1	64±0.00	8	65±0.57	8.7	51±1.15	9.5	56±1.00
7.2	75±0.57	8.4	73±0.57	8.3	76±0.00	9	65±0.57	9.8	64±0.00
7.5	80±0.00	8.7	84±0.00	8.6	87±0.57	9.1	79±0.57	10.1	80±0.00
Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00	Control	0±0.00

*statistical significance ($P < 0.05$)

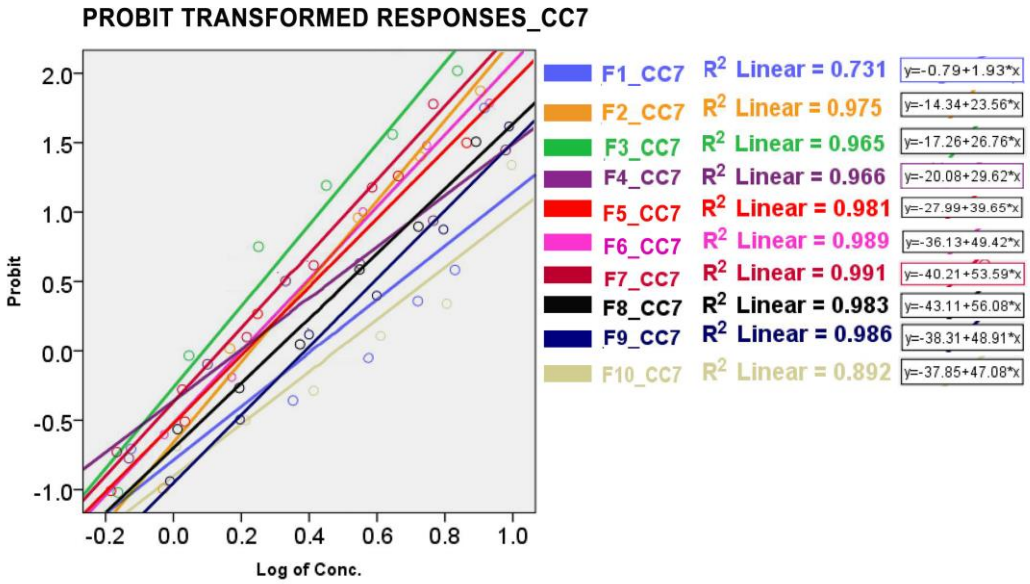


Fig. 3.1. Probit regression analysis of different generations of *Aedes aegypti* fourth instar larvae to the bioactive element CC7 selection pressure

Table 3.4. Bioassay results of bioactive element CC7 selection experiment on fourth instar larvae of *Aedes aegypti*

Sl. No	Generation	LC_{50} (In ppm)	Fiducial limit (In ppm)	LC_{90} (In ppm)	Fiducial limit (In ppm)	Chi-Square	df[b]	RR
1	F_1	2.771	0.879-5.141	14.971	7.066 - 828.271	7.55	4	-
2	F_2	4.059	3.968-4.160	4.606	4.399-5.251	0.40	4	1.46
3	F_3	4.416	4.335-4.535	4.942	4.731-5.570	0.61	4	1.59
4	F_4	4.761	4.677-4.854	5.267	5.082-5.788	0.66	4	1.71
5	F_5	5.079	5.014-5.152	5.472	5.340-5.764	0.59	4	1.83
6	F_6	5.383	5.327-5.445	5.714	5.610-5.920	0.41	4	1.94
7	F_7	5.628	5.575- 5.696	5.943	5.837-6.150	0.34	4	2.03
8	F_8	5.876	5.822- 5.935	6.193	6.095-6.380	0.64	4	2.12
9	F_9	6.072	6.009-6.143	6.452	6.327-6.721	0.43	4	2.19
10	F_{10}	6.375	6.305-6.456	6.799	6.652-7.145	2.7	4	2.30

*statistical significance ($P < 0.05$)

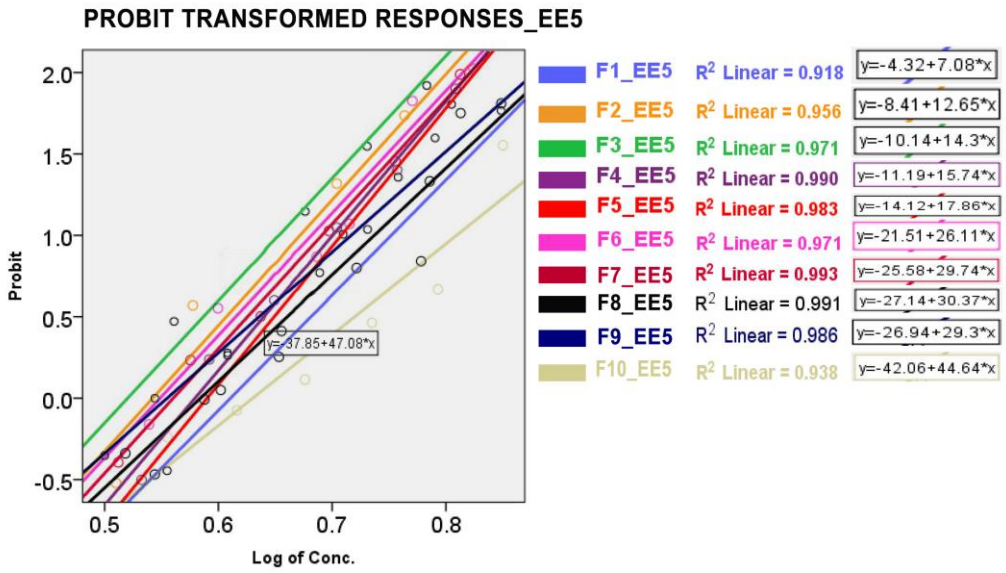


Fig. 3.2. Probit regression analysis of different generations of *Aedes aegypti* fourth instar larvae to the bioactive element EE5 selection pressure

Table 3.5. Bioassay results of bioactive element EE5 selection experiment on fourth instar larvae of *Aedes aegypti*

Sl No	Generation	LC_{50} (In ppm)	Fiducial limit (In ppm)	LC_{90} (In ppm)	Fiducial limit (In ppm)	Chi-Square	df^b	RR
1	F_1	4.071	3.603-4.407	6.383	5.698-8.020	1.94	4	-
2	F_2	4.618	4.246-4.799	5.824	5.423-7.331	0.62	4	1.13
3	F_3	5.113	4.827-5.287	6.285	5.879-7.707	0.43	4	1.25
4	F_4	5.543	5.249-5.706	6.600	6.270-7.607	0.17	4	1.36
5	F_5	6.172	5.977-6.361	7.289	6.873-8.619	0.28	4	1.51
6	F_6	6.664	6.502-6.792	7.467	7.230-8.000	0.85	4	1.63
7	F_7	7.248	7.112-7.374	8.009	7.775-8.510	0.23	4	1.78
8	F_8	7.827	7.696-7.970	8.625	8.360-9.215	0.24	4	1.92
9	F_9	8.306	8.137-8.448	9.184	8.907-9.852	0.32	4	2.04
10	F_{10}	8.760	8.639-8.861	9.375	9.193-9.768	1.8	4	2.15

*statistical significance ($P < 0.05$)

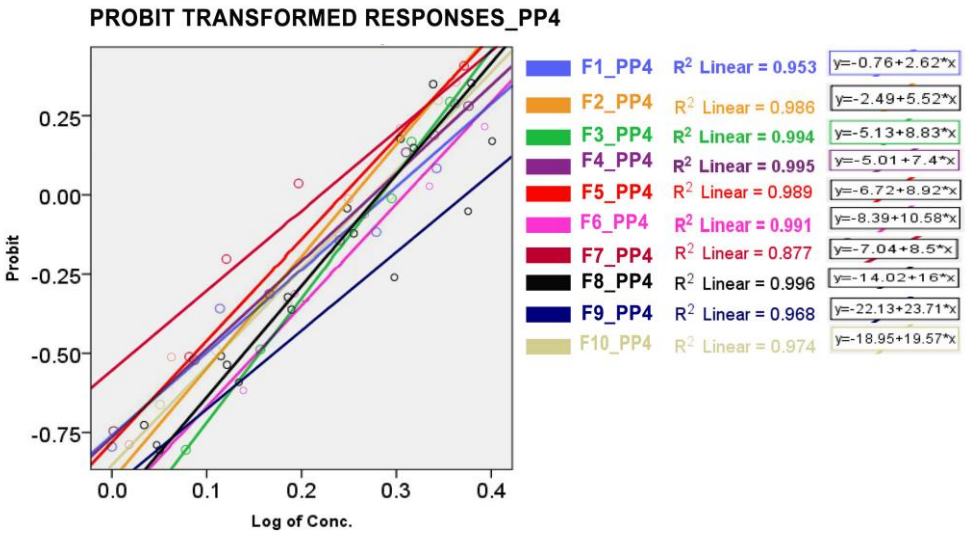


Fig. 3.3. Probit regression analysis of different generations of *Aedes aegypti* fourth instar larvae to the bioactive element PP4 selection pressure

Table 3.6. Bioassay results of bioactive element PP4 selection experiment on fourth instar larvae of *Aedes aegypti*

Sl No	Generation	LC_{50} (In ppm)	Fiducial limit (In ppm)	LC_{90} (In ppm)	Fiducial limit (In ppm)	Chi-Square	df[b]	RR
1	F_1	1.952	1.625-2.885	6.068	3.606-59.390	0.51	4	-
2	F_2	2.834	2.598-3.226	4.814	3.915-8.363	0.26	4	1.45
3	F_3	3.815	3.598-4.080	5.330	4.710-7.562	0.10	4	1.95
4	F_4	4.758	4.409-5.171	7.088	6.042-13.017	0.05	4	2.43
5	F_5	5.658	5.198-5.973	7.881	7.067-10.939	0.16	4	2.89
6	F_6	6.215	5.443-6.520	8.206	7.533-11.337	0.10	4	3.18
7	F_7	6.720	6.133-7.116	9.520	8.574-12.508	2.65	4	3.44
8	F_8	7.524	7.149-7.757	9.054	8.570-10.456	0.07	4	3.85
9	F_9	8.579	8.395-8.785	9.723	9.333-10.628	0.87	4	4.39
10	F_{10}	9.302	9.048-9.561	10.827	10.295-12.207	0.56	4	4.76

*statistical significance ($P < 0.05$)

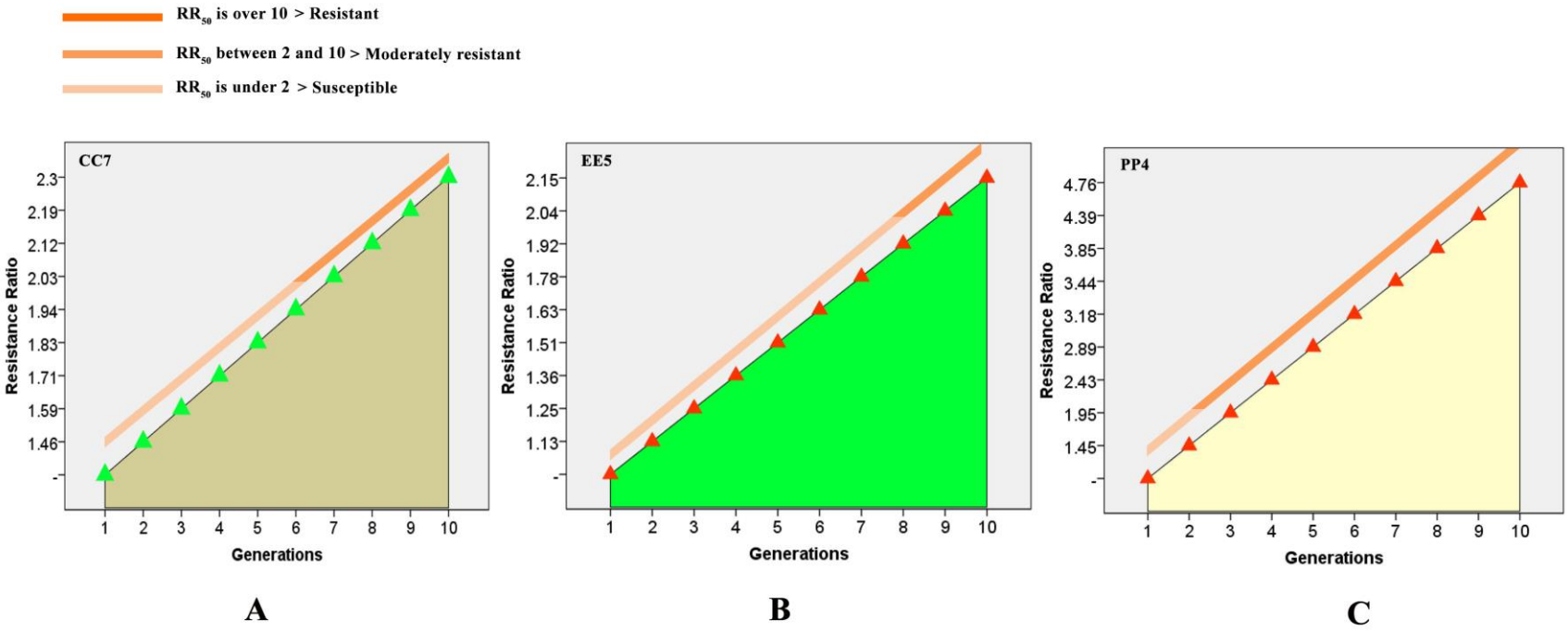


Fig. 3.4. Moderate level of resistance emerged in *Aedes aegypti* fourth instar larvae to the bioactive elements CC7, EE5 and PP4 isolated from *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius* respectively.

3.4. DISCUSSION

A striking increase in the morbidity and mortality allied with dengue infection has been reported in the current scenario in contrast to a slight drop in other vector-borne diseases. The *Aedes* spp. are prominent vectors of several severe infectious diseases such as dengue, chikungunya, yellow fever, Zika, and Rift Valley and this made the species a vital element in the global burden of mosquito-borne disease. The invasion of the Zika virus has exposed severe health impacts such as GBS and congenital ZIKV infection syndrome. The infection rate of dengue is rapidly increasing day by day and approximately 390 million peoples are infected every year. Due to this, the health officials of various national and international agencies have urged people to assure precautions to prevent mosquito bites. The expansion of dengue infections has been primarily associated with certain favouring conditions such as an unplanned urbanization, global trade, inefficient implementation of mosquito control programs, erratic water supply, lack of community engagement, and poor water storage practices (Horstick, Runge-Ranzinger, Nathan, & Kroeger, 2010). The global economic burden of dengue induced threats is still unclear. However, the economic losses instigated by dengue fever infection all over the world have been estimated to be around 9 billion US$ per annum (Stanaway et al., 2016).

There exists no effective vaccine against dengue fever, however, the Dengvaxia vaccine has now been known to be used against dengue in several countries. However, the lack of availability of the Dengvaxia vaccine against dengue is

gaining alarm in the current scenario (Su et al., 2019). Moreover, the use of prophylaxis for dengue is not available since the dengue virus transmission cannot be prevented during the time of the human infection phase. Mosquito control using insecticides coupled with habitat destruction in its larval stage is therefore assuredly vital in the control of various vector-borne diseases including dengue fever. Currently, the control of *Aedes aegypti* mosquito vectors is principally based on community engagement and synthetic insecticides (Su et al., 2019).

The practice alternative approaches including the use of natural insecticides of botanical origin is promising but the studies based on the bioassay-guided chromatographic fractionation of plant extracts are scanty in the current scenario. Only a few bioactive elements are reported previously regarding the potential applications of them in mosquito control with special emphasis on their mode of action. The prolonged period required for new vector control strategies mean that present natural insecticide-based strategies will play a vital role for many years to come. Prevention of the rapid proliferation of *Aedes aegypti* using synthetic insecticides, principally through IRS of organophosphate and pyrethroids, is fraught with numerous impediments including slow operational response and high expense (Esu, Lenhart, Smith, & Horstick, 2010).

The routine use of synthetic insecticides in health programmes as well as in vector control programmes have instigated several harmful effects such as hazardous effects to human beings, ecosystem destabilization, and environmental pollution. A key concern regarding the impediments induced by the synthetic insecticides is the rapid emergence of insecticide resistance with the capacity to diminish the

effectiveness of present insecticide-based mosquito control approaches. Resistance to various synthetic insecticides in mosquitoes can drastically impede the mosquito control programmes. In the era of emerging insecticide resistance, it is significant to study and develop natural insecticides to compete against the threat from synthetic products. During the outbreaks of vector-borne diseases, the emergence of insecticide resistance turns out to be a grievous risk to public health. Due to the afore-mentioned facts, the demand for natural insecticides for mosquito control has rapidly enhanced in the current scenario (Anoopkumar et al., 2017b). However, like synthetic insecticides, there has been a probability to emerge insecticide resistance against natural insecticides if the public community has frequently used natural insecticides for mosquito control. For that reason, determining the level of insecticide emergence in *Aedes aegypti* mosquitoes is paramount for effective vector control during the mosquito-borne disease outbreaks (Kandel et al., 2019). In addition to this, the data regarding the mechanism of insecticide resistance with special reference to isolated bioactive elements from medicinal plants are scanty. The afore-mentioned facts indicated that the emergence of resistance to insecticides in *Aedes aegypti* vector is a key challenge for mosquito control. Therefore, the investigations on the susceptibility status of mosquito vectors towards the insecticides will provide sufficient figure to aid novel insecticide choices for preventing dengue outbreaks. In this context, considering all the afore-mentioned aspects, the present study was evaluated the susceptibility status of 4^{th} instar larvae of *Aedes aegypti* towards the plant isolates by employing the standard larval bioassay procedure prescribed by WHO.

The present investigation revealed for the first time moderate resistance to bioactive elements isolated from three medicinal plants towards the fourth instar larvae of *Aedes aegypti*. It nurtures the discussions concerning the significance of the use of natural insecticide for mosquito control with particular relevance to public health interest and insecticide resistance. The objective of this investigation was to evaluate the candidate bioactive elements CC7, EE5, and PP4 with special reference to the susceptibility status of *Aedes aegypti* fourth instar larvae. In this objective, none of the examined *Aedes aegypti* strains were found to be highly resistant to the isolated bioactive elements. However, this study delivers the confirmation that the tested strains are moderately resistant to the three bioactive elements with low resistance ratio (RR).

This study has observed that the bioactive elements isolated from three medicinal plants such as *Jasminum brevilobum, Aglaia edulis,* and *Pogostemon auricularius* doesn't instigate threat towards mosquito control in terms of insecticide resistance since their higher concentration causes a moderate range of resistance in *Aedes aegypti* fourth instar larvae. One of the foremost explanations for the afore-mentioned statement to support the results of this study could be the lowest resistance ratio, compared to the previous investigation on synthetic insecticides. For example, a pyrethroid-selected strain of *Anopheles stephensi* has displayed 182 fold resistance against permethin (Enayati, Vatandoost, Ladonni, Townson, & Hemingway, 2003). Their results are in line with those of deltamethrin exposed *Anopheles sinensis* which showed 130 to190 folds as reported by Wang (2000). Similarly, Gayathri and Murthy (2006) have reported that the selection of *Anopheles culicifacies* against deltamethrin in Tamil Nadu, India, gives rise to

higher resistance. Kumar et al. (2002) reported that the continuous selection of *Aedes aegypti* for 40 generations with deltamethrin yields 703 fold resistance.

Previous studies have validated the emergence of resistance to pyrethroid and organophosphate in the dengue fever vector *Aedes aegypti*. For example, Kumar et al. (2002) reported the resistance emerged in *Aedes aegypti* to temephos and cypermethrin. The authors also discussed the gradual increase of resistance ratio (RR) from 9.0 to 192.7 in Brazil. In corroboration with their findings, Rodríguez, Bisset, Ruiz and Soca (2002) reported the emergence of high resistance against temephos in *Aedes aegypti* (six generations) with special reference to the use of the afore-mentioned organophosphate for *Aedes aegypti* control. A similar pattern was studied by Lima et al. (2003) who reported the emergence of insecticide resistance in *Aedes aegypti* against temephos. Similarly, Harris, Rajatileka and Ranson (2010) reported the high insecticide resistance of *Aedes aegypti* to pyrethroid insecticides and DDT. According to them, the resistance ratio for lambda-cyhalothrin, deltamethrin, and permethrin are >41.2, 29, and 434 respectively. Jirakanjanakit et al. (2014) suggested that it is essential to develop an alternative strategy for synthetic insecticides since the deltamethrin and permethrin have developed resistance in *Aedes aegypti* mosquitoes.

From the above-mentioned studies, it was clearly observed that most of all the synthetic insecticides exhibited a prominent range of resistance in various mosquito species especially in *Aedes aegypti* mosquitoes. The most commonly used synthetic insecticides such as deltamethrin, permethrin, lambda-cyhalothrinand, pirimiphos-methyl, and temephos exhibited a prominent range of

resistance ratio in various mosquito species in the least concentrations. In contrast with their results, the present study has reported for the first time that, the isolated bioactive elements such as CC7, EE, and PP4 only exhibited a moderate range of resistance (RR – under five for all the tested strains). For example, the RR value of CC7 exposed *Aedes aegypti* strain is 2.30 in F_{10} generation, while the RR value of EE5 is 2.15 in F_{10} generation. However, the RR value of PP4 exposed *Aedes aegypti* strain was found to be 4.76, which is comparatively higher than that of CC7 and EE5 exposed strains.

It is significant to keep in mind that getting information on the susceptibility status *of Aedes aegypti* larvae towards the bioactive elements isolated from medicinal plants is difficult in the current scenario. One of the probable reasons for the afore-mentioned situation is that almost all the studies have focussed on the preliminary studies rather than bio-assay guided chromatographic fractionation and susceptibility testing. However, the mechanism of resistance to synthetic insecticides is effectively addressed in many scientific publications. For example, as discussed earlier, the control of *Aedes aegypti* at larval and adult stages is mainly accomplished by the use of organophosphates like malathion and temephos; and their frequent use instigated the emergence of insecticide resistance in *Aedes aegypti*.

The LC_{50} values for temephos are rapidly being increased from one generation to another in insecticide susceptibility testing (Corbel et al., 2016). The result of this objective complements those from the latest investigation revealing that malathion showed higher insecticide resistance in *Aedes aegypti* in Magelang. The

susceptibility status of *Aedes aegypti* mosquito vectors is principally allied with biological, operational and genetic factors as described by (Georghiou & Taylor, 1977).

A study in 2012 suggested that insecticide resistance against permethrin and malathion may be due to their routine use in indoor residual spraying devoid of awareness concerning the susceptibility status (Bigoga et al., 2012).

More recently, several studies have confirmed that the high level of resistance may be due to the frequent use of synthetic insecticides for dengue vector by the Government and non-governmental agencies including vector management agencies. There are a large number of related reports concerning the insecticide resistance to various insecticides (propoxur) in mosquitoes from different regions of the world. In India, so far the propoxur is not a popular mosquitocidal tool; but, recent reports indicate that the resistance to propoxur has emerged in various mosquitoes (Rai, Bharati, Subba, & Saha, 2019). They also suggested that the extensive use of malathion and other insecticides in agriculture may open the way for generating insecticide resistance in *Culex quinquefasciatus* mosquitoes.

Another insecticide resistance-based study in *Aedes aegypti* in India also verified the same concept and they explained the indirect exposure of insecticides against mosquitoes. According to them, the contamination of mosquito breeding habitats by the accumulation of synthetic insecticides which is commonly used in agricultural sectors instigates indirect exposure which in turn emerge the insecticide resistance (Bharati & Saha, 2018a). Similar findings concerning the

routine use of insecticides in the agricultural sector validated the insecticide resistance in *Aedes aegypti* against malathion and other insecticides.

In the persisitent struggles to address this insecticide resistance-related problem, current research has been boosted by a novel focus on the bioactive element-based mosquito control. Based on the earlier discussed studies, it was interesting to mention that the RR values of CC7, EE5, and PP4 were lower than those (RR values of previously reported insecticides) reported in the scientific literature. Altogether, the data concerning the lower resistance of bioactive elements reported in this study corroborate the usefulness of natural insecticides for the accomplishment of rational management of insecticide resistance-based threats. So, in order to diminish the synthetic insecticides induced threats in the environment, it is imperative that plant-based products are used whenever applicable.

The results gathered from this investigation has suggested that mosquito control needs to be rethought with the development of novel natural insecticides that go beyond IRS and synthetic insecticide-treated bed nets. The natural insecticides usually possessed several benefits than that of synthetic products and several synthetic insecticides were withdrawn from the commercial market due to the adverse effects induced by them in the environment. In a resistant development context, the present study has validated the beneficial role of bioactive elements in mosquito control. One of the major benefits of using a natural insecticide is the least chance of emerging insecticide resistance in the target organism. As discussed earlier, several synthetic insecticides such as deltamethrin, permethrin,

lambda-cyhalothrinand, pirimiphos-methyl, and temephos instigated the highest insecticide resistance level with high RR value. The susceptibility status of fourth instar larvae of *Aedes aegypti* has verified that the frequent use of the isolated bioactive elements such as CC7, EE5, and PP4 for 10 generations has only developed a low level of insecticide resistance. Therefore, the present study has suggested that the afore-mentioned compounds can be used as an alternative to synthetic insecticides in the future to prevent the dengue infection by preventing the rapid proliferation of *Aedes aegypti* mosquitoes. However, practical assumptions on the afore-mentioned concept need to be studied.

According to Regnault-Roger, Philogène and Vincent (2002), one of the major reasons for evolving the insecticide resistance towards synthetic insecticides is the frequent use of the limited number of synthetic insecticides. Studies concerning the emergence of insecticide resistance against plant-based products are scanty in the current scenario. However, this study suggests that the management of insecticide resistance to plant-based products is vital for a long-term vision of mosquito control. Recent studies suggest that, in order to prevent the emergence of insecticide resistance against plant products, the applicability of genetic engineering can be employed (Qi, Lan, Ma, Yu, & Zhao, 2011). The authors also mention that the new strategies are promising to fight against the insecticide resistance since they exhibited different modes of action. The isolated bioactive elements in this study are a future solution to replace the synthetic insecticides and their adverse effects. Previous studies have verified that the natural approaches are comparatively acceptable and can be used for the treatment of larval breeding habitats. However, as stated earlier, the existing evidence for assessing the

practical implications of plant-based approaches for mosquito control is inadequate (Roiz et al., 2018). The phytochemical constituent-based insecticides are thought to exhibit fewer problems to the users and the environment than synthetic products. The consumers always tend to favour natural products lacking harsh chemical compounds.

According to the previous reports, the selection of an effective bioactive element is recognized as one of the most significant considerations in the development of insect repellent/natural insecticide. Therefore, the effective bioactive elements isolated in this study can be considered for the afore-mentioned attention with special reference to their low level of insecticide resistance emergence. There is a sturdy perception that the insecticides of botanical origin are safer than synthetic insecticides. Previous studies have verified that natural insecticides offer maximum safety in terms of a variety of significant factors than synthetic insecticides (Coats, 1994; Maia & Moore, 2011; Tavares et al., 2010). In support of their results, this objective validated that the isolated bioactive elements such as CC7 (Jatamansone), EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) and PP4 (3-hydroxy- 2,2,4- trimethylpentyl isobutyrate) are safer than synthetic insecticides in terms of insecticide resistance emergence, biodegradability, plant-based origin, eco-attractive and environmental sustainability.

3.5. SUMMARY

The health officials of both international and national agencies are confronted by a burden of arboviral diseases despite significant efforts in mosquito control programmes. Approximately, more than 4 billion peoples over the world faced severe threats from dengue fever infection every year. Insecticides are considered as one of the significant arsenals to combat mosquito-borne diseases to save millions of lives every year. The existing interventions for mosquito control are known to exhibit success in some instances, however, the level to which the current approaches may have prevented the disease epidemics is not well assumed due to the lack of prominent evidence.

For effective mosquito control, growing attention has been paid to community involvement and plant-based approaches including integrated vector management. Despite well-established existing strategies, the disease epidemics and rapid spread of infectious diseases continue in the current scenario. There are a number of significant factors that are considered as reasons for the afore-mentioned threats. They are i) implementation of complex and inadequate programs, ii) lack of human and infrastructural capacity, iii) insecticide resistance, and iv) inability to scale.

Forecasting the threats of insecticide resistance in *Aedes aegypti* towards synthetic insecticides is a vital part of a public health emergency. Therefore, integrated approaches and research based on alternative strategies for mosquito control have been of renewed attention. It is also kept in mind that, the studies concerning the

influence of insecticide resistance in disease transmission are of paramount significance in the current scenario since the insecticide resistance drives the epidemics of mosquito-borne diseases. The experiments performed in this study were principally used to find out the emergence of insecticide resistance in *Aedes aegypti* towards the three isolated plant isolates such as CC7, EE5, and PP4.

While following the WHO criteria, the present study has reported that all the *Aedes aegypti* strains were moderately resistant to the isolated bioactive elements (CC7 - Jatamansone; EE5 -2,2,4-Trimethyl-1,3-pentanediol diisobutyrateand; and PP4 -3-hydroxy-2,2,4-trimethylpentyl isobutyrate), indicating the significance of bioactive elements from plants in mosquito vector control. The lower resistance ratio (RR) towards higher concentrations of CC7, EE5, and PP4 is considered as the highlight of this study. Among the three isolated bioactive elements, the EE5 showed the least RR value of 2.15. The PP4 comparatively showed higher RR value than the two isolated bioactive elements. However, the foremost fact is that all the *Aedes aegypti* strains are exhibited moderate resistance. The RR value of previously reported synthetic insecticides is greater than the present study, confirming the effectiveness of natural insecticides in mosquito control. Previous studies have reported the higher insecticide resistance of various synthetic insecticides in mosquitoes. However, none have piloted a study concerning the susceptibility status of *Aedes aegypti* fourth instar larvae towards the bioactive elements isolated from *Jasminum brevilobum, Aglaia edulis* and *Pogostemon auricularius*. This has made the study unique and significant. Therefore, the results from this study suggest that the isolated bioactive elements from three medicinal plants have diminished the risk of resistance in *Aedes* mosquito control.

As a result, various mosquito-borne diseases especially, the dengue fever, can be prevented in an eco-attractive way.

CHAPTER IV

Qualitative and Quantitative Analysis of a Few Detoxifying Enzymes in Susceptible and Phytochemical Selected Lines of *Aedes aegypti*

4.1. INTRODUCTION

The major sector of integrated vector management includes the use of synthetic larvicides, entomological surveillance, use of larvivouros fish, indoor residual spray using 2% pyrethrum and 5% malathion for fogging. In addition to this, some of the major compounds belonging to the pyrethroid group have also been used for personal protection as a strong mosquito repellent product (Bharati & Saha, 2018b). Despite the environmental problems, the above-mentioned synthetic compounds including DDT remained an effective weapon against mosquito-borne diseases in endemic provinces of the world. The extensive use of synthetic insecticides has led to striking decreases in mosquito-borne disease epidemics (Morou et al., 2010).

As discussed in the earlier objective, the routine and indiscriminate use of the synthetic insecticides for mosquito control would result in the emergence of insecticide resistance in mosquitoes. The emergence of resistance against the synthetic insecticides is primarily influenced by either direct or indirect exposure (from the agricultural field) of insecticides (Bharati, Saha, & Saha, 2018). Mechanism of insecticide resistance and the mode of action of synthetic insecticides have been the theme of current research interest in the scientific community since resistance to synthetic insecticide is considered as the major obstacle in mosquito control programmes (Morou et al., 2010). Previous studies have indicated that several biochemical mechanisms are responsible for the emergence of resistance in mosquitoes towards various synthetic insecticides.

With regard to *Aedes aegypti*, it has been believed as one of the important mosquito species, which exhibited a prominent range of resistance towards popular insecticides as those coming under the category of organophosphates, carbamates, pyrethroids, and organochlorines (Brengues et al., 2003).

The overproduction of detoxification enzymes such as Cytochrome P450 monooxygenases, G6PD, esterases are known to instigate certain metabolism that is allied with the emergence of insecticide resistance. For example, it has been previously reported that the increased level of esterases is allied with the emergence of insecticide resistance in mosquito vectors towards the various synthetic insecticides such as organophosphates, carbamates, pyrethroids and organochlorines (Fonseca-González, Quiñones, McAllister, & Brogdon, 2009).

The P450s are usually encompassed with haem-thiolate enzymes that are actively involved in the wide range of metabolism. In insects, the cytochrome P450s are actively involved in the various metabolic processes linked with insecticide resistance. In mosquitoes, the activities concerned with P450s have been of specific interest since they are vital for the detoxification of various elements including chemical carcinogens, pesticides, mutagens, and drugs. They are also known to instigate metabolizing compounds such as steroids, fatty acids and hormones. Most of all the studies discussed that one of the major characteristic features of cytochrome P450s in mosquitoes is the detoxification of insecticides by increasing its level in the target body. It has been implicated that the overexpression of the genes responsible for cytochrome P450s activity offers the target insect to develop insecticide resistance against insecticides. The role of

G6PD and other enzymes in emerging insecticide resistance in mosquitoes have been also reported in previous studies (Mourya, Hemingway, & Leake, 1993).

Most of all the previous studies have indicated that understanding the mechanisms concerned with the emergence of insecticide resistance is a significant prerequisite for the successful eradication of mosquito-borne diseases spread by *Aedes aegypti* (Rawlins, 1998). Attempts to study the mechanisms concerned with the insecticide resistance that would tolerate the threats from synthetic insecticides prompt the development of effective mosquito control strategies of high attention. It has been also found that the rationale of most of all the studies has arisen from the afore-mentioned concepts. What rests unknown is whether the bioactive elements are allied with the insecticide emergence in *Aedes aegypti*. Therefore, studies concerning the above-mentioned concepts with a particular interest in plant-based products will gain uniqueness and novelty. All the earlier discussed studies have indicated that the investigation on insecticide resistance with a particular interest in its mechanism has significant impacts on mosquito control.

The production of free radicals has been shown to possess a significant role in various metabolic processes. The excessive production of free radicals has instigated several drastic effects such as damages in nucleic acids and proteins including DNA damage, changes in enzyme concentration, and cell damage (Sohal, Sohal, & Orr, 1995). Few studies have reported the role of free radicals in insecticide resistance. However, studies concerning the production of free radicals with a particular interest in the mode of action in the target insect are scanty.

The free radicals are produced as vital molecules in a wide variety of physiological and pathological processes. The proliferation of cells, immunity, cell signalling together with the differentiation of cells have recognized as the significant concerns allied with the physiological as well as pathological processes (Janssen-Heininger et al., 2008). The biological actions of free radicals are primarily influenced by the capacity to receive or donate electrons from various molecules, inducing a various set of processes linked with the regular activities of cells. However, the increased levels of free radicals may instigate adverse effects in various signalling pathways leading to oxidative stress (Jones, 2006). Hence, the precise balance between the ROS-detoxifying reactions and ROS-generating systems is vital for retaining the homeostasis in organisms. The present investigation employed this loophole as a novel intervention to prevent the rapid proliferation of *Aedes aegypti* mosquitoes by inducing bioactive elements that prompted reactive oxygen species production. In addition to this, a concept on studying the influence of microbiomes annihilation in arboviral disease transmission with a particular interest in microbial consortia have been discussed in this study. The main rationale to investigate the afore-mentioned concept in *Aedes aegypti* mosquito larvae is the significant link between various microorganisms and *Aedes aegypti* mosquitoes. For example, the microbial consortia that reside inside the mosquitoes have been known to show a prominent role in the mosquito life cycle by providing give-and-take multifarious collaborations (Sharon, Segal, Zilber-Rosenberg, & Rosenberg, 2011). The various microorganisms such as *Bacillus subtilis, Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumonia,* and *Escherichia coli* are known to

exhibit host-parasite interaction with mosquitoes. The present investigation also aimed to find out the probable mode of action of bioactive elements with particular interest on the microbiomes annihilation in mosquitoes. One of the major reasons for the microbe-based investigation is the larval stages of mosquitoes strongly linked with the microbial consortia, and the life cycle of larvae becomes damaged when the extermination of microbial consortia happened.

There have been no investigations concerned with the qualitative and quantitative changes of certain detoxifying enzymes such as alcohol dehydrogenase, monooxygenases, and esterases towards the isolated bioactive elements from *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius*. Despite the point that there exist several studies on the mode of action of synthetic insecticides on the *Aedes aegypti* mosquito vectors, none of them have discussed the mode of action of bioactive elements with special reference to the free radicals radical formation and microbiome annihilation. The present objective is the first attempt to investigate the qualitative and quantitative changes of certain detoxifying enzymes such as esterases, dehydrogenases, and monooxygenases if any, due to the effect of botanicals. Subsequently, the influence of the extermination of microbial consortia and the excessive production of free radicals was undertaken as a novel concept to enlighten the development of effective mosquito control strategy. In order to accomplish these two additional concepts, the present study has risen two concerns, 1) Evaluate the toxicity of isolated bioactive elements such as CC7, PP4, and EE5 towards the microorganisms that maintain a symbiotic link with *Aedes aegypti* mosquitoes; 2) evaluate the isolated

compounds mediated free radicals formation in the *Aedes aegypti* mosquito larvae.

4.2. MATERIALS AND METHODS

4.2.1. Bioactive elements and other chemicals

As stated earlier, the three bioactive elements such as CC7 (Jatamansone), PP4 (3-hydroxy-2,2,4-trimethylpentyl isobutyrate), and EE5 (2,2,4- Trimethyl-1,3-pentanediol diisobutyrate) used in this objective were isolated from *Jasminum brevilobum*, *Pogostemon auricularius*, and *Aglaia edulis*. All the other chemicals employed for the executed experiments were purchased from Merck Co. (Germany) (HPLC grade).

4.2.2. Mosquito colony

The *Aedes aegypti* colony was maintained as explained in chapter 1 (Page No:88).

4.2.3. Qualitative enzyme assay

Qualitative analysis of changes in certain detoxifying enzymes such as esterases, dehydrogenases and monooxygenases if any, due to the effect of isolated bioactive elements such as CC7, EE5 and PP4 were investigated using PAGE. *Aedes aegypti* fourth instar larvae were used for all executed experiments that are made for determining the qualitative changes in enzymes induced by the isolated bioactive elements.

4.2.3.1. Native gel electrophoresis

Native PAGE was done using a vertical slab. Gels having a size of 0.7 mm thickness and teflon spacers were used. The separating gels for esterases were prepared with 7.5% acrylamide, whereas the stacking gels for the same were prepared with 5% acrylamide. For cytochrome P450 and G6PD, 4% stacking and

5% separating gels were employed. The gel buffer for separating gels was prepared using prepared with Tris (hydroxyl methyl aminomethane) with a pH of 8.8, while the pH was maintained at 6.8 for stacking gels. An eight-slot Teflon comb was used to create the sample wells. The constant power supply was provided during the experiment.

4.2.3.1a. Sample preparation and electrophoretic study

Aedes *aegypti* fourth instar larvae were homogenized in an eppendorf containing 25 μL of 40% (w/v) sucrose solution. The homogenized samples were then centrifuged for 5 min at 2,400 rpm with a constant temperature of 4ºC. For G6PD, a mixture prepared using 20% sucrose and NADP (2mg/mL) together with 0.1 M Tris-HCl (pH 8.5) were used for homogenization. The samples were then centrifuged for 5 min at 14,000 rpm. To respective wells, an equal volume of supernatant was attentively loaded. The gels were initially run for 20-30 min at 40 V at room temperature. The electricity was then amplified to 65 V at 4º C.

4.2.3.1b. Tray buffer preparation for different enzymes

For cytochrome P450 and esterases, 0.3M Sodium borate buffer having a pH of 8.65 was used. To prepare sodium borate buffer, 0.24 g of NaOH and 18.55 g of boric acid were dissolved in 100 mL of distilled water. The EDTA-borate buffer having a pH of 9.0 was prepared using 66.4 mg NAD, 37.2 mg EDTA, Tris, and 53.8 mg Boric acid, with distilled water. The final volume was made up to 100 mL using distilled water.

4.2.3.1c. α-esterase (-A)/β-esterase (Est-B)

The following solutions (i) and (ii) were thoroughly mixed and transferred to a glass box containing the gel and kept for 20 min at 37ºC for incubation.

i. In 1 mL of acetone, 25mg of α/β naphthol acetate was dissolved. To this mixture, 0.5 mL of 0.1 M sodium phosphate buffer having a pH of 5.9 was added.
ii. To 2.5 mL of 0.1 M phosphate buffer having a pH of 6.5, 25 mg of Fast Blue RR salt was dissolved.

4.2.3.1d. Cytochrome p450

According to the method of Thomas, Ryan and Levin (1976), the gels were incubated in TMBZ (, 3', 5, 5' tetramethyl benzidine) in methanol and sodium acetate buffer at dark at room temperature for 2 hours. The 30 mM of Hydrogen peroxide (H_2O_2) was added and the gel bands appeared in 5 min.

4.2.3.1e. Glucose 6-phosphate dehydrogenase (G6PD)

A method suggested by Bakay and Nyhan (1969) with slight modifications was used to stain the Glucose 6-phosphate dehydrogenase (G6PD) bands.

i) 7.5 mg NBT, 2.5 mg MTT, 5 mL distilled water and 0.5 mg PMS were dissolved in Tris-HCl buffer (0.1 M - pH 8.5);
ii) 12.5 mg NADP, 250 µL $MgCl_2$ (10% w/v), and 10 mg Glucose-6-phosphate were dissolved 0.1 M Tris-HCl having pH of 8.5.

The above-mentioned solutions such as (i) and (ii) were thoroughly mixed and the gels were immersed in the resulting solution. For incubation, the gels were kept

for 2 hours at room temperature. The size and intensity of the gel remain steady for a long time if it is stored at 4ºC in 7% acetic acid.

4.2.3.1f. Fixation and zymogram plotting

After the formation of gel bands, the gels were fixed in glacial acetic acid (7%) and the zymograms were generated to reveal the respective bands. The commonest gel bands found in both the lines were marked as 1.00. Bands that move faster were marked with the numbers greater than 1.00. Moreover, the bands that move slowly were marked with decreasing order of numbers. The number for each band was provided based on the distance travelled from the wells.

4.2.4. Microplate assay for the quantitative estimation of enzymatic changes induced by the isolated bioactive elements

The experiment was done as per the protocol suggested by Hemingway and Karunaratne (1998), usually regarded as a plate assay method.

4.2.4.1. Preparation of sample solution

Thirty early fourth instar larvae of *Aedes aegypti* were homogenized as separate samples in 200 µL of distilled water was taken in an eppendorf tube having 1.5 mL capacity. The tube was kept on ice. The prepared homogenate proceeded for centrifugation at 5,000 rpm for 10 min. The temperature was maintained at 4ºC during centrifugation. About 20 µL supernatant from the sample solution was transferred to another eppendorf tube. The final volume of the sample was then made to 100 µL using 0.1 M potassium phosphate (KPO_4) buffer having a pH of

7.2. The above-mentioned procedure was repeated for the preparation of samples for all the executed experiments.

4.2.4.2. Esterase assay

The mosquito homogenates (100 µL, CC7, EE5, and PP4 exposed) were loaded on the wells of the microplate. To this, 100 µL of α/β naphthol acetate was added. The α/β naphthol acetate (80 mL phosphate buffer plus 56 mg/20 mL acetone) was first dissolved in acetone before the addition of phosphate buffer. After the incubation (10 min) at room temperature, using a multichannel pipet, 100 µL dianisidine -Fast Blue was added and kept at room temperature for 2 min. The absorbance was read at 540 nm for ß-naphthol and 620 nm for α-naphthol. The optical densities recorded in this study were compared with those of known concentration of α/ß-naphthol, by plotting a standard curve. The activity of esterases were registered with respect to the 'nm' of product formed/min/mg protein.

4.2.4.3. Monooxygenase assay (Cytochrome P450)

Heme-peroxidase assay was used to measure the total quantity of heme-containing protein. The reaction mixture of the respective wells contained 25 µL of hydrogen peroxide (3%), 100 µL mosquito homogenates, and 200 µL of TMBZ (3, 3`, 5, 5` tetramethyl benzidine). The TMBZ solution was prepared using 10 mg TMBZ, 5 mL methanol and 15 mL of 0.25M sodium acetate buffer having a pH of 5.0. Then it is allowed to expose room temperature for 2 hours. and the optical density was measured at 450 nm. The values correspond to absorbance were compared with a standard curve.

4.2.4.4. Glucose 6-phosphate dehydrogenase assay

The Glucose 6-phosphate dehydrogenase assay is carried out as per the protocol suggested by Kumar, Thomas, and Pillai (1991) with slight modifications. Each well of the plate contained a reaction mixture which is prepared using 200 μL of reagent mixture and 100 μL of prepared mosquito homogenates. One mL of the reagent mixture contained 400 μL of Tris-HCl (0.15 M) having a pH of 7.5, 3.8×10^{-4} M NADP, 500 μL of 0.03 M D-glucose 6-phosphate and 0.01 mL of 0.3 M MgCl2. The optical density was measured at 340 nm. Based on the coefficient (6.22×10^{6}) the quantity of NADP reduced was calculated.

4.2.4.5. Estimation of protein

The protein content of the CC7, EE5 and PP4 selected lines of *Aedes aegypti* was estimated using the Lowry's method.

i) Folin ceacalteu's reagent: Equal volumes of distilled water and Folin Ceacalteu's reagent were mixed toughly and freshly at the time of the experiment.

ii) Lowry's reagent: 2% Copper sulphate, 2% Sodium potassium tartarate, 4% Sodium carbonate is dissolved in 1:1:98 ratio. 10 μL of the mosquito homogenate was made up to a final volume of 1 mL using distilled water. To this 5 mL of the reagent ii was added and kept for 15 min and then 0.5 mL of the (i) reagent was added. Kept the solution for 20-30 min and the optical density was measured at 660 nm. A standard curve was prepared using bovine serum albumin (BSA). The total protein content of the respective samples were determined by inferring the optical density values with the standard protein curve.

4.2.5. Toxicity of isolated towards microbial consortia

The toxic effects of three bioactive elements such as CC7, PP4, and EE5 against *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Klebsiella pneumonia* (MTCC 661), *Proteus mirabilis* (MTCC 442), and *Staphylococcus aureus* (MTCC 3160) were analyzed based on the method prescribed by Wiegand et al. (2008) and Bussmann et al. (2010). The different concentrations of each bioactive elements were loaded on the wells prepared in the microbe plated agar plates. Controls were maintained for all the executed experiments and the plates were left for 15 to 18 hours in the incubator at 37ºC. The zone of inhibition for respective samples were measured. For positive control, Chloramphenicol was used. The data gained have registered as a mean ± standard deviation. Minimum inhibitory concentrations (MIC) for all the bioactive elements towards the microbial consortia were determined using the method suggested by Wiegand et al. (2008).

4.2.6. Phytochemicals prompted free radical generation

The ability of the bioactive elements such as CC7, PP4, and EE5 to instigate the excess production of reactive oxygen species results in cell damage and oxidative stress was verified using the modified ammonium molybdate method suggested by Prieto et al. (1999). Twenty *Aedes aegypti* fourth instar larvae which are treated with the bioactive elements CC7, PP4 and EE5 were homogenized. The larvae that are unexposed with the bioactive elements are considered as the control group. The level of reactive oxygen species formed in the untreated and bioactive elements exposed lines was determined by the method proposed by Prieto et al. (1999) with slight modifications. The optical density of the test samples was read

at 695 nm using Ultraviolet-visible spectroscopy. The morphological anomalies (in the head, abdomen and anal gills region of *Aedes aegypti*) developed in response to the continuous exposure of phytochemical prompted reactive oxygen species in the *Aedes aegypti* larvae were verified using the Olympus CH -20i Microscope connected to the canon digital camera.

4.2.7. Statistical analysis

Based on the mobility of various dehydrogenase and monooxygenases enzymes including p450 from anode to cathode, the isozymes were designated as α-EST I, II, III and so on. The same strategy was implemented for β-Esterases, P450 and G6PD. T-test was employed to analyze the quantitative changes of selected enzymes and protein level between the phytocompounds exposed strains and susceptible lines of *Aedes aegypti* ($P<0.05$). The present objective used Bio Image Advance Quantifier Version 4.3.8 by Bio image systems. Inc and image J 1.5.2 by National Institutes of Health, USA for PAGE analysis.

4.3. RESULTS

4.3.1. Qualitative and quantitative analysis of detoxifying enzymes

The qualitative analysis of certain detoxifying enzymes such as glucose 6-phosphate dehydrogenase (G6PD), α- esterases (A-est), β- esterases (B-est), and cytochrome P450 using PAGE are given in Table 4.1 (Page No. 231). The densitometric profile of the phytochemicals exposed strains of *Aedes aegypti* was also provided in the Figs. 4.1-4.12 (Page No. 228-230). Each band in the zymogram characterizes the product of the respective allele. The different alleles of each detoxifying enzymes are characterized by providing a number (superscript) for their abbreviated form.

The profile of α-esterase in CC7 selected lines revealed three bands such as α-est$^{1.0}$, α-est$^{0.95}$, and α-est$^{0.80}$ (Fig. 4.1; Page No. 228). Similarly, the EE5 strain (Fig. 4.5; Page No. 229) showed three bands such as α-est$^{1.0}$, α-est$^{1.05}$, and α-est$^{0.93}$; while the PP4 strain (Fig. 4.9; Page No. 230) showed two bands such as α-est$^{1.0}$ and α-est $^{0.77}$. The CC7 strain showed three bands in β-esterase profile such as β-est$^{0.8}$, β-est$^{1.0}$, β-est$^{1.06}$ (Fig. 4.2; Page No. 228). The EE5 (β-est$^{0.76}$, β-est$^{1.0}$, β-est$^{1.03}$) and PP4 (β-est$^{0.80}$ and β-est$^{1.0}$) lines also revealed three and two bands respectively. The electromorph together with respective densitometric profiles of Cytochrome P450 enzyme in the CC7, EE5, and PP4 selected lines of *Aedes aegypti* are given in Figs. 4.4 (Page No. 228), 4.8 (Page No. 229), and 4.12 (Page No. 230) respectively. However, no additional bands appeared in the electromorph of CC7, EE5, and PP4 selected lines of *Aedes aegypti* indicating that

the enzyme was found to be monomorphic. An extra allele in G6PD profiles was noticed in the CC7 selected strains of *Aedes aegypti*. Fig. 4.7 (Page No. 229) depicts the electromorph profile of the G6PD enzyme in the EE5 selected strains of *Aedes aegypti*. The densitometric profiles of F_{10} strains of most of all the selected lines revealed the appearance of the extra band as an additional peak in the plot. The G6PD enzyme revealed an extra band in EE5 selected lines of *Aedes aegypti*. For, G6PD only one additional band was observed in the PP4 selected lines of *Aedes aegypti* ($G6PD^{1,2}$).

The quantitative analysis of detoxifying enzymes such as glucose 6-phosphate dehydrogenase, cytochrome P450, α-esterases, and β-esterase in CC7, EE5, and PP4 selected strains of *Aedes aegypti* are provided in Figs. 4.13-4.16 (Page No. 234-235). The experiments were done on the susceptible (F_1) and the selected (F_{10}) generations of *Aedes aegypti*. Thirty, fourth instar larvae were used for each of the executed experiments. The activity of the dehydrogenases and esterases (α-est and β-est) are found to be increased in phytochemicals exposed lines of *Aedes aegypti*.

The mean value of α-esterase activity in the susceptible strain of *Aedes aegypti* was found to be 0.065±0.0308 nM of α-naphthol produced/min/mg protein while it was 0.108±0.00642 nM in the F_{10} strain (CC7 exposed line) (Table 4.2; Page No. 231). The fold increase value for the α-esterase activity in CC7 exposed strains was 1.65 times more than that of the susceptible strain. The PP4 selected strain showed α-esterase activity with a mean value of 0.028± 0.00642 while its susceptible line has shown 0.0176± 0.0308 (Table 4.3; Page No. 232). The α-

esterase activity for the PP4 selected strain was 1.60 times more than that of the susceptible strain. Fig. 4.13 (Page No. 234) revealed the α-esterase activity of the afore-mentioned strains (CC7, EE5, and PP4 selected lines) of *Aedes aegypti*. The α-esterase activity in the EE5 selected lines of *Aedes aegypti* was observed as 0.055±0.0015 while its susceptible line exhibited 0.033 ±0.001 activity with a fold increase of 1.68 (significant at 0.002; ($P<0.05$, t-25.70)) (Table 4.4; Page No. 233).

The mean value of β-esterase activity in the CC7 selected line was found to be 0.029±0.0005nM of β-naphthol produced/min/mg protein while the susceptible line has exhibited 0.018±0.0005nM of β-naphthol produced/min/mg protein. The β-esterase activity for the CC7 selected line was 1.61 ($P<0.05$-0.001; t-32.00) more than that of the susceptible lines as given in Table 4.2 (Page No. 231). The PP4 selected lines of *Aedes aegypti* in F^{10} produced 0.73±0.0005 nM β-naphthol produced/min/mg protein with a fold increase of 1.71 ($P<0.05$-0.001; t-34.77). Similarly, the β-esterase activity for the EE5 selected line was found to be 0.067±0.0005 nM β-naphthol produced/min/mg protein. The β-esterase activity in the EE5 selected line was 1.56 times greater than that of the susceptible strain ($P<0.05$-0.003; t-18.25).

The mean value of G6PD activity in the CC7 selected strains of *Aedes aegypti* was found to be 0.027±0.0005 nM NADP produced/ min/ mg protein with a fold increase of 1.80. The values were statistically significant ($P<0.05$; 0.002, t-25.00). The EE5 (0.061±0.001; ($P<0.05$; 0.003, t-17.67)) and PP4 (0.117±0.0008; $P<0.05$; 0.000, t-16.60) selected strains of *Aedes aegypti* has also produced G6PD activity.

The G6PD activity in the EE5 and PP4 selected strains was 1.79 and 1.89 times more than that of the susceptible lines respectively.

The mean value of Cytochrome P450 activity in the CC7selected strains of *Aedes aegypti* was observed as 0.005±0.00047 with a fold increase of 1.77 ($P<0.05$; 0.015, t-8). The mean value for PP4 and EE5 selected strains of *Aedes aegypti* have been provided in Table 4.3 and 4.4 (Page No. 232, 233). The Cytochrome P450 activity in the EE5 and PP4 selected strains of *Aedes aegypti* were 1.81 and 1.80 times greater than that of the susceptible lines respectively. The α- esterase, β esterase, G6PD, Cytochrome P450 activity CC7, EE5, and PP4 selected lines of *Aedes aegypti* were provided in the Figs 4.13-4.16 (Page No. 234-235).

4.3.2. Extermination of microbial consortia

The toxic potential of the plant extracts (various extracts of *Aglaia edulis*, *Pogostemon auricularius*, and *Jasminum brevilobum*) and isolated bioactive elements against five bacterial species were assessed (Fig. 4.18; Page No. 240). As reported in our published article, (A novel intervention on the inhibiting effects of *Catunaregam spinosa* induced free radical formation and DNA damage *in Aedes aegypti* (Diptera: Culicidae): a verdict for new perspectives on microorganism targeted vector control approach, *International Journal Of Tropical Insect Science* (2020), Springer, Singapore) all the isolated bioactive elements and the plant extracts exhibited a prominent range of toxicity against *Proteus mirabilis* and *Staphylococcus aureus* (MTCC 3160) with strong MIC values (0.08-1.65 mg/mL). The water and acetone extracts of *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius* have showed strong

toxicity against *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160), *Klebsiella pneumonia* (MTCC 661), *Escherichia coli* (MTCC 443) and *P. mirabilis* (MTCC 442) (MIC- 0.08-1.06 mg/mL).

The various extracts prepared from the medicinal plants *Jasminum brevilobum* and *Pogostemon auricularius* remarkably exhibited strong impeding activity against tested microbial consortia (MIC 0.42-1.65 mg/mL). However, the petroleum ether and methanol extracts of *Jasminum brevilobum, Aglaia edulis*, and *Pogostemon auricularius* have comparatively shown a considerable range of impeding activity against the tested bacterial species (MIC 0.4-1.65 mg/mL).

The toxic potential of the isolated bioactive elements was also verified with special reference to the mode of action in *Aedes aegypti* fourth instar larvae and the results were published in the article entitled "Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases" [*International Journal of Tropical Insect Science*, Springer, Singapore].

Among the three isolated bioactive elements, the EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) showed strong toxicity against *Staphylococcus aureus* with MIC value of 0.04 (Zone of inhibition: 20.42±1.598). The same compound also exhibited strong toxic potential against *Proteus mirabilis* (MIC=0.14, Zone of inhibition (19.95±1.530) (Table 4.5-4.6; Page No. 236-238). The bioactive element Jatamansone isolated from *Jasminum brevilobum* also showed strong impeding activity against *Escherichia coli* (MIC=0.0765 mg/mL; Zone of inhibition: 20.42±1.598) and *Staphylococcus aureus* (MIC=0.0565 mg/mL; Zone

of inhibition: 21.72±0.571). The bioactive element PP4 (3-hydroxy-2,2,4-trimethylpentyl isobutyrate) isolated from *Pogostemon auricularius* verified strong inhibiting potential together with effective MIC values (0.08-0.46).

In addition to this, the present study also verified the toxic potential of *Catunaegam spinosa* seed extracts against the above-mentioned bacterial strains (Table 4.7a-b (Page No. 239, 241); Fig. 4.19 (Page No. 241)). The results from this investigation concerning the extermination of microbial consortia indicated that the bioactive elements isolated from the three medicinal plants sustained great toxic potential against the microbial consortia that reside inside the *Aedes aegypti* mosquitoes.

4.3.3. Mode of action of cc7, ee5, and pp4 prompted reactive oxygen species (ROS) in *Aedes aegypti*

This study assessed the bioactive elements induced ROS formation towards the dengue fever vector *Aedes aegypti* (Table 4.8-4.9; Page No. 242, 245). The CC7, EE5, and PP4 were considered as a double-edged weapon since the excessive production of free radicals has instigated the oxidative stress in the target insect (Fig. 4.20 (Page No. 242); Table 4.9 (Page No. 245)). The level of ROS production in the water and acetone extracts of *Aglaia edulis* significantly augmented than the control strains. The control groups possess a relatively low level of ROS. The petroleum ether, methanol, acetone and water extracts of *Jasminum brevilobum, Pogostemon auricularius* and *Aglaia edulis* were found to produce a large amounts of ROS and this has drawn the concern to further analyze the bioactive-element specific oxidative stress. The present study has verified that

the three bioactive elements such as CC7 (Jatamansone), EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate), and PP4 (3-hydroxy-2,2,4-trimethylpentyl isobutyrate) exposure in *Aedes aegypti* resulted in excessive production of ROS inside the larvae, that also appear to be interlinked with various physiological processes.

In addition to this, the present study also inferred the novel insights of *Catunaragam spinosa* induced reactive oxygen species formation and oxidative stress with special emphasis on the mechanism of larvicidal action (Fig. 4.21 (Page No. 243); Table 4.10 (Page No. 245)). The afore-mentioned concepts can be also recognized as a verdict for new perspectives since the study also discussed the toxic potential of *Catunaragam spinosa* seed extracts towards the microbial consortia in context with DNA damage (Table 4.7 (Page No. 239, 241); Fig. 4.22-4.23 (Page No. 244)).

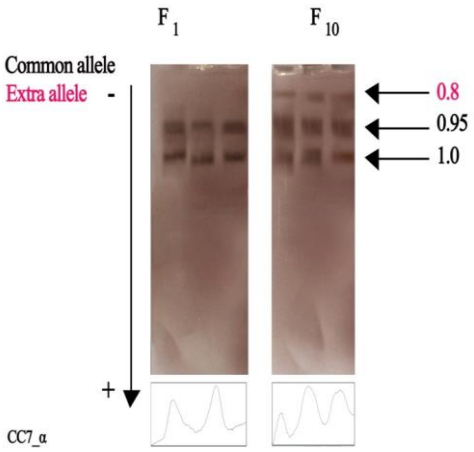


Fig. 4.1. α-esterase isozymes and densitometric profile of CC7 selected strains of *Aedes aegypti* fourth instar larvae

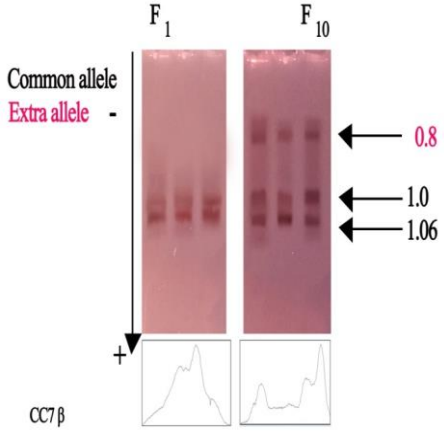


Fig. 4.2. β-esterase isozymes and densitometric profile of CC7 selected strains of *Aedes aegypti* fourth instar larvae

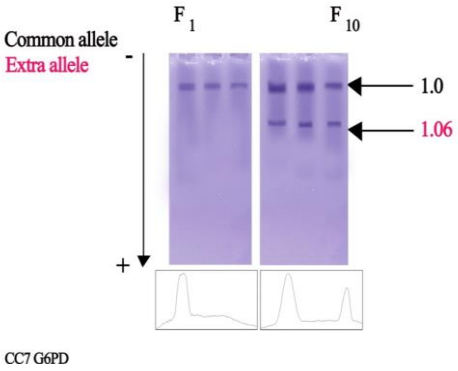


Fig. 4.3. G6PD isozymes and densitometric profile of CC7 selected strains of *Aedes aegypti* fourth instar larvae

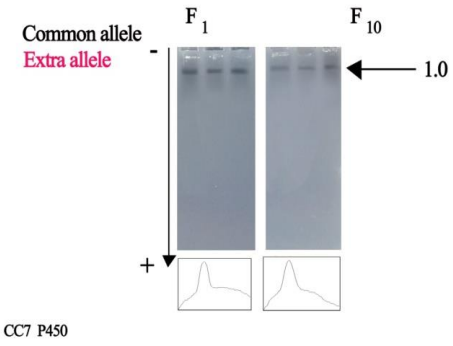


Fig. 4.4. Cytochrome P450 isozymes and densitometric profile of CC7 selected strains of *Aedes aegypti* fourth instar larvae

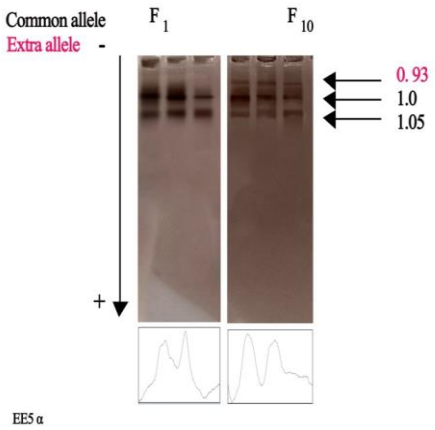


Fig. 4.5. α-isozymes and densitometric profile of EE5 selected strains of *Aedes aegypti* fourth instar larvae

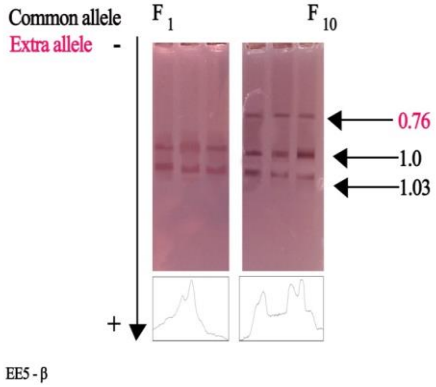


Fig. 4.6. β-isozymes and densitometric profile of EE5 selected strains of *Aedes aegypti f*ourth instar larvae

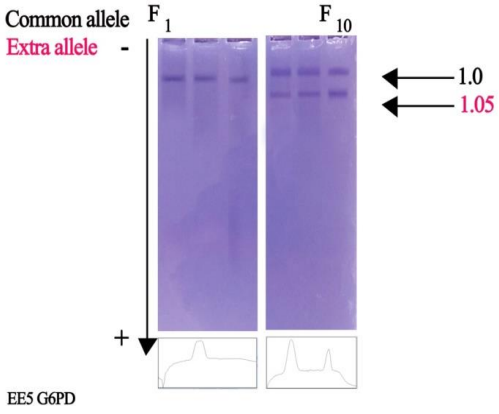


Fig. 4.7. G6PD-isozymes and densitometric profile of EE5 selected strains of *Aedes aegypti* fourth instar larvae

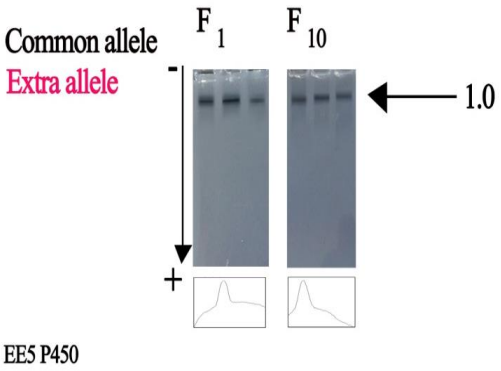


Fig. 4.8. Cytochrome P 450-isozymes and densitometric profile of EE5 selected strains of *Aedes aegypti* fourth instar larvae

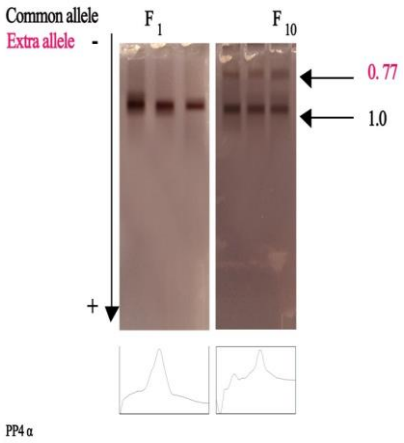


Fig. 4.9. α-isozymes and densitometric profile of PP4 selected strains of *Aedes aegypti* fourth instar larvae

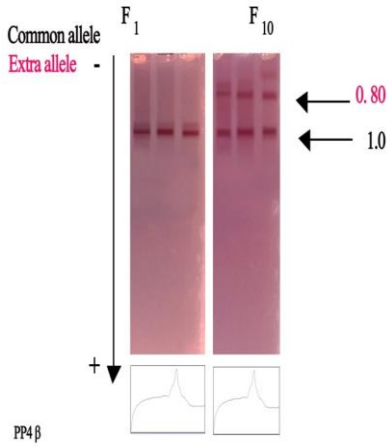


Fig. 4.10. β-isozymes and densitometric profile of PP4 selected strains of *Aedes aegypti* fourth instar larvae

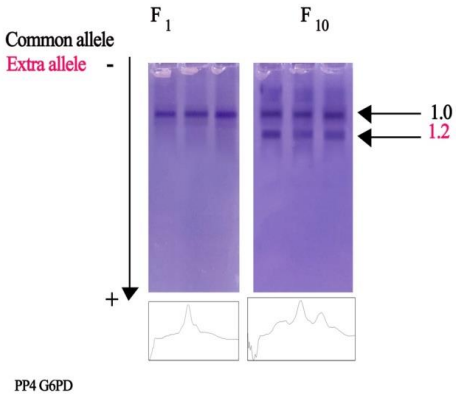


Fig. 4.11. G6PD- isozymes and densitometric profile of PP4 selected strains of *Aedes aegypti* fourth instar larvae

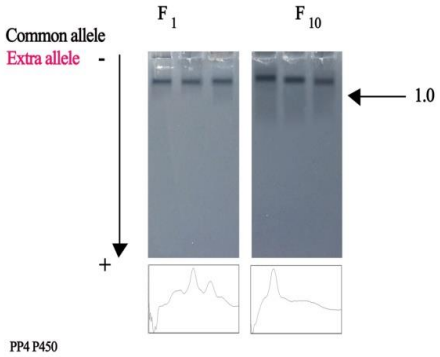


Fig. 4.12. Cytochrome P450- isozymes and densitometric profile of PP4 selected strains of *Aedes aegypti* fourth instar larvae

Table 4.1. Different α, β- esterases, dehydrogenases and Cytochrome P450 in CC7, PP4 and EE5 selected lines of *Aedes aegypti* population.

Sl. No.	Enzymes	CC7 exposed strains	PP4 exposed strains	EE5 exposed strains
1	α-EST I	+	+	+
2	α-EST II	+	+	+
3	α-EST III	+	+	+
4	β-EST I	+	+	+
5	β-EST II	+	+	+
6	β-EST III	+	-	+
7	G6PD I	+	+	+
8	G6PD II	+	+	+
9	P450 I	+	+	+

Table 4.2. Differential activity of four detoxifying enzymes in susceptible and CC7 selected lines of *Aedes aegypti*

Sl. No.	Enzyme	Susceptible Mean±SD	Selected Mean±SD	't' value	Fold increase	Sig. (2-tailed)	df
1	α-esterase[b] nM-naphthol produced/min/mg protein	0.065±0.031	0.108±0.00642	2.26	1.65[a]	0.156	2
2	β-esterase[b] nM-naphthol produced/min/mg protein	0.018±0.001	0.029±0.0005	32.00	1.61[a]	0.001	2
3	G6PD[c] nM NADP produced/min/ mg protein	0.015±0.001	0.027±0.0005	25.00	1.80[a]	0.002	2
4	Cyt P450[d] nM Cyto.-C produced/min/mg protein	0.003±0.001	0.005±0.00047	8.00	1.77[a]	0.015	2
5	Protein	0.170±0.200	0.36±0.200	16.454	2.1	0.004	2

*statistical significance ($P < 0.05$)

[a] Difference is significant at 5% level (paired t-test); [b] Activity stated as μg naphthol produced/min/mg protein; [c] Activity represented as μmoles/min/mg protein; [d]Activity conveyed as equivalent units of cytochrome C

Table 4.3. Differential activity of four detoxifying enzymes in susceptible and PP4 selected lines of *Aedes aegypti*

Sl. No.	Enzyme	Susceptible Mean ±SD	Selected Mean ±SD	't' value	Fold increase	Sig. (2- tailed)	df
1	α-esterase[b] nM-naphthol produced/min/mg protein	0.0176±0.030	0.028±0.0064	12.09	1.60[a]	0.007	2
2	β-esterase[b] nM-naphthol produced/min/mg protein	0.043± 0.073	0.73±0.0005	34.77	1.71[a]	0.001	2
3	G6PD[c] nM NADP produced/min/mg protein	0.061±0.0011	0.117±0.0008	16.60	1.89[a]	0.0000	2
4	Cyt P450[d] nM Cyto.-C produced/min/mg protein	0.003±0.0008	0.006±0.00	8.00	1.80[a]	0.015	2
5	Protein	0.1567±0.0058	0.3800±0.0100	33.50	2.42	0.001	2

*statistical significance ($P < 0.05$)

[a]Difference is significant at 5% level (paired t-test)

[b]Activity stated as µg naphthol produced/min/mg protein

[c]Activity represented as µmoles/min/mg protein

[d]Activity conveyed as equivalent units of cytochrome C.

Table 4.4. Differential activity of four detoxifying enzymes in susceptible and EE5 selected lines of *Aedes aegypti*

Sl. No.	Enzyme	Susceptible Mean ±SD	Selected Mean ±SD	't' value	Fold increase	Sig. (2-tailed)	df
1	α-esterase[b] nM -naphthol produced/min/mg protein	0.033 ±0.001	0.055±0.0015	25.70	1.68[a]	0.002	2
2	β-esterase[b] nM -naphthol produced/min/mg protein	0.043±0.0020	0.067 ±0.0005	18.25	1.56[a]	0.003	2
3	G6PD[c] nM NADP produced/ min/mg protein	0.034 ±0.0081	0.061 ± 0.001	17.67	1.79[a]	0.003	2
4	Cyt P450[d] nM Cyto.-C produced/min/ mg protein	0.005 ±0.0004	0.009±0.0004	19.00	1.81[a]	0.003	2
5	Protein	0.1133±0.0115	0.3333±0.0351	6.08	2.9	0.026	2

*statistical significance ($P < 0.05$)

[a] Difference is significant at 5% level (paired t-test)

[b] Activity stated as μg naphthol produced/min/mg protein

[c] Activity represented as μmoles/min/mg protein

[d] Activity conveyed as equivalent units of cytochrome C

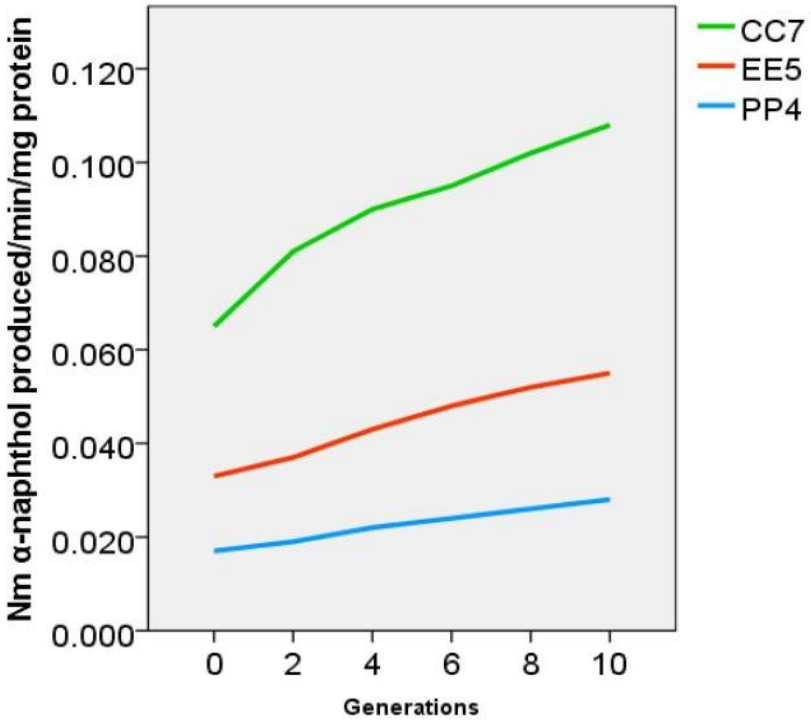


Fig. 4.13. α-esterase activity in the CC7, EE5, and PP4 selected lines of *Aedes aegypti* fourth instar larvae

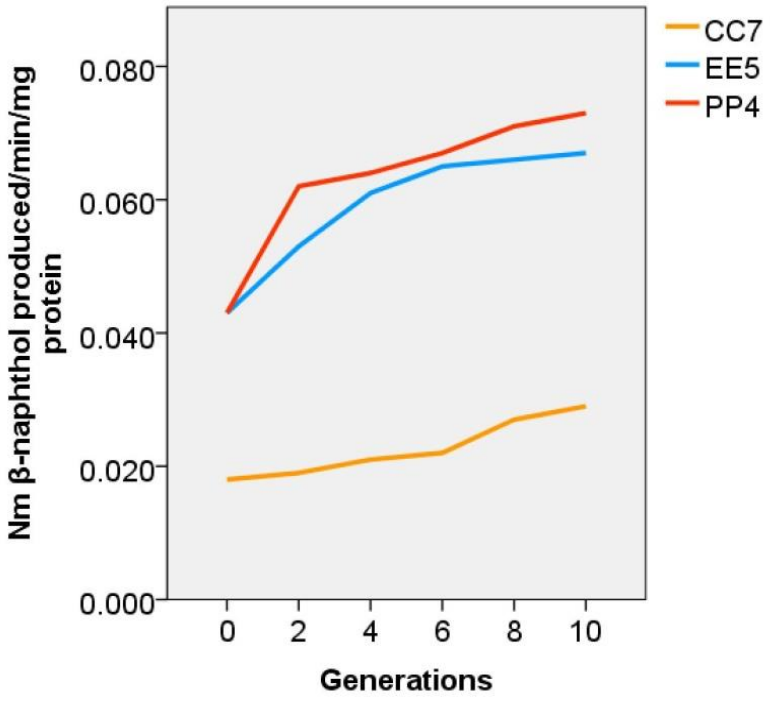


Fig. 4.14. β-esterase activity in the CC7, EE5, and PP4 selected lines of *Aedes aegypti* fourth instar larvae

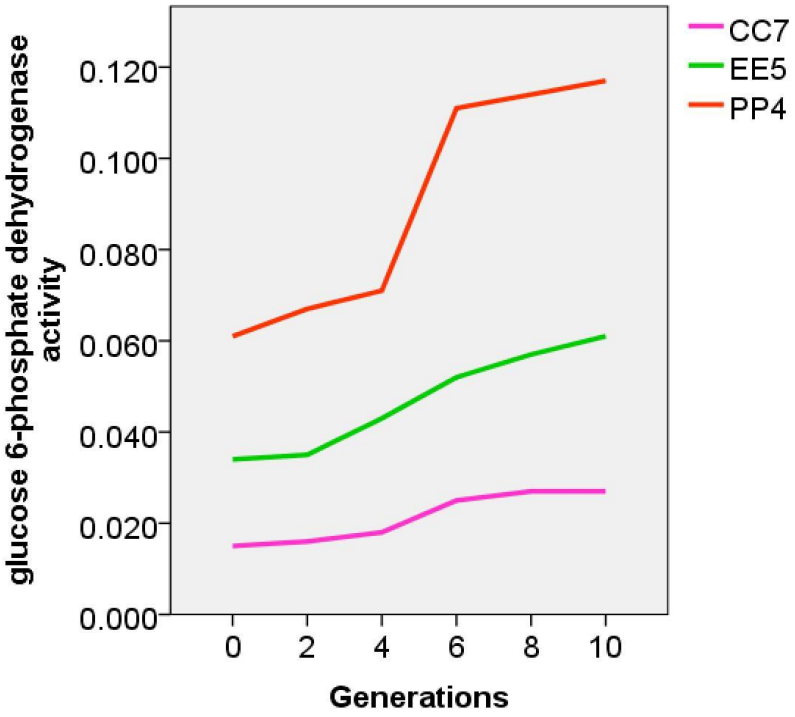


Fig. 4.15. Glucose 6-phosphate dehydrogenase activity in the CC7, EE5, and PP4 selected lines of *Aedes aegypti* fourth instar larvae

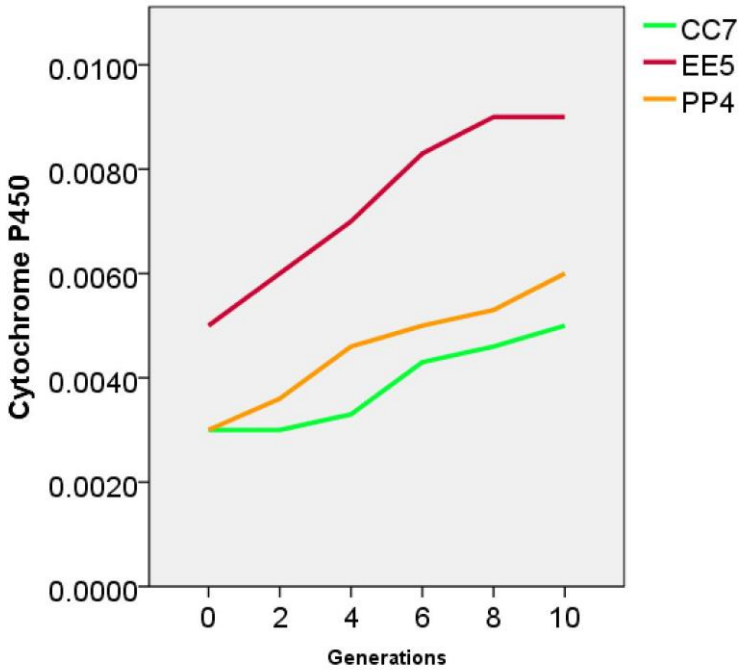


Fig. 4.16. Cytochrome P450 activity in CC7, EE5, and PP4 selected lines of *Aedes aegypti* fourth instar larvae

Table 4.5. Toxic effects of plant extracts and isolated compounds against microbial consortia. (Zone of inhibition measured in mm)

Plant	Solvent used for extraction	Zone of inhibition (mm)				
		Gram-positive		Gram-Negative		
		Staphylococcus aureus Mean ±SD	*Bacillus subtilis* Mean ±SD	*Escherichia coli* Mean ±SD	*Klebsiella pneumonia* Mean ±SD	*Proteus mirabilis* Mean ±SD
Aglaia deeded	Acetone	20.04±1.440	18.00 ± 0.535	16.73±0.679	16.99± 0.432	18.22 ± 0.997
	Water	18.42±0.212	17.56 ± 0.336	15.84±0.545	15.42± 0.650	17.44 ± 0.887
	Methanol	14.32±0.156	16.56 ± 0.210	14.52±0.469	16.40± 0.333	16.22 ± 0.998
	Petroleum ether	12.25±0.351	13.41 ± 0.210	10.16±0.548	15.07± 0.432	14.43 ± 0.564
Jasminum brevilobum	Acetone	17.41±0.364	17.74 ± 1.365	14.53±0.578	16.46± 0.333	17.03 ± 0.350
	Water	14.40±0.169	15.60 ± 0.335	10.32±0.465	15.48± 0.241	16.44 ± 0.560
	Methanol	11.62±0.374	13.42 ± 0.460	12.36±0.659	14.67± 0.550	15.03 ± 0.833
	Petroleum ether	9.18±0.444	10.55 ± 0.865	12.44±0.643	13.57± 0.420	14.00 ± 0.660

Pogostemon auricularius	Acetone	15.00±0.555	17.56 ± 0.433	16.37±0.769	16.68± 0.222	16.38 ± 0.157
	Water	12.10±0.250	13.02 ± 0.137	14.30±0.580	13.00± 0.100	14.34 ± 0.336
	Methanol	10.74±0.300	13.78 ± 0.555	12.05±0.560	12.05± 0.258	13.32 ± 0.025
	Petroleum ether	8.05±0.246	12.37 ± 0.778	10.22±0.464	10.46± 0.466	13.04 ± 0.360
Isolated compounds		22.65±0.700	20.56 ± 0.478	18.68±0.579	18.55± 0.507	19.95 ± 1.530
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate						
Jatamansone		21.72±0.571	19.55 ± 0.689	20.42±1.598	19.75± 0.458	20.03 ± 0.444
3-hydroxy-2,2,4-trimethylpentyl isobutyrate		17.91±0.456	18.55 ± 0.100	18.59±0.760	17.98± 0.123	19.57 ± 0.268

*statistical significance ($P < 0.05$)

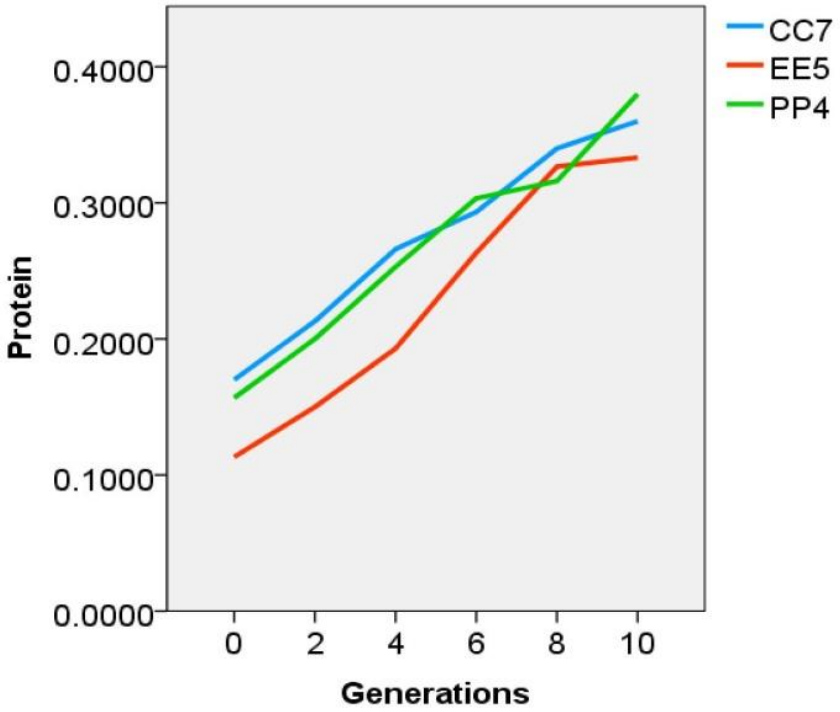


Fig. 4.17 Changes in protein level in the CC7, EE5, and PP4 selected lines of *Aedes aegypti* fourth instar larvae

Table 4.6. Minimum inhibitory concentrations of plant extracts and isolated compounds against microbial consortia

Plant	Solvent used for extraction	Minimum Inhibitory Concentration (In µg/mL)				
		Gram positive		Gram Negative		
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*
Aglaia edulis	Acetone	0.08	0.26	0.58	0.51	0.29
	Water	0.29	0.60	0.58	0.61	0.33
	Methanol	0.44	0.72	0.89	0.71	0.80
	Petroleum ether	1.03	0.91	1.26	0.66	0.92
Jasminum brevilobum	Acetone	0.44	0.42	0.89	0.54	0.49
	Water	0.92	0.62	1.24	0.66	0.51
	Methanol	1.17	1.04	1.09	0.95	0.60
	Petroleum ether	1.56	1.36	1.08	1.00	0.92

Pogostemon auricularius	Acetone	0.66	0.44	0.54	0.53	0.54
	Water	1.03	1.02	0.94	1.06	0.96
	Methanol	1.30	1.02	1.08	1.05	1.03
	Petroleum ether	1.65	1.08	1.36	1.42	1.16
Isolated compounds						
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		0.04	0.06	0.26	0.28	0.14
Jatamansone		0.05	0.15	0.07	0.14	0.09
3-hydroxy-2,2,4-trimethylpentyl isobutyrate		0.08	0.23	0.25	0.46	0.17

*Statistical significance (P < 0.05)

*The microdilution method was used for MIC determination. 3 biological replicates were maintained for all executed experiments.

Table 4.7a. Determination of toxic effects of *Catunaregam spinosa* seed extracts against microbial consortia at 50 µl. (Zone of inhibition measured in mm)

Sl. No.	Bacteria Sp.	*Type*	*Catunaregam spinosa*seed (Zone of inhibition (mm)			
			Petroleum ether Mean ±SD	Ethanol Mean ±SD	Acetone Mean ±SD	Water Mean ±SD
1	*Staphylococcus aureus*	Gram +ve	9.02±0.462	13.56±0.224	12.01±1.24	19.25±0.430
2	*Bacillus subtilis*	Gram +ve	10.20±0.900	14.45±0.338	15.57±1.020	19.00±1.732
3	*Escherichia coli*	Gram −ve	10.34±0.820	9.87±0.240	13.45±1.34	15.83±0.886
4	*Klebsiella pneumonia*	Gram −ve	9.66±0.678	10.91±0.543	12.01±0.210	17.67±0.577
5	*Proteus mirabilis*	Gram −ve	16.67±1.528	14.24±0.166	10.44±0.590	16.33±1.155

*statistical significance (P < 0.05)

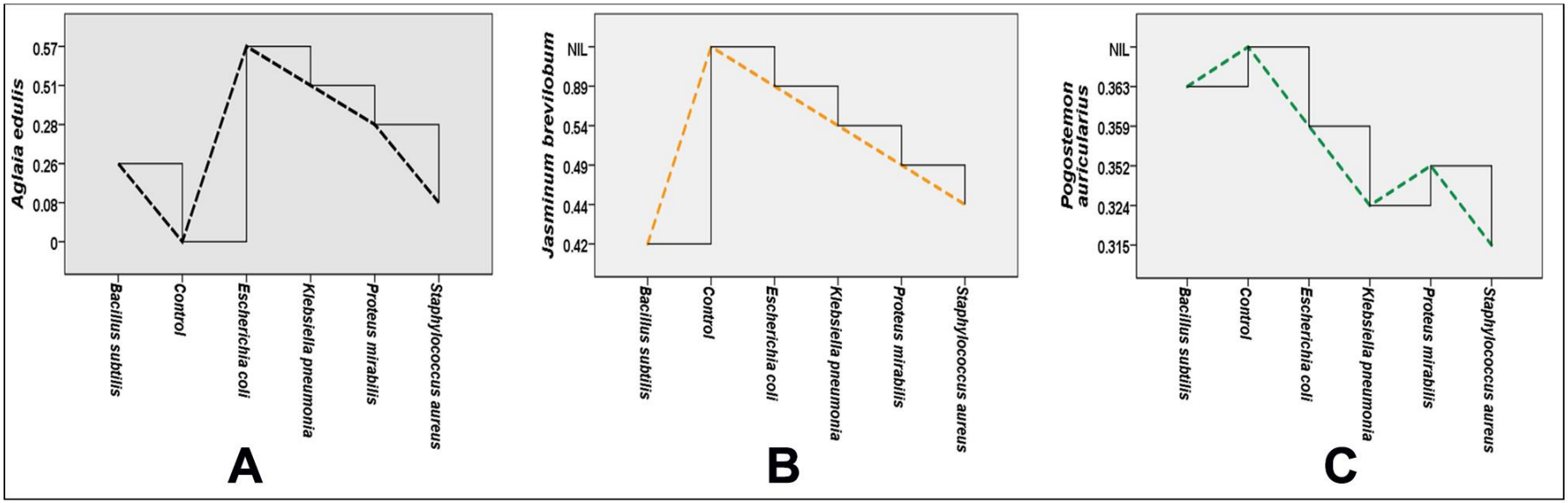


Fig. 4.18. Level of toxicity induced by the various extracts prepared from the medicinal plants *Aglaia edulis*, *Pogostemon auricularius*, and *Jasminum brevilobum* against the microbial consortia

Table 4.7b. Minimum inhibitory concentrations of *Catunaregam spinosa* seed extracts of increasing polarity against tested bacteria

Sl. No.	Sample solution (*Catunaregam spinosa* seed)	*Staphylococcus aureus* (In µg/mL)	*Bacillus subtilis* (In µg/mL)	*Escherichia coli* (In µg/mL)	*Klebsiella pneumonia* (In µg/mL)	*Proteus mirabilis* (In µg/mL)
1	Petroleum ether extract	1.04	1.32	1.9	5.9	0.81
2	Ethanol extract	0. 40	0.80	1.83	2.1	0.96
3	Acetone extract	0.50	0.60	0.90	3.2	1.3
4	Water extract	0. 07	0.08	0.88	3	0.73

*statistical significance ($P < 0.05$)

*MIC = minimum inhibitory concentration mg/mL.

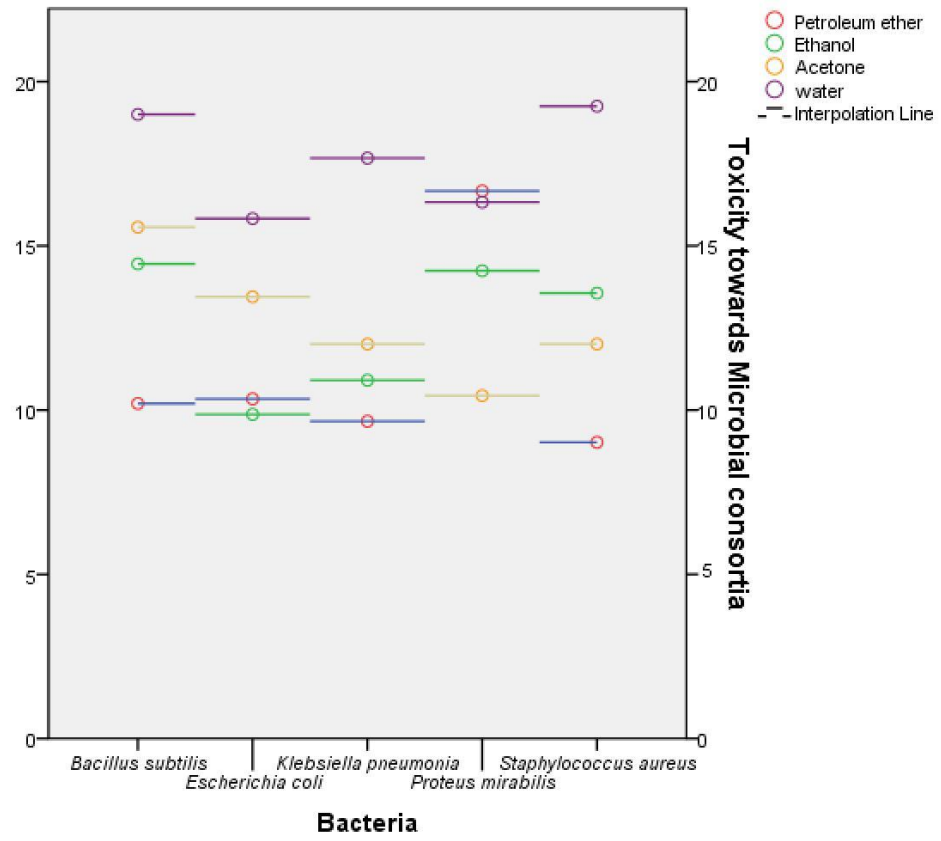


Fig. 4.19. Level of toxicity induced by the various extracts prepared from the medicinal plant *Catunaregam spinosa*

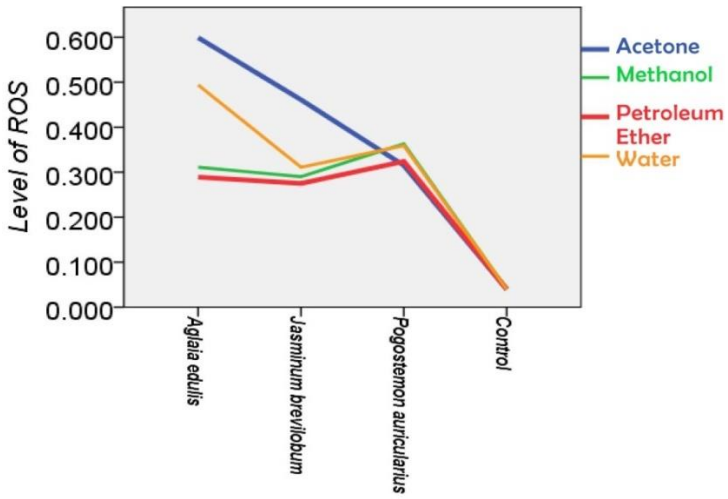


Fig. 4.20. Level of plant extracts prompted reactive oxygen species in the *Aedes aegypti* fourth instar larvae

Table 4.8. Phytochemicals prompted reactive oxygen species in the *Aedes aegypti larvae*

Sl. No.	Sample/ Solvent used for extraction of the plant sample (µg/mL)	Extract Dose (µg/mL)	Absorbance Mean ± SD		
			Aglaia edulis	*Jasminum brevilobum*	*Pogostemon auricularius*
1	*Aedes aegypti* Larvae+Distilled water	125	0.006±0.004	0.005±0.002	0.006±0.000
		250	0.017±0.004	0.018±0.002	0.018±0.000
		500	0.023±0.004	0.023±0.000	0.020±0.000
		1000	0.041± 0.004	0.040±0.004	0.041±0.000
2	Acetone extract	125	0.224±0.004	0.171±0.000	0.100±0.000
		250	0.395±0.006	0.265±0.000	0.221±0.002
		500	0.459±0.004	0.366±0.000	0.299±0.004
		1000	0.599±0.000	0.461±0.000	0.315±0.000
3	Water extract	125	0.141±0.001	0.123±0.000	0.103 ±0.000
		250	0.296±0.000	0.198±0.002	0.200±0.000
		500	0.378±0.000	0.247±0.002	0.299±0.000
		1000	0.495±0.004	0.311±0.002	0.359±0.000
4	Methanol extract	125	0.114±0.001	0.028±0.004	0.111±0.004
		250	0.239±0.000	0.173±0.004	0.216±0.002
		500	0.263±0.004	0.241±0.004	0.279±0.002
		1000	0.311±0.002	0.290±0.004	0.363±0.002
5	Petroleum ether extract	125	0.111±0.002	0.099±0.000	0.147±0.000
		250	0.212±0.002	0.134±0.000	0.194±0.000
		500	0.259±0.016	0.199±0.000	0.267±0.000
		1000	0.289±0.006	0.275±0.000	0.324±0.004

*statistical significance ($P < 0.05$)

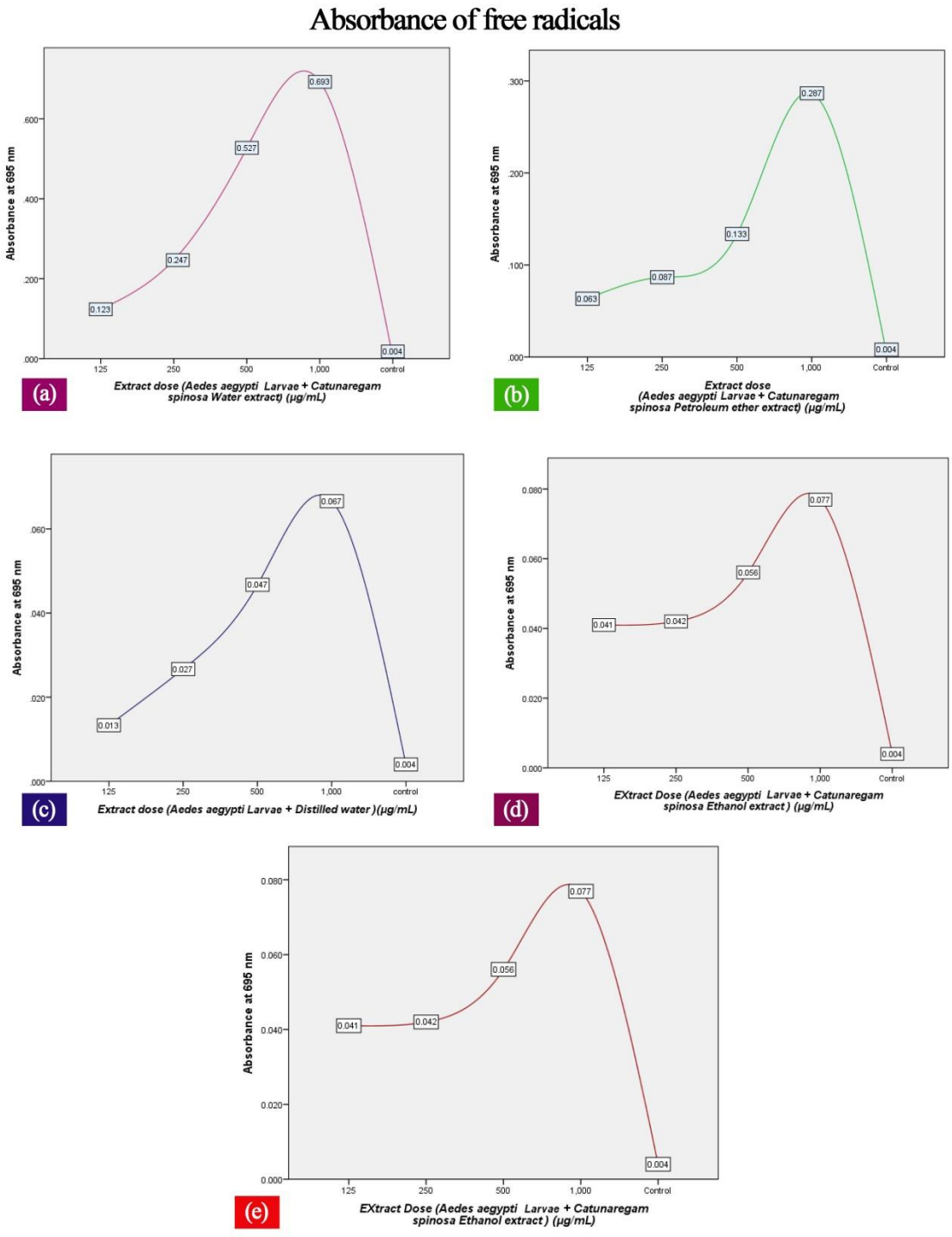


Fig. 4.21. Mechanism of larvicidal action. Determination of free radicals of *Catunaregam spinosa* seed extract exposed *Aedes aegypti* larvae.

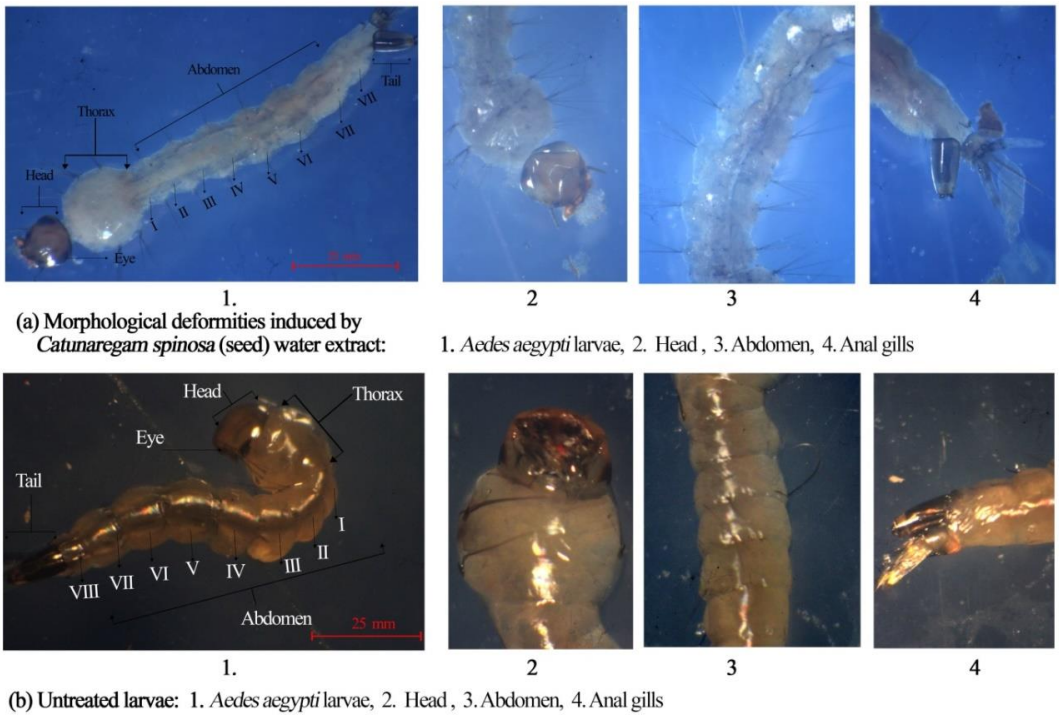
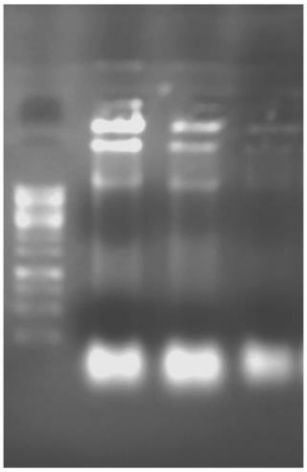


Fig. 4.22. *Catunaregam spinosa* seed extract induces histological modifications on *Aedes aegypti* larvae fourth instar larvae. Light micrographs of head, thorax, abdomen, and anal gills regions of *Aedes aegypti* fourth instar larvae (a) with treatment and(b) without X40 magnification.



Lane 1 :1 kb DNA Ladder
Lane 2 : Untreated DNA
Lane 3 : Hydrogen peroxide treated DNA
Lane 4 : *Catunaregam spinosa* treated DNA

Fig. 4.23. Mechanism of larvicidal action in the *Catunaregam spinosa* seed extract exposed *Aedes aegypti* in context with DNA damage

Table 4.9. Mode of action of isolated bioactive compounds prompted reactive oxygen species as oxidative stress in *Aedes aegypti* larvae

Sl. No.	Compound	Dose (µg/mL)	Absorbance Mean ± SD
1	*Aedes aegypti* Larvae + Distilled water (µg/mL)	125 250 500 1000	0.005 ± 0.000 0.016 ± 0.000 0.022 ± 0.002 0.040 ± 0.000
2	**EE5-** 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	125 250 500 1000	0.301 ± 0.000 0.400 ± 0.000 0.572 ± 0.002 0.600 ± 0.000
3	**CC7-** Jatamansone	125 250 500 1000	0.203 ± 0.000 0.371 ± 0.004 0.433 ± 0.000 0.552 ± 0.006
4	**PP4-** 3-hydroxy-2,2,4-trimethylpentyl isobutyrate	125 250 500 1000	0.240 ± 0.000 0.356 ± 0.002 0.460 ± 0.006 0.559 ± 0.004

*statistical significance ($P < 0.05$)

Table 4.10. Mode of action of *Catunaregam spinosa* seed extracts prompted reactive oxygen species in *Aedes aegypti* fourth instar larave

Sl. No.	Sample	Extract Dose (µg/mL)	Absorbance Mean ± SD
1	*Aedes aegypti* Larvae + Distilled water (µg/mL)	125 250 500 1000	0.013±0.006 0.027±0.006 0.047±0.006 0.067±0.006
2	*Aedes aegypti*Larvae + *Catunaregam spinosa* Acetone extract (µg/mL)	125 250 500 1000	0.063±0.005 0.087±0.005 0.133±0.010 0.287±0.005
3	*Aedes aegypti*Larvae + *Catunaregam spinosa* Water extract (µg/mL)	125 250 500 1000	0.123±0.006 0.247±0.005 0.527±0.005 0.693±0.006
4	*Aedes aegypti*Larvae + *Catunaregam spinosa*Ethanol extract (µg/mL)	125 250 500 1000	0.017±0.05 0.024±0.002 0.037±0.002 0.051±0.005
5	*Aedes aegypti*Larvae + *Catunaregam spinosa* Petroleum ether extract (µg/mL)	125 250 500 1000	0.041±0.002 0.042±0.002 0.056±0.016 0.077±0.006

*statistical significance ($P < 0.05$)

4.4. DISCUSSION

From the moment that the first report regarding the emergence of insecticide resistance in the mosquitoes during the 1950s came out, the scientific community throughout the world has been focusing on ways to find out the mode of action of insecticides with special emphasis on the emergence of resistance. Investigations unveiling the mechanisms and mode of actions concerned with the resistance hold significance by having been directed towards novel strategies against the mosquitoes (Rai et al., 2019). It is significant to keep in mind that a large number of investigations have been carried out previously to find out the insecticide resistance mechanisms with special emphasis on the common classes of insecticides. For instance, previous studies have reported the emergence of resistance in mosquitoes towards the major synthetic insecticides such as permethrin, DDT, malathion, and deltamethrin (Thanispong, Sathantriphop, & Chareonviriyaphap, 2008).

The previous studies have provided evidence that the *Aedes aegypti* mosquito vectors are resistant to several synthetic insecticides and none have piloted a study on the bioactive elements isolated from the traditionally used medicinal plants such as *Aglaia edulis*, *Jasminum brevilobum*, and *Pogostemon auricularius* with special reference to the insecticide resistance, mode of action, microbial extermination and phytochemical prompted reactive oxygen species. Moreover, majority of the studies reported that the emergence of insecticide resistance at a high level can severely hamper the effective mosquito control programmes during

the arboviral disease outbreaks. Hence, analyzing the level of insecticide resistance in *Aedes aegypti* mosquitoes is of paramount importance for effective vector control programme during emergency situations like dengue and zika fever outbreaks.

Based on the afore-mentioned aspects, the present study sets out to determine the qualitative and quantitative analysis of certain detoxifying enzymes (esterases, G6PD, and Cytochrome P450) towards the isolated bioactive elements CC7, PP4, and EE5. In addition to this, the role of reactive oxygen species (bioactive elements prompted ROS) in mosquito control with special emphasis on the extermination of microbial consortia that resides inside the *Aedes aegypti* larvae were discussed in this study.

The mechanism behind the resistance towards the bioactive compounds was qualitatively analyzed by gel profiles while its quantitative analysis was performed using biochemical assays. As stated in the results section, the gel profiles of α-esterases, β-esterases, P450, and G6PD show differences in selected (CC7, EE5 and PP4) and susceptibility strains in terms of the number of bands and mobility. The additional bands are shown in the densitometric profiles of respective strains as additional peaks. A similar pattern was observed in previous studies by (Jagadeshwaran & Vijayan, 2009).

Poly Acrylamide Gel Electrophoresis is an effective technique in revealing the mechanism behind the emergence of insecticide resistance as any change in the pattern of enzymes can be recognized as being directly influenced by the gene function (Hemingway & Karunaratne, 1998; Jagadeshwaran & Vijayan, 2009).

The α-esterase profile of CC7 and EE5 selected strains of *Aedes aegypti* in F_{10} generation shows 3 bands as compared to three in the susceptible strain. A similar pattern was observed in the EE5 and PP4 selected strains of *Aedes aegypti* in F_{10} generation. The PP4 selected strains of *Aedes aegypti* has also shown an extra band in the F_{10} generation compared to susceptible strains. Likewise, Ganesh, Vijayan, Urmila, Gopalan and Prakash (2002) reported that the deltamethrin selected lines of *Anopheles stephensi* revealed 3 bands for α-esterases. Aside from the number of bands and the nature of the insecticide, the afore-mentioned results are similar to those reported in the other mosquito species, *Anopheles stephensi* (Hariprasad & Shetty, 2016).

The β-esterase profile of CC7 and EE5 have revealed three bands as reported in *Anopheles stephensi* by (Ganesh et al., 2002). However, the densitometric profiles of β-esterase reported in this study have shown variations as compared to the profile reported by them. One explanation for this condition could be the nature of insecticide since the present study used bioactive elements isolated from traditionally used medicinal plants rather than synthetic insecticides.

The G6PD profiles of CC7, EE5, and PP4 selected strains of *Aedes aegypti* in F_{10} shows additional bands as compared to susceptible strains. This is supported by another study in *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti* by Kumar et al. (1991). The present study also validated that the CYTP450 is monomorphic as studied by Suwanchaichinda and Brattsten (2002) in *Aedes albopictus* strains. The CYTP450 (monooxygenases) is primarily known for their active participation in the metabolic system concerning the anabolism and

catabolism of endogenous compounds and xenobiotics. In addition, the monooxygenase linked detoxification is primarily recognized as an important mechanism that is responsible for the emergence of insecticide resistance in mosquitoes (Scott, 1999).

The previous studies reported that the oxidation arbitrated by monooxygenase enzymes is recognized as the significant pathway responsible for the detoxification of toxic elements in insects. All the afore-mentioned studies have validated that the CYTP450s are an important metabolic system observed in all insects. They need NADP as a cofactor to involve various metabolic processes and the NADP was usually generated by G6PD. Therefore, an upsurge in the level of the afore-mentioned monooxygenases and dehydrogenases is considered as a significant factor in insecticide resistance emergence as opined by previous reports. In addition to this, due to the drawbacks linked with the quantitative determination of monohydogenases and dehydrogenases using conventional strategies, the spectrophotometric coupled with microtitre plate assay has gained importance for the quantitative determination of detoxifying enzymes in mosquitoes. Previous studies have validated the accuracy of enzyme assay techniques in determining the quantitative estimation of monooxygenases, dehydrogenases, and esterases (α-esterases and β-esterases) with special reference to the emergence of resistance against common classes of synthetic insecticides as discussed earlier.

The enzyme assay techniques turn into apparent that it can hold accuracy of results. In addition to this, due to the drawbacks linked with the quantitative

determination of monohydogenases and dehydrogenases using conventional strategies, the spectrophotometric coupled with microtitre plate assay has gained importance for the quantitative determination of detoxifying enzymes in mosquitoes. This has enhanced the significance of the quantitative analysis of esterases (α-esterases and β-esterases) CYTP450 and G6PD in the CC7, PP4 and EE5 selected lines of *Aedes aegypti* mosquitoes in the present study.

The results from the present investigation regarding the quantitative analysis of detoxifying enzymes such as α-esterases, β-esterases, cytochrome P450, and G6PD upon statistical analysis ($P < 0.05$) revealed a significant level of activities in CC7, EE5 and PP4 selected strains of *Aedes aegypti*. This has revealed the fear that the continuous exposure of insecticides may lead to the emergence of resistance in *Aedes aegypti* at a moderate level as discussed in the previous objective. The results from this investigation are in pact with the previously published reports concerning the insecticide resistance in synthetic insecticides (Chakravorthy & Kalyanasundaram, 1992). However, the level of insecticide resistance that emerged in this study against the bioactive elements is relatively lower than that of the synthetic insecticides. For instance, the pyrethroid exposed strain of *Anopheles stephensi* exhibited a fold increase of 182 to permethrin (Enayati et al., 2003). In line with their results, a study from China revealed that 12 generations of deltamethrin selected strains of *Anopheles stephensi* showed 190 fold increase (Ranson et al., 2000). Similar results were also reported by (Kumar et al., 2004). Similarly, the continuous exposure of deltamethrin in *Aedes aegypti* for 40 generations showed 703 fold resistance. According to previous studies, α-esterases and β-esterases are tremendously important in emerging insecticide

resistance (organophosphates). For instance, the elevated level of carboxylesterases in mosquitoes is allied with the emergence of resistance against various common synthetic insecticides such as temephos, malathion, permethrin, lambdacyhalothrin, deltamethrin, and DDT (Rai et al., 2019).

The hydrolysis of certain toxic compounds (pyrethroids and other insecticides) is alleged to act as a reason for emerging resistance in mosquitoes (Vulule et al., 1999). Besides this, previous studies have reported a high level of fold increase for esterases activity. Further support for the afore-mentioned statement is provided by Macoris et al. (2003) and Gokhale, Jacob and Mourya (2000). According to them, the esterase activity in DDT selected *Aedes aegypti* strains was 3-4 times more than that of the susceptible lines. In accordance with their results, the present study has reported a low level of α-esterases activity for CC7, EE5 and PP4 selected strains with low fold increase values ranging from 1.56 to 1.71. The values were significant at $P<0.05$. The fold increase value for the α-esterase activity in CC7 exposed strains was 1.65 times more than that of the susceptible strains. The EE5 selected strain also possessed α-esterase activity with a fold increase of 1.68. Similarly, the PP4 selected strain was known to produce α-esterase activity with a fold increase of 1.60.

The influence of β-esterase activity in insecticide resistance in various mosquito vectors has been reported previously by (Liu, Xu, Zhang, & Liu, 2005). In accordance with their results, several studies have verified the link between the emergence of insecticide resistance and β-esterase activity (Ganesh et al., 2002; Vulule et al., 1999). In addition to this, Rai et al. (2019) have reported that the

elevated level of carboxylesterases in mosquitoes is allied with the emergence of resistance against various common synthetic insecticides such as temephos, malathion, permethrin, lambdacyhalothrin, deltamethrin, and DDT (Rai et al., 2019). A study by Poupardin et al. (2012) reported that the level of β-esterase activity was dramatically increased in the field populations of *Anopheles stephensi*. Similarly, the present study has reported the moderate level of β-esterase activity. Among the three strains investigated, the CC7 selected lines of *Aedes aegypti* showed a relatively low level of β-esterase activity with a fold increase value of 1.65. However, while comparing the level of β-esterase activity towards the synthetic insecticides, it was observed that the level of β-esterase activity induced by the EE5 (Fold increase - 1.56) and PP4 (Fold increase - 1.71) bioactive elements was frail. The values were significant at $P<0.05$. Conclusively, the data regarding the α-esterase and β-esterase activity supported by the afore-mentioned studies clearly analysed the influence of enzymatic metabolism in emerging moderate levels of resistance against the bioactive elements in CC7, PP4, and EE5 selected lines of *Aedes aegypti.*

The monooxygenases have long been of special interest since they are vital for the detoxification of drugs, chemical carcinogens, plant toxins, mutagens, and pesticides. They are also known to involve in the metabolization of several endogenous elements such as steroids, fatty acids, and hormones. A prominent characteristic feature of P450s allied with the detoxification of pesticides and other elements is the increase in the level of cytochrome P450 activity by the increased expression of CYTP450 genes in target organisms, which has drawn the emergence of resistance to insecticides (Marcombe et al., 2012).

The following previous studies have reported the role of CYTP450s in insecticide resistance emergence towards Permethrinand deltamethrin in *Aedes aegypti* populations; (Marcombe et al., 2012; Marcombe et al., 2009; Poupardin et al., 2012; Strode et al., 2008). Similarly there exists several studies concerning the role of Cytochrome P450s in insecticide resistance emergence towards various insecticides including DDT, deltamethrin, and permethrin in *Anopheles* spp., *Culex* sp. (Awolola et al., 2009; David et al., 2005; Djouaka et al., 2008; Liu, Li, Reid, Yang, & Zhang, 2011; Mueller et al., 2008; Stevenson et al., 2011; Vontas et al., 2005). In accordance with their results, previous studies have validated the role of P450s in detoxifying various synthetic insecticide like permethrin as important primary enzymes conferring resistance in mosquito vectors (Liu et al., 2005; Xu, Liu, Zhang, & Liu, 2005). The present study also reported the monooxygenase activity in three selected lines of *Aedes aegypti* with fold increase values ranging from 1.70 to 1.81. The values were significant at $P<0.05$. In context with the earlier discussed studies, the afore-mentioned findings suggest that the monooxygenases have a prominent role in the emergence of insecticide resistance. However, the comparison of the level of monooxygenases activity towards the previously published reports on synthetic insecticides has revealed that the bioactive elements are safer than that of the synthetic ones owing to their low level of capacity to develop resistance in *Aedes aegypti* mosquitoes.

The previous studies have made attempts to correlate the potential of certain detoxifying enzymes including glucose-6-phosphate dehydrogenase (G6PD) with the emergence of insecticide resistance in mosquito vectors. The quantitative estimation revealed that the CC7, EE5, and PP4 selected strains of *Aedes aegypti*

have revealed G6PD activity with a fold increase ranging from 1.79-1.89. The values were significant at $P<0.05$. In addition to this, the present study also determined the total protein content of the CC7, EE5 and PP4 selected strains of *Aedes aegypti* with a fold increase ranging from 2.1 to 2.9 ($P < 0.05$) (Fig. 4.17; Page No. 238). Previous laboratory studies have reported the link between the emergence of insecticide resistance and G6PD (Ganesh et al., 2002; Kumar et al., 1991). In comparison with their results, the present study has reported a reduced chance of emerging insecticide resistance in *Aedes aegypti* as verified in the previous objective. One of the major explanations for the reduced level of emerging insecticide resistance is the low level of esterase, G6PD and PP4 activity instigated by the bioactive elements CC7, EE5, and PP4. The afore-mentioned facts imply that all the bioactive elements used in this study can be used for effective mosquito control since they reduce the risk of emerging insecticide resistance in *Aedes aegypti* mosquitoes than that of synthetic insecticides. The increasing resistance ratio (RR) from the parent generation to F_{10} generation has indicated that there exists a prominent role of natural selection in exposed strains of *Aedes aegypti*.

The results from this objective also revealed that the phytochemical constituents retrieved from traditionally used medicinal plants are biodegradable, eco-attractive and reputed to have effective toxic potential towards the various mosquito vectors. Previous studies have supported these facts; and due to this reason, several medicinal plants and insecticides of botanical origin have been principally used for mosquito vector control. Due to the presence of several phytochemicals constituents like phenolic compounds, alkaloids, organic acids

and flavonoids, the insecticides of botanical origin and their significant role in producing the excessive level of reactive oxygen species, the medicinal plants have taken a prominent role in the area where the humans are threatened by arthropod-borne diseases (Anoopkumar, Aneesh, & Sudhikumar, 2020).

The plant extracts induced reactive oxygen species have been principally known for their significance to initiate important processes that make the reactive oxygen species as stable. The plant phytochemical constituents have been principally known for their significance to initiate important processes that make the reactive oxygen species as stable. The initiated reactions have made a disparity between ROS and various defense processes thereby leading to abnormalities in amino acids, proteins, cell constituents, and eventually causing oxidative stress and death. In this study, the the researcher has chosen this loophole to generate an excessive amount of free radicals inside the *Aedes aegypti* fourth instar larvae.

The present objective has verified that the various extracts and compounds retrieved from the three medicinal plants have produced a high level of reactive oxygen species in the target organism, specifically in the *Aedes aegypti* fourth instar larvae. It is significant to keep in mind that the mode of action of phytochemicals induced reactive oxygen species with special reference to the mosquito control is scanty in the present scenario.

The present objective revealed that one of the probable reasons for the death of *Aedes aegypti* fourth-instar larvae towards the continuous exposure of phytochemicals is the excessive production of ROS instigated by bioactive elements isolated from the three medicinal plants such as *Jasminum brevilobum*,

Pogostemon auricularius, and *Aglaia edulis.* In specific, the elevated level of free radicals induced by the CC7, EE5, and PP4 has recognized as the probable mode of action in the target insect, *Aedes aegypti* fourth instar larvae. In agreement with the findings of this study, Oliveira et al. (2017) reported that the excess production of free radicals might interrupt several signalling pathways that lead to p53-dependent apoptosis along with deformities in numerous physiological activities.

The augmented production of free radicals may also instigate damages to nucleic acids and proteins including the cell organelles thereby leading to the death of *Aedes aegypti* mosquito larvae (Anoopkumar et al., 2020; Kodrík, Bednářová, Zemanová, & Krishnan, 2015). In view of the afore-mentioned fact, this study assumed that the excess production of ROS by the bioactive elements (CC7, EE5, and PP4) and various extracts prepared from *Jasminum brevilobum, Aglaia edulis,* and *Pogostemon auricularius* would be responsible for larval death. Additionally, this study has verified the potential of various extracts of *Catunaregam spinosa* seeds to generate an excessive amount of free radicals in the *Aedes aegypti* fourth instar larvae. Based on the earlier reported and discussed findings, the researcher has assumed that the excessive production of free radicals induced by the various extracts of *Catunaregam spinosa* could be recognized as one of the prominent reasons behind the death of *Aedes aegypti* fourth instar larvae.

The structural damages in the *Aedes aegypti* mosquito larvae instigated by the continuous exposure of phytochemical constituents in *Catunregam spinosa* have been analyzed using the microscopic examination. The severe damages in the head, abdomen, and thorax as photographed in Fig. 4.22 (Page No. 244) indicated

that the free radicals have a significant role in inducing damages in cells and tissues.

After the analysis of the role of ROS in the mode of action of the phytochemical constituents, the present investigation has invented the influence of the extermination of microbial consortia in mosquito control. The significance of microorganism-based techniques to analyze their profits to humans has intensely augmented in the present research. Important efforts have been made to find out the link between microorganisms and mosquito vectors in recent epochs. A recent report revealed that *Aedes aegypti* species failed to complete their life cycle without the presence of microorganisms in their body during their larval stage (Valzania, Coon, Vogel, Brown, & Strand, 2018a; Valzania et al., 2018b). According to their inference, the insects have maintained a proper give-and-take multifarious link with microorganisms; this interaction has offered many advantages for the insect host. One of the major beneficial impacts that the host has received from the microbial consortia is the sufficient amount of nutrients. The microbial consortia also help the host to provide resistance against pathogens and parasitoids (Valzania et al., 2018b). They also mentioned that the reason behind the death of *Aedes aegypti* larvae is the interrupted bacteria-induced hypoxia signal. The microbial consortia that reside inside the *Aedes aegypti* larvae produce a signal which is important to activate the hypoxia-induced transcription factors that are allied with the growth-related activities of larvae. Here this study chooses this loophole as an effective way to prevent the life cycle stages of *Aedes aegypti* since many of the phytochemical constituents including CC7, EE5 and PP4 are known to exhibit potent toxicity against microorganisms.

The prevention of hypoxia-induced transcription factors can be accomplished through the destruction of microbial consortia that reside in the *Aedes aegypti* larvae. The present study validated that the extermination of *Klebsiella pneumonia*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, and *Bacillus subtilis* by the direct administration of various extracts and purified compounds. In which *Klebsiella pneumonia*, *Staphylococcus aureus*, *Bacillus* sp, and *Escherichia coli* exist in the *Aedes aegypti* are previously reported for their host-parasite interaction. These microorganisms also provide essential growth factors and most of the microbial consortia can modulate the vectorial capacity and vector immunity (Azambuja, Garcia, & Ratcliffe, 2005; Yadav et al., 2015). In context with the previous findings, the present study assumed that the extermination of microbial consortia are regarded as one of the probable reasons for the death of *Aedes aegypti* larvae. Therefore, the results of this investigation suggest that all the isolated compounds (CC7 -Jatamansone; EE5 -2,2,4-Trimethyl-1,3-pentanediol diisobutyrateand; PP4-3-hydroxy-2,2,4-trimethylpentyl isobutyrate) can be used to eliminate the microbial consortia that reside inside the *Aedes aegypti* thereby providing an intervention into the microbe based mosquito control strategy.

4.5. SUMMARY

The scientific research concerning the natural control approaches for mosquito control in context with the mode of action of bioactive elements, insecticide resistance, and evolutionary perspectives in *Aedes aegypti* would be helpful for designing the efficient vector control protocols. Early detection of emerging insecticide resistance is a key factor for the sustainable management of mosquito control. The insecticide resistance in mosquitoes is mainly linked with metabolic resistance and target site modifications. The latter one befalls through the augmented bio-degradation of toxic elements like insecticides, typically through the excessive production of certain detoxifying enzymes such as dehydrogenases, monooxygenases, esterases, cytochrome P450s and glucose-6-phosphate dehydrogenase.

The resistance to some of the commonly used insecticides is a major evolutionary consequence that happens through natural selection. The emergence of resistance against active and frequently used mosquito controlling products is an environmental aspect since the mutation and enzymatic changes in *Aedes aegypti* mosquitoes allow them to persist against synthetic products. Moreover, the trait responsible for the insecticide resistance has been transferred from one generation to the next generation and thereby forms a new strain of mosquito vector with increased insecticide resistance capacity (F_1-F_{10} generation) than the parent strain as described in this chapter.

Any damage in the DNA of mosquitoes may also cause many alterations in certain encoded proteins thereby instigating the inhibition of protein production. The strand breakage, deoxyribose oxidation followed by nucleotide removal may also develop severe abnormal processes that lead to oxidative stress which is elaborately discussed in our published work entitled "A Novel Intervention on the Inhibiting Effects of *Catunaregam spinosa* Induced Free Radical Formation and DNA Damage in *Aedes aegypti* (Diptera: Culicidae): A Verdict for New Perspectives on Microorganism Targeted Vector Control Approach", in the "International Journal of Tropical Insect Science, Springer, Singapore".

The overexpression of concerned genes has been reported in association with the emergence of insecticide resistance in many mosquitoes. Previous studies have reported the emergence of insecticide resistance in mosquito vectors with special reference to the influence of detoxifying enzymes. However, none have piloted a study concerning the emergence of resistance towards phytochemical constituents with special implications on the free radicals production, extermination of microbial inhabitants, evolutionary perspectives and natural selection.

The first approach to this investigation was to qualitatively estimate the presence of certain detoxifying enzymes such as α-esterases, β-esterases, G6PD, and cytochrome P450s in CC7 (Jatamansone), EE5 (2,2,4-Trimethyl-1,3-pentanedioldiisobutyrate) and PP4 (3-hydroxy-2,2,4-trimethylpentyl isobutyrate) selected strains of *Aedes aegypti* fourth star larvae. The qualitative analysis of the selected strains was accomplished using PAGE. It revealed that the profile of α-esterase, β-esterase, G6PD and cytochrome P450s profile of phytochemicals

selected strains showed specific bands. The electromorph along with the respective densitometric profiles of CC7, EE5, and PP4 selected strains revealed that the cytochrome P450 was monomorphic. The quantitative estimation of the above said enzymes were performed using microplate assay. The mean value of α-esterase, β-esterases, G6PD, and cytochrome P450s activity was recognized to be statistically significant. A comparison of the level of α-esterases, β-esterases, G6PD, and P450s of the afore-mentioned strains with the previously published reports concerning the synthetic insecticides and resistance in mosquitoes revealed that the bioactive elements are safer than that of synthetic insecticides.

The results suggest that the investigated plant products have instigated less level of insecticide resistance in the *Aedes aegypti* mosquitoes, while the synthetic insecticides develop an extreme level of insecticide resistance in the target insect.

Moreover, this investigation has also unveiled the fact that resistance ratio of *Aedes aegypti* has been increased from F_1-F_{10} generation indicating the concern that the trait which was responsible for insecticide resistance has been transferred from parent strain to exposed strains through natural selection, and this has illustrated the evolutionary significance of this study.

In addition to this, the present study has explored the mode of action of the bioactive elements CC7 (Jatamansone), PP4 (3-hydroxy-2,2,4- trimethylpentyl isobutyrate) and EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) with special reference to the formation of reactive oxygen species and extermination of microbial consortia. The afore-mentioned perspective has been clearly discussed in our article entitled “Exploring the Mode of Action of Isolated Bioactive

Compounds by Induced Reactive Oxygen Species Generation in *Aedes aegypti*: A Microbes-based Double-edged Weapon to Fight against Arboviral Diseases" published in the "International Journal of Tropical Insect Science, Springer, Singapore".

The three isolated compounds from the present study have prominently generated excessive amounts of free radicals inside the treated *Aedes aegypti* larvae thereby causing an imbalance in homeostasis. In context with the afore-mentioned aspects and due to the low level of esterases and dehydrogenases activity, the bioactive elements such as CC7 (Jatamansone), EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) and PP4 (3-hydroxy-2,2,4-trimethylpentyl isobutyrate) can be recognized as effective raw materials for the preparation of natural insecticide against *Aedes aegypti* mosquitoes in future. These findings recommend that the use of bioactive elements for mosquito control is likely to be a vital factor governing the prevention of rapid proliferation of mosquito-borne diseases. The evolutionary perspective that emerged in this investigation could be considered as a base for future studies concerning the influence of natural selection and evolution in mosquito-borne disease eradication.

GENERAL SUMMARY

GENERAL SUMMARY

Mosquito control is a daunting problem confronted by both the research community and the public health experts all over the world. *Aedes aegypti* is recognized as an important vector of several arboviruses which are responsible for the transmission of various deadly diseases such as dengue, Zika, chikungunya and yellow fever. Among them, dengue is considered as one of the most widely spread arbo-viral disease; prevalent in more than 100 nations all over the world.

The DENV (I-V) in humans instigates a broad spectrum of symptoms ranging from mild illness to fatal cases of hemorrhagic fever. In tropical and sub-tropical provinces of the world where the infection is severely prevalent, the dengue becomes hyper-endemic, resulting in foremost public health issues. In the absence of effective vaccine against the dengue infection, it is necessary that the rapid proliferation of dengue vector *Aedes aegypti* should be prevented. it is prominent that the research community act against the illnesses and the threat of local transmission in an efficient way by ensuing mosquito control, principally by using natural insecticides to prevent the proliferation of *Aedes aegypti* mosquitoes. Moreover, there exists a paucity of understanding regarding the evolutionary aspects of insecticide resistance in *Aedes aegypti* and the topics on the mode of action and natural selection based on the afore-mentioned concepts remain a considerable focus of this investigation.

Finding new aspects of natural insecticides development and the mode of action will definitely assist reducing the chance of resistance in mosquitoes, which is a

growing problem in mosquito control. The frequent and routine use of synthetic insecticides or the chance of developing insecticide resistance should be reduced to prevent the threat to public health. In fact, the development of insecticide resistance from parent strain to exposed strains (F_1-F_{10} generation) as described in the fourth chapter is an evolutionary phenomenon that happens through natural selection and mutation in *Aedes aegypti* mosquitoes.

The present investigation was carried out using fifty traditionally used medicinal plants against *Aedes aegypti* mosquitoes. Most of all the medicinal plants exhibited potent larvicidal efficacy. Among them, 21 plants exhibit more than 41% of toxicity, 4 plants show 31-40 of toxicity and 8 plants show 20-30% of mortality. The medicinal plants such as *Euphorbia thymifolia*, *Catunaregam spinosa*, *Cardiospermum halicacabum*, *Persicaria hydropiper*, *Ageratum conyzoides*, *Glycosmis pentaphylla*, *Plectranthus hadiensis*, *Asclepias curassavica*, *Mimosa pudica*, *Toddalia asiatica*, *Scoparia dulcis*, *Jasminum brevilobum*, *Aglaia edulis*, and *Pogostemon auricularius* exhibited maximum range of larvicidal potential against *Aedes aegypti* fourth instar larvae.

Despite the fact that there exist several investigations on the larvicidal potential of plants with mosquito vectors, only a few studies have reported the isolation and identification of bioactive elements against *Aedes aegypti* mosquitoes. The present study described the isolation and characterization of three bioactive elements such as "2,2,4-Trimethyl-1,3-pentanediol diisobutyrate" (from *Aglaia edulis*), "Jatamansone" (from *Jasminum brevilobum*), "3-hydroxy-2,2,4-trimethylpentyl isobutyrate" (from *Pogostemon auricularius*). All the bioactive

fractions had reported prominent range of larvicidal efficacy. The EE5 (2,2,4-Trimethyl-1,3-pentanediol diisobutyrate) isolated from *Aglaia edulis* showed most effective larvicidal efficacy against *Aedes aegypti* fourth instar larvae with LC50 value of 1.724 (0.834-2.479) mg/l. The results gathered from the second chapter have recommended that the plant-based products for mosquito control would mitigate the threat to humans.

Further investigations with reference to the susceptibility status of isolated bioactive elements should be studied to find out the chance of emerging resistance in the F_1-F_{10} generations of phytochemical exposed strains of *Aedes aegypti* mosquitoes. Analysing the chance of emerging resistance towards insecticides is paramount in public health emergencies. Therefore, the third chapter has evaluated the susceptibility status of 4^{th} instar larvae of *Aedes aegypti* towards the plant isolates by employing the standard larval bioassay procedure prescribed by WHO. This chapter has reported that all the *Aedes aegypti* strains were moderately resistant to the isolated bioactive elements (CC7 – Jatamansone; EE5 - 2,2,4-Trimethyl-1,3-pentanediol diisobutyrateand; and PP4 - 3-hydroxy-2,2,4-trimethylpentyl isobutyrate), exposing the importance of phytochemical constituents in mosquito control. All the experiments showed lower resistance ratio (RR) towards higher concentrations of CC7, EE5, and PP4 that makes the study unique and specific.

According to the literature, none have piloted a study on the mode of action and mechanism behind the emergence of a moderate level of resistance with special reference to the microbial consortia, natural selection and evolution.

Therefore the afore-mentioned concept has been investigated in the final chapter with special reference to the qualitative and quantitative changes of certain detoxifying enzymes such as esterases, dehydrogenases, and monooxygenases if any, due to the effect of botanicals. A comparison of the level of α-esterases, β-esterases, G6PD, and P450s of the phytochemical selected strains against the previously published reports concerning the synthetic insecticides and resistance in mosquitoes verified that botanical products are safer than that of synthetic insecticides. The isolated bioactive elements in this study prominently instigated the production of Reactive Oxygen Species in the target insect *Aedes aegypti* larvae, thereby resulting imbalance in homeostasis. The increase in the Resistance Ratio (RR) for phytochemicals exposed strains from F_1-F_{10} generation illustrates the role of natural selection in insecticide resistance emergence.

The final chapter has also explored the role of the extermination of microbial consortia and natural selection in mosquito control in context with DNA damage as explained in our published article entitled as "A Novel Intervention on the Inhibiting Effects of *Catunaregam spinosa* Induced Free Radical Formation and DNA Damage in *Aedes aegypti* (Diptera: Culicidae): A Verdict for New Perspectives on Microorganism Targeted Vector Control Approach", in "International Journal of Tropical Insect Science, Springer, Singapore". The importance of microorganism-based techniques to explore their benefits to humans has intensely been augmented in the final chapter. All the isolated bioactive elements such as (CC7 – Jatamansone; EE5 -2,2,4-Trimethyl-1,3-pentanediol diisobutyrateand; and PP4 - 3-hydroxy-2,2,4-trimethylpentyl

isobutyrate exhibited a prominent range of toxicity against the microbial consortia that shares a symbiotic relationship with the mosquito vector.

In context with the previous findings, the final chapter has revealed that the extermination of microbial consortia can principally be recognized as one of the probable reasons for the death of *Aedes aegypti* larvae. This study verified that the bioactive elements isolated from the three medicinal plants have been in the centre of interest for natural insecticide discovery in the future.

REFERENCES

Abirami, P. & Rajendran A. (2012. GC-MS analysis of methanol extracts of *Vernonia cinerea*. *European Journal of Experimental Biology*, *2*(1), 9-12.

Abbott WS. A. (1925). method of computing of the effectiveness of an insecticide. *J Econ Entomol,* 8(2):265-267.

Aiub, C. A. F., Coelho, E. C. A., Sodré, E., Pinto, L. F. R., & Felzenszwalb, I. (2002). Genotoxic evaluation of the organophosphorus pesticide temephos. *Genetics and Molecular Research, 1*(2), 159-166.

Aiton, Hort. Kew.(1789). [W. Aiton] 3: 20.

Aktar, W., Sengupta, D., & Chowdhury, A. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary Toxicology, 2*(1), 1-12.

Akula, R., & Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior, 6*(11), 1720-1731.

Amer, A., & Mehlhorn, H. (2006a). Larvicidal effects of various essential oils against *Aedes, Anopheles,* and *Culex* larvae (Diptera, Culicidae). *Parasitology Research, 99*(4), 466-472.

Amer, A., & Mehlhorn, H. (2006b). Persistency of larvicidal effects of plant oil extracts under different storage conditions. *Parasitology Research, 99*(4), 473-477.

Andrade-Ochoa, S., Correa-Basurto, J., Rodríguez-Valdez, L., Sánchez-Torres, L., Nogueda-Torres, B., & Nevárez-Moorillón, G. (2018). In vitro and in silico studies of terpenes, terpenoids and related compounds with larvicidal and pupaecidal activity against *Culex quinquefasciatus* Say (Diptera: Culicidae). *Chemistry Central Journal, 12*(1), 53.

Anjana, S., & Thoppil, J. E. (2013). Analysis of cytotoxic potential of the aqueous leaf extracts of *Pogostemon auricularius* (L.) Hassk. using *Allium cepa*

root tip assay. *International Journal of Research and Development in Pharmacy and Life Sciences, 2*(5), 562-566.

Anoopkumar, A. N., Aneesh, E. M., & Sudhikumar, A. V. (2020). Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases. *International Journal of Tropical Insect Science*, 40, 573-585.

Anoopkumar, A. N., Puthur, S., Rebello, S., & Aneesh, E. M. (2017b). Screening of a few traditionally used medicinal plants for their larvicidal efficacy against *Aedes aegypti* Linn (Diptera: Culicidae), a dengue fever vector. *SOJ Microbiology and Infectious Diseases, 5*(4), 1-5.

Anoopkumar, A. N., Puthur, S., Rebello, S., & Aneesh, E. M. (2019). Molecular characterization of *Aedes, Culex, Anopheles,* and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis. *Biologia*, *74*, 1125-1138.

Anoopkumar, A., Puthur, S., Varghese, P., Rebello, S., & Aneesh, E. M. (2017a). Life cycle, bio-ecology and dna barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). *The Journal of Communicable Diseases, 49*(3), 32-41.

Arivoli, S., Divya, S., Arumugam, B., Meeran, M., Jayakumar, M., Raveen, R., & Samuel, T. (2018). Phytochemical constituents of *Jasminum fluminense* Linnaeus (Oleaceae): An additional tool in the ecofriendly management of mosquitoes? *Journal of Pharmacognosy and Phytochemistry, 7*(1), 548-556.

Arivoli, S., Tennyson, S., & Martin, J. J. (2011). Larvicidal efficacy of *Vernonia cinerea* (L.) (Asteraceae) leaf extracts against the filarial vector *Culex quinquefasciatus* Say (Diptera: Culicidae). *Journal of Biopesticides, 4*(1), 37.

Astill, B., Terhaar, C., & Fassett, D. (1972). The toxicology and fate of 2, 2, 4-trimethyl-1, 3-pentanediol diisobutyrate. *Toxicology and applied pharmacology, 22*(3), 387-399.

Atangwho, I., Ebong, P., Eyong, E., Williams, I., Eten, M., & Egbung, G. (2009). Comparative chemical composition of leaves of some antidiabetic medicinal plants: *Azadirachta indica*, *Vernonia amygdalina* and *Gongronema latifolium*. *African Journal of Biotechnology*, *8*(18). 4685-4689.

Ault, S. K. (1994). Environmental management: a re-emerging vector control strategy. *The American journal of tropical medicine and hygiene, 50*(6 Suppl), 35-49.

Auwal, S., Atiku, M., Wudil, A., & Sule, M. (2012). Phytochemical composition and acute toxicity evaluation of aqueous root bark extract of *Securidaca longipedunculata* (Linn). *Bayero Journal of Pure and Applied Sciences, 5*(2), 67-72.

Awolola, T., Oduola, O., Strode, C., Koekemoer, L., Brooke, B., & Ranson, H. (2009). Evidence of multiple pyrethroid resistance mechanisms in the malaria vector *Anopheles gambiae* sensu stricto from Nigeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103*(11), 1139-1145.

Azambuja, P., Garcia, E. S., & Ratcliffe, N. A. (2005). Gut microbiota and parasite transmission by insect vectors. *Trends in Parasitology, 21*(12), 568-572.

Bacher, M., Hofer, O., Brader, G., Vajrodaya, S., & Greger, H. (1999). Thapsakins: possible biogenetic intermediates towards insecticidal cyclopenta [b] benzofurans from *Aglaia edulis*. *Phytochemistry, 52*(2), 253-263.

Bakay, B., & Nyhan, W. L. (1969). An improved technique for the separation of glucose 6-phosphate dehydrogenase isoenzymes by disc electrophoresis on polyacrylamide gel. *Biochemical Genetics, 3*(6), 571-582.

Bandyopadhyay, S., Jain, D., & Datta, K. (1996). Reported incidence of dengue/DHF in India 1991-1995. *Dengue Bulletin, 20*, 33-34.

Belinato, T. A., Martins, A. J., & Valle, D. (2012). Fitness evaluation of two Brazilian *Aedes aegypti* field populations with distinct levels of resistance to the organophosphate temephos. *Memorias do Instituto Oswaldo Cruz, 107*(7), 916-922.

Benelli, G. (2015). Plant-borne ovicides in the fight against mosquito vectors of medical and veterinary importance: a systematic review. *Parasitology Research, 114*(9), 3201-3212.

Benelli, G., Govindarajan, M., Senthilmurugan, S., Vijayan, P., Kadaikunnan, S., Alharbi, N. S., & Khaled, J. M. (2018). Fabrication of highly effective mosquito nanolarvicides using an Asian plant of ethno-pharmacological interest, Priyangu (*Aglaia elaeagnoidea*): toxicity on non-target mosquito natural enemies. *Environmental Science and Pollution Research, 25*(11), 10283-10293.

Benelli, G., Jeffries, C., & Walker, T. (2016). Biological control of mosquito vectors: past, present, and future. *Insects, 7*(4), 52.

Benelli, G., Pavela, R., Drenaggi, E., & Maggi, F. (2019). Insecticidal efficacy of the essential oil of jambú (*Acmella oleracea* (L.) RK Jansen) cultivated in central Italy against filariasis mosquito vectors, houseflies and moth pests. *Journal of Ethnopharmacology, 229*, 272-279.

Bentley, C. A. (1911). *Report of an investigation into the causes of malaria in Bombay and the measures necessary for its control*. Bombay, India, Government Central Press.

Bergé, J., Feyereisen, R., & Amichot, M. (1998). Cytochrome P450 monooxygenases and insecticide resistance in insects. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 353*(1376), 1701-1705.

Berling, M. (2009). *Durabilité du contrôle du carpocapse des pommes et des poires (Cydia pomonella) avec le virus de la granulose (CpGV): Résistance versus Virulence* (PhD Thesis). Université de Pau et des Pays de l'Adour, Français.

Bharati, M., & Saha, D. (2018a). Assessment of insecticide resistance in primary dengue vector, *Aedes aegypti* (Linn.) from Northern districts of West Bengal, India. *Acta Tropica*, *187*, 78-86.

Bharati, M., & Saha, D. (2018b). Multiple insecticide resistance mechanisms in primary dengue vector, *Aedes aegypti* (Linn.) from dengue endemic districts of sub-Himalayan West Bengal, India. *PLoS One, 13*(9).

Bharati, M., Saha, P., & Saha, D. (2018). Variation in esterase activity among different *Aedes aegypti* L. populations from the Dooars and Terai Regions of West Bengal, India. *Proceedings of the Zoological Society, 71*, 239-247.

Bhatt, R. P., & Khanal, S. N. (2009). Environmental impact assessment system in Nepal–An overview of policy, legal instruments and process. *Kathmandu University Journal of Science, Engineering and Technology, 5*(2), 160-170.

Bhatt, S., Weiss, D., Cameron, E., Bisanzio, D., Mappin, B., Dalrymple, U., ... Eckhoff, P. (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature, 526*(7572), 207.

Bigoga, J. D., Ndangoh, D. N., Awono-Ambene, P. H., Patchoke, S., Fondjo, E., & Leke, R. G. (2012). Pyrethroid resistance in *Anopheles gambiae* from the rubber cultivated area of Niete, South region of Cameroon. *Acta Tropica, 124*(3), 210-214.

Bouhdid, S., Skali, S., Idaomar, M., Zhiri, A., Baudoux, D., Amensour, M., & Abrini, J. (2008). Antibacterial and antioxidant activities of *Origanum compactum* essential oil. *African Journal of Biotechnology, 7*(10), 1563-1570.

Bowers, M. D., & Puttick, G. M. (1989). Iridoid glycosides and insect feeding preferences: gypsy moths (*Lymantria dispar*, Lymantriidae) and buckeyes (Junonia coenia, Nymphalidae). *Ecological Entomology, 14*(3), 247-256.

Bowsher, C., Steer, M., & Tobin, A. (2008). *Plant Biochemistry* (15th Edn). New York, Garland Science.

Brengues, C., Hawkes, N. J., Chandre, F., McCarroll, L., Duchon, S., Guillet, P., ... Hemingway, J. (2003). Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. *Medical and Veterinary Entomology, 17*(1), 87-9

Brewer, K. K., & Keil, C. B. (1989). A mixed function oxidase factor contributing to permethrin and dichlorvos resistance in *Lycoriella mali* (Fitch) (Diptera: Sciaridae). *Pesticide Science, 26*(1), 29-39.

Briskin, D. P. (2000). Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiology, 124*(2), 507-514.

Britch, S. C., Linthicum, K. J., Wynn, W. W., Walker, T. W., Farooq, M., Smith, V. L., ... & Lothrop, H. D. (2010). Evaluation of ULV and thermal fog mosquito control applications in temperate and desert environments. *Journal of the American Mosquito Control Association*, *26*(2), 183-197.

Brown, A. (1986). Insecticide resistance in mosquitoes: a pragmatic review. *Journal of the American Mosquito Control Association, 2*(2), 123-140.

Bruneton, J. (1996). *Plantes toxiques: vegetaux dangereux pour l'home et les animaux*. Paris, Editions Techniques et Documentation Lavoisier.

Burm.f. DC. (1834) Contr. Bot. India [Wight] 14.

Burm.f. DC. Burman, N. L. (1768). Flora indica. apud Cornelium Haek.

Bussmann, R., Malca-García, G., Glenn, A., Sharon, D., Chait, G., Díaz, D., ... Guardado, G. (2010). Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. *Journal of Ethnopharmacology, 132*(1), 101-108.

Cam, B., Fonsmark, L., Hue, N., Phuong, N., Poulsen, A., & Heegaard, E. (2001). Prospective case-control study of encephalopathy in children with dengue hemorrhagic fever. *The American Journal of Tropical Medicine and Hygiene, 65*(6), 848-851.

Campbell, F. L., Sullivan, W. N., & Smith, C. R. (1933). The relative toxicity of nicotine, anabasine, methyl anabasine, and lupinine for culicine mosquito larvae. *Journal of Economic Entomology, 26*(2), 500-509.

Candido, L. P., Cavalcanti, M. T., & Beserra, E. B. (2013). Bioactivity of plant extracts on the larval and pupal stages of *Aedes aegypti* (Diptera, Culicidea). *Revista da Sociedade Brasileira de Medicina Tropical, 46*(4), 420-425.

Carey, D. E. (1971). Chikungunya and dengue: a case of mistaken identity? *Journal of the History of Medicine and Allied Sciences, 26*(3), 243-262.

Céspedes, C. L., Salazar, J. R., Martínez, M., & Aranda, E. (2005). Insect growth regulatory effects of some extracts and sterols from *Myrtillocactus geometrizans* (Cactaceae) against *Spodoptera frugiperda* and *Tenebrio molitor*. *Phytochemistry, 66*(20), 2481-2493.

Cetin, H., & Yanikoglu, A. (2006). A study of the larvicidal activity of *Origanum* (Labiatae) species from southwest Turkey. *Journal of Vector Ecology, 31*(1), 118-123.

Chakravorthy, B., & Kalyanasundaram, M. (1992). Selection of permethrin resistance in the malaria vector *Anopheles stephensi*. *Indian Journal of Malariology*, *29*(3), 161-165.

Champion, C. J., & Xu, J. (2018). Redox state affects fecundity and insecticide susceptibility in *Anopheles gambiae*. *Scientific Reports*, *8*(1), 1-11.

Chandor-Proust, A., Bibby, J., Régent-Kloeckner, M., Roux, J., Guittard-Crilat, E., Poupardin, R., ... Reynaud, S. (2013). The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling. *Biochemical Journal*, *455*(1), 75-85.

Chapagain, B. P., Saharan, V., & Wiesman, Z. (2008). Larvicidal activity of saponins from *Balanites aegyptiaca* callus against *Aedes aegypti* mosquito. *Bioresource Technology*, *99*(5), 1165-1168.

Chareonviriyaphap, T., Bangs, M. J., Suwonkerd, W., Kongmee, M., Corbel, V., & Ngoen-Klan, R. (2013). Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand. *Parasites and Vectors*, *6*(1), 280.

Chen, Y., Xu, S. S., Chen, J. W., Wang, Y., Xu, H. Q., Fan, N. B., & Li, X. (2012). Anti-tumor activity of *Annona squamosa* seeds extract containing annonaceous acetogenin compounds. *Journal of Ethnopharmacology*, *142*(2), 462-466.

Cheng, S. S., Huang, C. G., Chen, W. J., Kuo, Y. H., & Chang, S. T. (2008). Larvicidal activity of tectoquinone isolated from red heartwood-type *Cryptomeria japonica* against two mosquito species. *Bioresource Technology*, *99*(9), 3617-3622.

Christophers, S. R. (1960). *Aedes aegypti: The yellow fever mosquito*. Cambridge, London, The Syndics of the Cambridge University Press.

Coats, J. R. (1994). Risks from natural versus synthetic insecticides. *Annual Review of Entomology, 39*(1), 489-515.

Collins, L. E., & Blackwell, A. (2000). The biology of *Toxorhynchites* mosquitoes and their potential as biocontrol'agents. *Biocontrol News and Information, 21*(4), 105N-116N.

Conti, B., Benelli, G., Flamini, G., Cioni, P. L., Profeti, R., Ceccarini, L., ... Canale, A. (2012). Larvicidal and repellent activity of *Hyptis suaveolens* (Lamiaceae) essential oil against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). *Parasitology Research, 110*(5), 2013-2021.

Cook, S., Diallo, M., Sall, A. A., Cooper, A., & Holmes, E. C. (2005). Mitochondrial markers for molecular identification of *Aedes* mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. *Journal of Medical Entomology, 42*(1), 19-28.

Corbel, V., Achee, N. L., Chandre, F., Coulibaly, M. B., Dusfour, I., Fonseca, D. M., ... Martins, A. J. (2016). Tracking insecticide resistance in mosquito vectors of arboviruses: the Worldwide Insecticide resistance Network (WIN). *PLoS Neglected Tropical Diseases, 10*(12), e0005054.

Croteau, R., Kutchan, T. M., & Lewis, N. G. (2000). Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants, 24*, 1250-1319.

Cui, S., Tan, S., Ouyang, G., Jiang, S., & Pawliszyn, J. (2009). Headspace solid-phase microextraction gas chromatography–mass spectrometry analysis of *Eupatorium odoratum* extract as an oviposition repellent. *Journal of Chromatography B, 877*(20-21), 1901-1906.

Cunha, L. C., de Morais, S. A., de Aquino, F. J., Chang, R., de Oliveira, A., Martins, M. M., ... da Silva, C. V. (2017). Bioassay-guided fractionation and antimicrobial and cytotoxic activities of *Cassia bakeriana* extracts. *Revista Brasileira de Farmacognosia, 27*(1), 91-98.

da Rocha-Lima, H. (1912). Zur pathologischen Anatomie des Gelbfiebers. G. Fischer.

Das, N., Dhiman, S., Talukdar, P., Rabha, B., Goswami, D., & Veer, V. (2015). Synergistic mosquito-repellent activity of *Curcuma longa, Pogostemon heyneanus* and *Zanthoxylum limonella* essential oils. *Journal of Infection and Public Health, 8*(4), 323-328.

Dash, V., & Kashyap, V. (1999). *Ayurveda Materia Medica*. India, Concept Publishing Company.

Daudé, É., Mazumdar, S., & Solanki, V. (2017). Widespread fear of dengue transmission but poor practices of dengue prevention: A study in the slums of Delhi, India. *PLoS One, 12*(2), e0171543.

Dave, H., & Ledwani, L. (2012). A review on anthraquinones isolated from *Cassia* species and their applications. *Indian Journal of Natural Products and Resources, 3*(3), 291-319

David, J. P., Strode, C., Vontas, J., Nikou, D., Vaughan, A., Pignatelli, P. M., ... Ranson, H. (2005). The *Anopheles gambiae* detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proceedings of the National Academy of Sciences, 102*(11), 4080-4084.

De Azambuja Garcia, G., David, M. R., de Jesus Martins, A., Maciel-de-Freitas, R., Linss, J. G. B., Araújo, S. C., ... Valle, D. (2018). The impact of insecticide applications on the dynamics of resistance: The case of four *Aedes aegypti* populations from different Brazilian regions. *PLoS Neglected Tropical Diseases, 12*(2), e0006227.

De Candolle. Prodr. [A. P. de Candolle]. (1836) 5: 522. [1-10 Oct 1836].

De Dieu Tamokou, J., Chouna, J. R., Fischer-Fodor, E., Chereches, G., Barbos, O., Damian, G., ... Wabo, H. K. (2013). Anticancer and antimicrobial

activities of some antioxidant-rich Cameroonian medicinal plants. *PLoS One, 8*(2), e55880.

De Lima Santos, N. D., de Moura, K. S., Napoleão, T. H., Santos, G. K. N., Coelho, L. C. B. B., Navarro, D. M. d. A. F., & Paiva, P. M. G. (2012). Oviposition-stimulant and ovicidal activities of *Moringa oleifera* lectin on *Aedes aegypti*. *PLoS One, 7*(9), e44840.

De Réaumur, R. A. F. (1738). *Mémoires pour servir à l'histoire des insectes.* Paris, De l'Imprimerie royale.

Del Carmen Recio, M., Giner, R. M., Manez, S., Gueho, J., Julien, H., Hostettmann, K., & Rios, J. (1995). Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. *Planta Medica, 61*(01), 9-12.

Deletre, E., Martin, T., Campagne, P., Bourguet, D., Cadin, A., Menut, C., ... Chandre, F. (2013). Repellent, irritant and toxic effects of 20 plant extracts on adults of the malaria vector *Anopheles gambiae* mosquito. *PLoS One, 8*(12), e82103.

Deletre, E., Martin, T., Duménil, C., & Chandre, F. (2019). Insecticide resistance modifies mosquito response to DEET and natural repellents. *Parasites and Vectors, 12*(1), 89.

Dembitsky, V. M. (2004). Chemistry and biod iversity of the biologically active natural glycosides. *Chemistry and Biodiversity, 1*(5), 673-781.

Desneux, N., Decourtye, A., & Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *The Annual of Review of Entomology, 52*, 81-106.

Dey, Y. N., Ota, S., Srikanth, N., Jamal, M., & Wanjari, M. (2012). A phytopharmacological review on an important medicinal plant-*Amorphophallus paeoniifolius*. *Ayu, 33*(1), 27.

Diaz-Ruiz, A., Zavala, C., Montes, S., Ortiz-Plata, A., Salgado-Ceballos, H., Orozco-Suarez, S., ... Ríos, C. (2008). Antioxidant, antiinflammatory and antiapoptotic effects of dapsone in a model of brain ischemia/reperfusion in rats. *Journal of Neuroscience Research, 86*(15), 3410-3419.

Djouaka, R. F., Bakare, A. A., Coulibaly, O. N., Akogbeto, M. C., Ranson, H., Hemingway, J., & Strode, C. (2008). Expression of the cytochrome P450s, CYP6P3 and CYP6M2 are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* ss. from Southern Benin and Nigeria. *BMC Genomics, 9*(1), 538.

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis, 22*(3), 296-302.

Dong, K. (2007). Insect sodium channels and insecticide resistance. *Invertebrate Neuroscience, 7*(1), 17.

Dória, G. A., Silva, W. J., Carvalho, G. A., Alves, P. B., & Cavalcanti, S. C. (2010). A study of the larvicidal activity of two Croton species from northeastern Brazil against *Aedes aegypti*. *Pharmaceutical Biology, 48*(6), 615-620.

Dougoud, J., Toepfer, S., Bateman, M., & Jenner, W. H. (2019). Efficacy of homemade botanical insecticides based on traditional knowledge. A review. *Agronomy for Sustainable Development, 39*(4), 37.

Dreyer, M., Nugroho, W., Bohnenstengel, I., Ebel, R., Wray, V., Witte, L., ... Hung, D. (2001). New insecticidal rocaglamide derivatives and related compounds from *Aglaia oligophylla*. *Journal of Natural Products, 64*(4), 415-420.

Dutta, S., Dey, P., & Chaudhuri, T. K. (2014). Phytochemical investigation and correlation study of *Croton bonplandianus* Baill. Stem. *Journal of Pharmacognosy and Phytochemistry, 3*(1), 142-148.

Eberhardt, T. L., Li, X., Shupe, T. F., & Hse, C. Y. (2007). Chinese tallow tree (*Sapium Sebiferum*) utilization: Characterization of extractives and cell-wall chemistry. *Wood and Fiber Science, 39*(2), 319-324.

Enayati, A. A., Vatandoost, H., Ladonni, H., Townson, H., & Hemingway, J. (2003). Molecular evidence for a kdr-like pyrethroid resistance mechanism in the malaria vector mosquito *Anopheles stephensi*. *Medical and Veterinary Entomology, 17*(2), 138-144.

Esu, E., Lenhart, A., Smith, L., & Horstick, O. (2010). Effectiveness of peridomestic space spraying with insecticide on dengue transmission; systematic review. *Tropical Medicine and International Health, 15*(5), 619-631.

Fallatah, S., & Khater, E. (2010). Potential of medicinal plants in mosquito control. *Journal of the Egyptian Society of Parasitology, 40*(1), 1-26.

Faucon, F., Dusfour, I., Gaude, T., Navratil, V., Boyer, F., Chandre, F., ... Poupardin, R. (2015). Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. *Genome Research, 25*(9), 1347-1359.

Fernando, S. D., Hapugoda, M., Perera, R., Saavedra-Rodriguez, K., Black, W. C., & De Silva, N. K. (2018). First report of V1016G and S989P knockdown resistant (kdr) mutations in pyrethroid-resistant Sri Lankan *Aedes aegypti* mosquitoes. *Parasites and Vectors, 11*(1), 526.

Finlay, C. J. (1886). Yellow fever. Its transmission by means of the *culex* mosquito. *American Journal of the Medical Sciences, 92*, 395400.

Fonseca-González, I., Quiñones, M. L., McAllister, J., & Brogdon, W. G. (2009). Mixed-function oxidases and esterases associated with cross-resistance

between DDT and lambda-cyhalothrin in *Anopheles darlingi* Root 1926 populations from Colombia. *Memorias do Instituto Oswaldo Cruz, 104*(1), 18-26.

Forssk. Schweinf. (1894). ex Sprenger. Wiener Ill. Gart.-Zeitung 19: 2.

Fortin, C., Maire, A., & Leclair, R. (1987). The residual effect of temephos (Abate 4-E) on nontarget communities. *Journal of the American Mosquito Control Association, 3*(2), 282-288.

Gandhi, G., Chapla, J., Reddya Naik, B., & Gujju Gandhi, C. (2017). Data mapping of vector borne disease with geographical information system & global position system technology: in tribal areas Khammam District, Telangana State. *International Journal of Mosquito Research, 39*, 39-43.

Ganesh, K., Vijayan, V., Urmila, J., Gopalan, N., & Prakash, S. (2002). Role of esterases and monooxygenase in the deltamethrin resistance in *Anopheles stephensi* Giles (1908), at Mysore. *Indian Journal of Experimental Biology, 40*(5), 583-588.

Gao, G., Lu, Z., Tao, S., Zhang, S., & Wang, F. (2011). Triterpenoid saponins with antifeedant activities from stem bark of *Catunaregam spinosa* (Rubiaceae) against *Plutella xylostella* (Plutellidae). *Carbohydrate Research, 346*(14), 2200-2205.

Gao, H., & Wang, Z. (2006). Triterpenoid saponins and phenylethanoid glycosides from stem of *Akebia trifoliata* var. australis. *Phytochemistry, 67*(24), 2697-2705.

Garcez, W. S., Garcez, F. R., da Silva, L. M., & Hamerski, L. (2009). Larvicidal activity against *Aedes aegypti* of some plants native to the West-Central region of Brazil. *Bioresource Technology, 100*(24), 6647-6650.

Garrett-Jones, C., & Shidrawi, G. (1969). Malaria vectorial capacity of a population of *Anopheles gambiae*: an exercise in epidemiological entomology. *Bulletin of the World Health Organization, 40*(4), 531.

Gayathri, V., & Murthy, P. B. (2006). Reduced susceptibility to deltamethrin and kdr mutation in *Anopheles stephensi* Liston, a malaria vector in India. *Journal of the American Mosquito Control Association*, *22*(4), 678-689.

Gemedo-Dalle, T., Maass, B. L., & Isselstein, J. (2005). Plant biodiversity and ethnobotany of *Borana pastoralists* in southern Oromia, Ethiopia. *Economic Botany*, *59*(1), 43-65.

Georghiou, G. P., & Taylor, C. E. (1977). Genetic and biological influences in the evolution of insecticide resistance. *Journal of Economic Entomology*, *70*(3), 319-323.

G. Forst. (1786). Pl. Esc. 76

Ghosh, A., & Chandra, G. (2006). Biocontrol efficacy of *Cestrum diurnum* L. (Solanaceae: Solanales) against the larval forms of *Anopheles stephensi*. *Natural Product Research, 20*(04), 371-379.

Ghosh, A., Chowdhury, N., & Chandra, G. (2012). Plant extracts as potential mosquito larvicides. *Indian Journal of Medical Research, 135*(5), 581.

Ghosh, S., Derle, A., Ahire, M., More, P., Jagtap, S., Phadatare, S. D., ... Chopade, B. A. (2013). Phytochemical analysis and free radical scavenging activity of medicinal plants *Gnidia glauca* and *Dioscorea bulbifera*. *PLoS One*, *8*(12), e82529.

Gibbons, S. (2004). Anti-staphylococcal plant natural products. *Natural Product Reports, 21*(2), 263-277.

Gibbs, E. P. J. (1981). *Virus diseases of food animals: a world geography of epidemiology and control*. India, Academic Press.

Gjullin, C., & Peters, R. F. (1952). Recent studies of mosquito resistance to insecticides in California. *Mosquito news, 12*(1), 1-7.

Godara, R., Verma, M. K., Katoch, R., Yadav, A., Dutt, P., Satti, N. K., & Katoch, M. (2018). In vitro acaricidal activity of *Piper nigrum* and *Piper longum*

fruit extracts and their active components against *Rhipicephalus* (*Boophilus*) *microplus* ticks. *Experimental and Applied Acarology, 75*(3), 333-343.

Gokhale, M. D., Jacob, P. G., & Mourya, D. T. (2000). Dengue virus and insecticide susceptibility status of *Aedes aegypti* mosquitoes from Belagola village, Mandya District, Karnataka state: during and post-epidemic investigations. *The Journal of communicable diseases, 32*(4), 247-253.

Gokulakrishnan, J., Kuppusamy, E., Shanmugam, D., Appavu, A., & Kaliyamoorthi, K. (2013). Pupicidal and repellent activities of *Pogostemon cablin* essential oil chemical compounds against medically important human vector mosquitoes. *Asian Pacific Journal of Tropical Disease, 3*(1), 26-31.

Goldberg, L. J., & Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitattus*, *Aedes aegypti* and *Culex pipiens*. *Mosquito News, 37*(3), 355-358.

Gopalan, N., Prakash, S., Bhattacharya, B. K., Anand, O. P., & Rao, K. M. (1996). Development of malathion resistance in *Culex quinquefasciatus* say (Diptera: Culicidae). *The Indian Journal of Medical Research, 103*, 84-90.

Gould, E. A., & Higgs, S. (2009). Impact of climate change and other factors on emerging arbovirus diseases. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 103*(2), 109-121.

Govindarajan, M., & Benelli, G. (2016). Eco-friendly larvicides from Indian plants: effectiveness of lavandulyl acetate and bicyclogermacrene on malaria, dengue and Japanese encephalitis mosquito vectors. *Ecotoxicology and Environmental Safety, 133*, 395-402.

Govindarajan, M., & Sivakumar, R. (2012). Repellent properties of *Cardiospermum halicacabum* Linn. (Family: Sapindaceae) plant leaf

extracts against three important vector mosquitoes. *Asian Pacific Journal of Tropical Biomedicine, 2*(8), 602-607.

Govindarajan, M., Mathivanan, T., Elumalai, K., Krishnappa, K., & Anandan, A. (2011). Mosquito larvicidal, ovicidal, and repellent properties of botanical extracts against *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* (Diptera: Culicidae). *Parasitology Research, 109*(2), 353-367.

Govindarajan, M., Rajeswary, M., & Amsath, A. (2013). Larvicidal properties of *Caesalpinia pulcherrima* (Family: Fabaceae) against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* (Diptera: Culicidae). *International Journal of Pure and Applied Zoology, 1*(1), 15-23.

Gratz, N., & Pal, R. (1988). Malaria vector control: larviciding. *Malaria: Principles and Practices of Malariology*, 1213-1226.

Gu, H. J., Cheng, S. S., Huang, C. G., Chen, W. J., & Chang, S. T. (2009). Mosquito larvicidal activities of extractives from black heartwood-type *Cryptomeria japonica*. *Parasitology Research, 105*(5), 1455.

Gupta, S., Bisht, S. S., Kukreti, R., Jain, S., & Brahmachari, S. K. (2007). Boolean network analysis of a neurotransmitter signaling pathway. *Journal of Theoretical Biology, 244*(3), 463-469.

Hamid, P. H., Prastowo, J., Ghiffari, A., Taubert, A., & Hermosilla, C. (2017). *Aedes aegypti* resistance development to commonly used insecticides in Jakarta, Indonesia. *PLoS One, 12*(12), e0189680.

Harborne, A. (1998). *Phytochemical methods a guide to modern techniques of plant analysis*: springer science & business media.

Hariprasad, T. P., & Shetty, N. J. (2016). Biochemical basis of alphamethrin resistance in different life stages of *Anopheles stephensi* strains of Bangalore, India. *Pest Management Science, 72*(9), 1689-1701.

Harris, A. F., Rajatileka, S., & Ranson, H. (2010). Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. *The American Journal of Tropical Medicine and Hygiene, 83*(2), 277-284.

Hartjes, L. B. (2011). Preventing and detecting malaria infections. *The Nurse Practitioner, 36*(6), 45.

Hemingway, J., & Karunaratne, S. (1998). 2008901. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and Veterinary Entomology, 12*(1), 39-45.

Hemingway, J., Hawkes, N. J., McCarroll, L., & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology, 34*(7), 653-665.

Hertlein, M. B., Mavrotas, C., Jousseaume, C., Lysandrou, M., Thompson, G. D., Jany, W., & Ritchie, S. A. (2010). A review of spinosad as a natural product for larval mosquito control. *Journal of the American Mosquito Control Association, 26*(1), 67-88.

Hirsch, A. (1883). *Handbook of geographical and historical pathology* (Vol. 1). London, The New Sydenham Society.

Horstick, O., Runge-Ranzinger, S., Nathan, M. B., & Kroeger, A. (2010). Dengue vector-control services: how do they work? A systematic literature review and country case studies. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 104*(6), 379-386.

Hossain, S., & Khalequzzaman, M. (2018). Repellent and oviposition deterrent activity of leaf extracts of *Azadirachta indica* A. Juss., *Persicaria hydropiper* (L.) Spach. and *Vitex negundo* Linn. against the melon fruit fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). *Journal of Entomology and Zoology Studies, 6*(2), 2291-2295.

Houghton, P. J. (1995). The role of plants in traditional medicine and current therapy. *The Journal of Alternative and Complementary Medicine, 1*(2), 131-143.

Howard, L. O. (1921). *A fifty-year sketch history of medical entomology*. Smithsonian Institution.

Hussaini, F. A., Agarwal, S., Roy, R., Prakash, O., & Shoeb, A. (1988). Novel cleistanthane diterpenoids from *Pogostemon auricularis*. *Journal of Natural Products, 51*(2), 212-216.

Hussaini, F. A., Roy, R., Shoeb, A., Pragya, Gajhede, M., Jacobsen, J. P., & Boll, P. M. (1993). Structure and configuration of a new cleistanthane diterpenoid from *Pogostemon auricularis*. *Natural Product Letters, 3*(1), 71-77.

Ikram, N. K., & Simonsen, H. T. (2017). A review of biotechnological artemisinin production in plants. *Frontiers in Plant Science, 8*, 1966.

India., N. E. G. O. (1909). Published under the authority of His Majesty's secretary of state for India in council. Vol. IV Administrative. *Oxford: Clarendon Press: The Imperial Gazetteer of India.*, 457–480.

Innocent, E., Hassanali, A., Kisinza, W. N., Mutalemwa, P. P., Magesa, S., & Kayombo, E. (2014). Anti-mosquito plants as an alternative or incremental method for malaria vector control among rural communities of Bagamoyo District, Tanzania. *Journal of Ethnobiology and Ethnomedicine, 10*(1), 56.

Innocent, E., Joseph, C. C., Gikonyo, N. K., Moshia, M. J., Nkunya, M. H., & Hassanali, A. (2008). Mosquito larvicidal constituents from *Lantana viburnoides* sp viburnoides var kisi (A. rich) Verdc (Verbenaceae). *Journal of Vector Brone Diseaese, 45*(3), 240-244.

Iqbal, J., Abbasi, B. A., Mahmood, T., Kanwal, S., Ahmad, R., & Ashraf, M. (2019). Plant-extract mediated green approach for the synthesis of

ZnONPs: Characterization and evaluation of cytotoxic, antimicrobial and antioxidant potentials. *Journal of Molecular Structure, 1189*, 315-327.

Islam, M. T., Das, P. R., Kabir, M. H., Akter, S., Khatun, Z., Haque, M. M., ... Rahmatullah, M. (2012). Acanthaceae and Asteraceae family plants used by folk medicinal practitioners for treatment of malaria in Chittagong and Sylhet divisions of Bangladesh. *American-Eurasian Journal of Sustainable Agriculture*, *6*(3), 146-152.

Isman, M. B. (1997). Neem and other botanical insecticides: barriers to commercialization. *Phytoparasitica, 25*(4), 339.

Isman, M. B., & Grieneisen, M. L. (2014). Botanical insecticide research: many publications, limited useful data. *Trends in Plant Science, 19*(3), 140-145.

Ito, M., Takasaki, T., Yamada, K. I., Nerome, R., Tajima, S., & Kurane, I. (2004). Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. *Journal of Clinical Microbiology*, *42*(12), 5935-5937.

Iwanaga, K., & Kanda, T. (1988). The effects of a juvenile hormone active oxime ether compound on the metamorphosis and reproduction of an anopheline vector, *Anopheles balabacensis* (Diptera: Culicidae). *Applied Entomology and Zoology*, *23*(2), 186-193.

Jacobson, M. (1975a). Insecticides from plants: US Government Printing Office.

Jacobson, M. (1975b). *Insecticides from plants, a review of the literature, 1954-1971*. United States, Agricultural Research Service, United States Department of Agriculture.

Jagadeshwaran, U., & Vijayan, V. (2009). Biochemical characterization of deltamethrin resistance in a laboratory-selected strain of *Aedes aegypti*. *Parasitology Research, 104*(6), 1431-1438.

James, S. (1906). *The Causation and Prevention of Malarial Fevers: A Statement of the Results of Research* (PhD Thesis). University of London.

Jamison, D. T., Jha, P., Malhotra, V., & Verguet, S. (1900). Human health: the twentieth century transformation of human health-its magnitude and value. In *How much have global problems cost the world? A scorecard from 1900 to 2050*. Cambridge, London, Cambridge University Press, pp. 207-246.

Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., ... van der Vliet, A. (2008). Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radical Biology and Medicine*, *45*(1), 1-17.

Jensen, K., Panagiotou, G., & Kouskoumvekaki, I. (2014). Integrated text mining and chemoinformatics analysis associates diet to health benefit at molecular level. *PLoS Computational Biology*, *10*(1), e1003432.

Jewitt, D., Agarwal, J., Weaver, H., Mutchler, M., & Larson, S. (2013). The extraordinary multi-tailed main-belt comet P/2013 P5. *The Astrophysical Journal Letters*, *778*(1), L21.

Jirakanjanakit, N., Rongnoparut, P., Saengtharatip, S., Chareonviriyaphap, T., Duchon, S., Bellec, C., & Yoksan, S. (2014). Insecticide susceptible/resistance status in *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* (Diptera: Culicidae) in Thailand during 2003-2005. *Journal of Economic Entomology*, *100*(2), 545-550.

Johnson, M., Maharajan, M., & Janakiraman, N. (2015). Floristic diversity and medicinal importance of South Vagaikulam Region Tirunelveli, Tamil Nadu, South India. *Journal of Medicinal Herbs and Ethnomedicine*, *1*, 125.

Jones, D. P. (2006). Redefining oxidative stress. *Antioxidants and Redox Signaling*, *8*(9-10), 1865-1879.

Kadir, H. A., Zakaria, M. B., Kechil, A. A., & Azirun, M. D. (1989). Toxicity and electrophysiological effects of *Spilanthes amella* Murr. extracts on *Periplaneta americana* L. *Pesticide Science, 25*(4), 329-335.

Kamaleswari, K., & Nandagopalan, V. (2017). Phytochemical analysis of secondary metabolites on *Pogostemon auricularis* (L.) Hassk and *Anisomeles malabarica* (L.) R. Br. ex Sims. *Journal of Pharmacognosy and Phytochemistry, 6*(6), 1942-1945.

Kandel, Y., Vulcan, J., Rodriguez, S. D., Moore, E., Chung, H. N., Mitra, S., ... Kulkarni, A. (2019). Widespread insecticide resistance in *Aedes aegypti* L. from New Mexico, USA. *PLoS One, 14*(2), e0212693.

Kantheti, P., Alapati, P., & Sulthana, S. K. (2018). Traditional medicinal practices for mosquito repellency by tribes of west central India: An overview. *Journal of Pharmacognosy and Phytochemistry, 7*(2), 2755-2759.

Kaushik, S., Sharma, N. R., Thomas, T., Sharma, A. K., & Bansal, A. (2019). Indigenous plants and their larvicidal potential against indian mosquito vectors: A review. *Journal of Communicable. Diseases, 51*(2), 59-72.

Kennedy, D. O., & Wightman, E. L. (2011). Herbal extracts and phytochemicals: plant secondary metabolites and the enhancement of human brain function. *Advances in Nutrition, 2*(1), 32-50.

Kennedy, D. O., Dodd, F. L., Robertson, B. C., Okello, E. J., Reay, J. L., Scholey, A. B., & Haskell, C. F. (2011). Monoterpenoid extract of sage (*Salvia lavandulaefolia*) with cholinesterase inhibiting properties improves cognitive performance and mood in healthy adults. *Journal of Psychopharmacology, 25*(8), 1088-1100.

Killeen, G. F., Tanner, M., Mukabana, W. R., Kalongolela, M. S., Kannady, K., Lindsay, S. W., ... de Castro, M. C. (2006). Habitat targeting for controlling aquatic stages of malaria vectors in Africa. *The American Journal of Tropical Medicine and Hygiene, 74*(4), 517-518.

Kim, S., Chin, Y. W., Su, B. N., Riswan, S., Kardono, L. B., Afriastini, J. J., ... Swanson, S. M. (2006). Cytotoxic flavaglines and bisamides from *Aglaia edulis*. *Journal of Natural Products, 69*(12), 1769-1775.

Kiprop, A. K., Kiprono, P. C., Rajab, M. S., & Kosgei, M. K. (2007). Limonoids as larvicidal components against mosquito larvae (*Aedes aegypti* Linn.). *Zeitschrift für Naturforschung C, 62*(11-12), 826-828.

Kishore, N., Mishra, B. B., Tiwari, V. K., Tripathi, V., & Lall, N. (2014). Natural products as leads to potential mosquitocides. *Phytochemistry Reviews, 13*(3), 587-627.

Kishore, N., Mishra, B., Tiwari, V., & Tripathi, V. (2011). *Opportunity, challenge and scope of natural products in medicinal chemistry*. Research Signpost.

Kitamura, T., Kobayashi, S., & Okada, M. (1996). Regional expression of the transcript encoding sterol carrier protein x-related thiolase and its regulation by homeotic genes in the midgut of *Drosophila* embryos. *Development, Growth and Differentiation, 38*(4), 373-381.

Kliebenstein, D. J., & Osbourn, A. (2012). Making new molecules–evolution of pathways for novel metabolites in plants. *Current Opinion in Plant Biology, 15*(4), 415-423.

Klowden, M. J., & Chambers, G. M. (1989). Ovarian development and adult mortality in *Aedes aegypti* treated with sucrose, juvenile hormone and methoprene. *Journal of Insect Physiology, 35*(6), 513-517.

Klungthong, C., Zhang, C., Mammen Jr, M. P., Ubol, S., & Holmes, E. C. (2004). The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. *Virology, 329*(1), 168-179.

Kodrík, D., Bednářová, A., Zemanová, M., & Krishnan, N. (2015). Hormonal regulation of response to oxidative stress in insects-an update. *International Journal of Molecular Sciences, 16*(10), 25788-25816.

Kolaczinski, K., Kolaczinski, J., Kilian, A., & Meek, S. (2007). Extension of indoor residual spraying for malaria control into high transmission settings in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 101*(9), 852-853.

Komalamisra, N., Trongtokit, Y., Rongsriyam, Y., & Apiwathnasorn, C. (2005). Screening for larvicidal activity in some Thai plants against four mosquito vector species. *Southeast Asian Journal of Tropical Medicine and Public Health, 36*(6), 1412.

Koul, O., Kaur, H., Goomber, S., & Wahab, S. (2004). Bioefficacy and mode of action of rocaglamide from *Aglaia elaeagnoidea* (syn. *A. roxburghiana*) against gram pod borer, *Helicoverpa armigera* (Hübner). *Journal of Applied Entomology, 128*(3), 177-181.

Koul, O., Shankar, J., Mehta, N., Taneja, S., Tripathi, A., & Dhar, K. (1997). Bioefficacy of crude extracts of *Aglaia* species (Meliaceae) and some active fractions against lepidopteran larvae. *Journal of Applied Entomology, 121*(1-5), 245-248.

Koul, O., Singh, G., Singh, R., & Multani, J. (2005). Bioefficacy and mode of action of aglaroxin A from *Aglaia elaeagnoidea* (*syn. A. roxburghiana*) against *Helicoverpa armigera* and *Spodoptera litura*. *Entomologia Experimentalis et Applicata*, *114*(3), 197-204.

Koul, O., Walia, S., & Dhaliwal, G. (2008). Essential oils as green pesticides: potential and constraints. *Biopesticides International, 4*(1), 63-84.

Kumar, B., Vijayakumar, M., Govindarajan, R., & Pushpangadan, P. (2007). Ethnopharmacological approaches to wound healing-exploring medicinal plants of India. *Journal of Ethnopharmacology, 114*(2), 103-113.

Kumar, S., Nair, G., Singh, A. P., Batra, S., Wahab, N., & Warikoo, R. (2012a). Evaluation of the larvicidal efficiency of stem, roots and leaves of the

weed, *Parthenium hysterophorus* (Family: Asteraceae) against *Aedes aegypti* L. *Asian Pacific Journal of Tropical Disease*, *2*(5), 395-400.

Kumar, S., Thomas, A., & Pillai, M. (1991). Involvement of mono-oxygenases as a major mechanism of deltamethrin-resistance in larvae of three species of mosquitoes. *Indian Journal of Experimental Biology*, *29*(4), 379-384.

Kumar, S., Thomas, A., Sahgal, A., Verma, A., Samuel, T., & Pillai, M. (2002). Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Archives of Insect Biochemistry and Physiology*, *50*(1), 1-8.

Kumar, S., Thomas, A., Sahgal, A., Verma, A., Samuel, T., & Pillai, M. (2004). Variations in the insecticide-resistance spectrum of *Anopheles stephensi* after selection with deltamethrin or a deltamethrin–piperonyl-butoxide combination. *Annals of Tropical Medicine and Parasitology*, *98*(8), 861-871.

Kumar, S., Wahab, N., Mishra, M., & Warikoo, R. (2012b). Evaluation of 15 local plant species as larvicidal agents against an Indian strain of dengue fever mosquito, *Aedes aegypti* L.(Diptera: Culicidae). *Frontiers in physiology*, *3*, 104.

Kumar, S., Warikoo, R., & Wahab, N. (2010). Larvicidal potential of ethanolic extracts of dried fruits of three species of peppercorns against different instars of an Indian strain of dengue fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Parasitology Research*, *107*(4), 901-907.

Kweka, E. J., Mosha, F., Lowassa, A., Mahande, A. M., Kitau, J., Matowo, J., ... Feston, E. (2008). Ethnobotanical study of some of mosquito repellent plants in north-eastern Tanzania. *Malaria Journal*, *7*(1), 152.

Lallawmawma, H., Sathishkumar, G., Sarathbabu, S., Ghatak, S., Sivaramakrishnan, S., Gurusubramanian, G., & Kumar, N. S. (2015).

Synthesis of silver and gold nanoparticles using *Jasminum nervosum* leaf extract and its larvicidal activity against filarial and arboviral vector *Culex quinquefasciatus* Say (Diptera: Culicidae). *Environmental Science and Pollution Research, 22*(22), 17753-17768.

Larson, R. T., Wessely, V., Jiang, Z., & Lan, Q. (2014). Larvicidal activity of sterol carrier protein-2 inhibitor in four species of mosquitoes. *Journal of Medical Entomology, 45*(3), 439-444.

Lee, H. (1990). A rapid and simple biochemical method for the detection of insecticide resistance due to elevated esterase activity in *Culex quinquefasciatus*. *Tropical Biomedicine, 7*(1), 21-28.

Lewu, F., & Afolayan, A. (2009). Ethnomedicine in South Africa: The role of weedy species. *African Journal of Biotechnology, 8*(6).

Li, T. S. (2000). *Medicinal plants: culture, utilization and phytopharmacology*. Boca Raton, Florida, USA, CRC press.

Li, T., & Liu, N. (2014). Inheritance of permethrin resistance in *Culex quinquefasciatus*. *Journal of Medical Entomology, 47*(6), 1127-1134.

Lima, J. B. P., Da-Cunha, M. P., Júnior, R. C. D. S., Galardo, A. K. R., Soares, S. D. S., Braga, I. A., ... Valle, D. (2003). Resistance of *Aedes aegypti* to organophosphates in several municipalities in the State of Rio de Janeiro and Espirito Santo, Brazil. *The American Journal of Tropical Medicine and Hygiene, 68*(3), 329-333.

Linnaeus, C. (1753). "Species PlaTltarum." Stockholm 2: 1762-1763.

Linnaeus, C. (1758). Systema naturae (Vol. 1, No. part 1, p. 532). Laurentii Salvii: Stockholm.

Linnaeus, C. (1759). Systema naturae. 10, 2, 940.

Linnaeus, C. von (1762). "Species Plantarum, ed. 2, vol. 1." *Impensis Laurentii Salvii, Holmiae*.

Linnaeus, C. (1762). Vol. 1. Species Plantarum. ed. 2. Stockholm.: i-xvi, 1-784.

Linnaeus, C. von filius (1782) Supplementum Plantarum : 442. Type: Habitat in Java. Thunberg.

Thunberg, C. P. (1784). Flora Japonica: Sistens Plantas Insualrum Japonicarum Secundum Systema Sexuale Emendatum Redactas. IG Mülleriano.

Linnaeus, Lam. (1797). Tabl. Encycl. 2: 116.

Linnaeus W. T. Aiton, (1811) Hortus Kew. ed. 2. 2: 78.

Liu, H., Xu, Q., Zhang, L., & Liu, N. (2005). Chlorpyrifos resistance in mosquito *Culex quinquefasciatus*. *Journal of Medical Entomology, 42*(5), 815-820.

Liu, N. (2015). Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. *Annual Review of Entomology, 60*, 537-559.

Liu, N., Li, T., Reid, W. R., Yang, T., & Zhang, L. (2011). Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *PLoS One, 6*(8).

Liu, N., Liu, H., Zhu, F., & Zhang, L. (2007). Differential expression of genes in pyrethroid resistant and susceptible mosquitoes, *Culex quinquefasciatus* (S.). *Gene, 394*(1-2), 61-68.

Liu, X. C., & Liu, Z. L. (2014). Evaluation of larvicidal activity of the essential oil of *Ageratum conyzoides* L. aerial parts and its major constituents against *Aedes albopictus*. *Journal of Entomology and Zoology Studies, 2*(4), 345-350.

Liu, Z. L., Liu, Q. Z., Du, S. S., & Deng, Z. W. (2012). Mosquito larvicidal activity of alkaloids and limonoids derived from *Evodia rutaecarpa* unripe fruits against *Aedes albopictus* (Diptera: Culicidae). *Parasitology Research, 111*(3), 991-996.

Londhe, J. S., Devasagayam, T. P., Foo, L. Y., & Ghaskadbi, S. S. (2008). Antioxidant activity of some polyphenol constituents of the medicinal plant *Phyllanthus amarus* Linn. *Redox Report, 13*(5), 199-207.

Lour., Fl. Cochinch. (1790) 2: 433.; ed. 2, 2: 525. 1793.

Low, V. L., Chen, C. D., Lee, H. L., Tan, T. K., Chen, C. F., Leong, C. S., ... Sofian-Azirun, M. (2013). Enzymatic characterization of insecticide resistance mechanisms in field populations of Malaysian *Culex quinquefasciatus* Say (Diptera: Culicidae). *PLoS One, 8*(11), e79928.

Macoris, M. d. L. G., Andrighetti, M. T. M., Takaku, L., Glasser, C. M., Garbeloto, V. C., & Bracco, J. E. (2003). Resistance of *Aedes aegypti* from the state of São Paulo, Brazil, to organophosphates insecticides. *Memorias do Instituto Oswaldo Cruz, 98*(5), 703-708.

Madeira, N. G., Macharelli, C. A., Pedras, J. F., & Delfino, M. C. (2002). Education in primary school as a strategy to control dengue. *Revista da Sociedade Brasileira de Medicina Tropical, 35*(3), 221-226.

Madhu, S., Shaukath, A., & Vijayan, V. (2010). Efficacy of bioactive compounds from *Curcuma aromatica* against mosquito larvae. *Acta Tropica, 113*(1), 7-11.

Maia, M. F., & Moore, S. J. (2011). Plant-based insect repellents: a review of their efficacy, development and testing. *Malaria Journal, 10*(1), S11.

Maniafu, B. M., Wilber, L., Ndiege, I. O., Wanjala, C. C., & Akenga, T. A. (2009). Larvicidal activity of extracts from three *Plumbago* spp against *Anopheles gambiae*. *Memorias do Instituto Oswaldo Cruz, 104*(6), 813-817.

Marcombe, S., Chonephetsarath, S., Thammavong, P., & Brey, P. T. (2018). Alternative insecticides for larval control of the dengue vector *Aedes aegypti* in Lao PDR: insecticide resistance and semi-field trial study. *Parasites and Vectors, 11*(1), 1-8.

Marcombe, S., Mathieu, R. B., Pocquet, N., Riaz, M. A., Poupardin, R., Sélior, S., ... Corbel, V. (2012). Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. *PLoS One, 7*(2).

Marcombe, S., Poupardin, R., Darriet, F., Reynaud, S., Bonnet, J., Strode, C., ... Corbel, V. (2009). Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti*: a case study in Martinique Island (French West Indies). *BMC Genomics, 10*(1), 494.

Marina, C. F., Bond, J. G., Muñoz, J., Valle, J., Chirino, N., & Williams, T. (2012). Spinosad: a biorational mosquito larvicide for use in car tires in southern Mexico. *Parasites and Vectors, 5*(1), 95.

Marjoribanks, M. J. (1914). Report on certain features of malaria in the island of Salsette. *Proceedings of the 3rd all-India sanitary conference* (January 19th to 27th, 1914). Lucknow, India

Martins, A. J., Bellinato, D. F., Peixoto, A. A., Valle, D., & Lima, J. B. P. (2012). Effect of insecticide resistance on development, longevity and reproduction of field or laboratory selected *Aedes aegypti* populations. *PLoS One, 7*(3), e31889.

Masi, M., van der Westhuyzen, A. E., Tabanca, N., Evidente, M., Cimmino, A., Green, I. R., ... van Otterlo, W. A. (2017). Sarniensine, a mesembrine-type alkaloid isolated from *Nerine sarniensis*, an indigenous South African Amaryllidaceae, with larvicidal and adulticidal activities against *Aedes aegypti*. *Fitoterapia, 116*, 34-38.

Matkowski, A., & Piotrowska, M. (2006). Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. *Fitoterapia, 77*(5), 346-353.

Matsuo, Y., Akagi, N., Hashimoto, C., Tachikawa, F., & Mimaki, Y. (2013). Steroidal glycosides from the bulbs of *Bessera elegans* and their cytotoxic activities. *Phytochemistry, 96*, 244-256.

Matthews, G., Zaim, M., Yadav, R. S., Soares, A., Hii, J., Ameneshewa, B., ... Tan, S. H. (2011). Status of legislation and regulatory control of public health pesticides in countries endemic with or at risk of major vector-borne diseases. *Environmental Health Perspectives, 119*(11), 1517-1522.

McCaffery, A. & Nauen, R. (2006). The Insecticide Resistance Action Committee (IRAC): Public responsibility and enlightened industrial self interest. *Outlooks on Pest Management, 7*(1), 11-14.

Melo-Santos, M., Varjal-Melo, J., Araújo, A., Gomes, T., Paiva, M., Regis, L., ... Andrighetti, M. (2010). Resistance to the organophosphate temephos: mechanisms, evolution and reversion in an *Aedes aegypti* laboratory strain from Brazil. *Acta Tropica, 113*(2), 180-189.

Mgbemena, I., Ebe, T., Nnadozie, A., & Ekeanyanwu, K. (2015). Repellent activities of the methanolic leaf extracts of *Moringa oleifera* and *Stachytarpheta indica* against *Aedes aegypti* mosquito. *Journal of Pharmacy and Biological Sciences, 10*(4), 77-81.

Mimaki, Y., Aoki, T., Jitsuno, M., Kiliç, C. S., & Coşkun, M. (2008). Steroidal glycosides from the rhizomes of *Ruscus hypophyllum*. *Phytochemistry, 69*(3), 729-737.

Mimica-Dukic, N., & Bozin, B. (2008). *Mentha* L. species (Lamiaceae) as promising sources of bioactive secondary metabolites. *Current Pharmaceutical Design, 14*(29), 3141-3150.

Miresmailli, S., Bradbury, R., & Isman, M. B. (2006). Comparative toxicity of *Rosmarinus officinalis* L. essential oil and blends of its major constituents against *Tetranychus urticae* Koch (Acari: Tetranychidae) on two different host plants. *Pest Management Science, 62*(4), 366-371.

Misni, N., Nor, Z. M., & Ahmad, R. (2016). New candidates for plant-based repellents against *Aedes aegypti*. *Journal of the American Mosquito Control Association, 32*(2), 117-124.

Mohanraj, K., Karthikeyan, B. S., Vivek-Ananth, R., Chand, R. B., Aparna, S., Mangalapandi, P., & Samal, A. (2018). IMPPAT: A curated database of Indian medicinal plants, phytochemistry and therapeutics. *Scientific Reports, 8*(1), 4329.

Monath, T. (1988). *The arboviruses: ecology and epidemiology*. Boca Raton, Florida, USA, CRC Press.

Montoya, L. (2003). Geo-data acquisition through mobile GIS and digital video: an urban disaster management perspective. *Environmental Modelling and Software, 18*(10), 869-876.

Morou, E., Dowd, A. J., Rajatileka, S., Steven, A., Hemingway, J., Ranson, H., ... Vontas, J. (2010). A simple colorimetric assay for specific detection of glutathione-S transferase activity associated with DDT resistance in mosquitoes. *PLoS Neglected Tropical Diseases, 4*(8).

Morrison, A. C., Zielinski-Gutierrez, E., Scott, T. W., & Rosenberg, R. (2008). Defining challenges and proposing solutions for control of the virus vector *Aedes aegypti*. *PLoS Medicine, 5*(3), e68.

Mouchet, J. (1972). Survey of potential vectors of yellow fever in Tanzania. *Bulletin of the World Health Organization, 46*(5), 675-684.

Mourya, D. T., Hemingway, J., & Leake, C. J. (1993). Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticide resistance. *Medical and Veterinary Entomology, 7*(1), 11-16.

Mueller, P., Chouaibou, M., Pignatelli, P., Etang, J., Walker, E. D., Donnelly, M. J., ... Ranson, H. (2008). Pyrethroid tolerance is associated with elevated expression of antioxidants and agricultural practice in *Anopheles*

arabiensis sampled from an area of cotton fields in Northern Cameroon. *Molecular Ecology, 17*(4), 1145-1155.

Muellner, A. N., Samuel, R., Chase, M. W., Pannell, C. M., & Greger, H. (2005). *Aglaia* (Meliaceae): an evaluation of taxonomic concepts based on DNA data and secondary metabolites. *American Journal of Botany, 92*(3), 534-543.

Murrell, S., Wu, S. C., & Butler, M. (2011). Review of dengue virus and the development of a vaccine. *Biotechnology Advances, 29*(2), 239-247.

Murugan, R., Mallavarapu, G. R., Padmashree, K. V., Rao, R. R., & Livingstone, C. (2010). Volatile oil composition of *Pogostemon heyneanus* and comparison of its composition with patchouli oil. *Natural Product Communications, 5*(12), 1934578X1000501228.

Mustafa, M., Rasotgi, V., Jain, S., & Gupta, V. (2015). Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. *Medical Journal Armed Forces India, 71*(1), 67-70.

Muthee, J., Gakuya, D., Mbaria, J., Kareru, P., Mulei, C. M., & Njonge, F. (2011). Ethnobotanical study of anthelmintic and other medicinal plants traditionally used in Loitoktok district of Kenya. *Journal of Ethnopharmacology, 135*(1), 15-21.

Myers, R., Carey, D., Banerjee, K., Reuben, R., & Ramamurti, D. (1968). Recovery of dengue type 3 virus from human serum and *Aedes aegypti* in South India. *Indian Journal of Medical Research, 56*(6), 781-787.

Nabeshima, T., Mori, A., Kozaki, T., Iwata, Y., Hidoh, O., Harada, S., ... Tomita, T. (2004). An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*. *Biochemical and Biophysical Research Communications, 313*(3), 794-801.

Nagpal, B., Gupta, S. K., Shamim, A., Vikram, K., Srivastava, A., Tuli, N., ... Bhagat, V. (2016). Control of *Aedes aegypti* breeding: a novel intervention for prevention and control of dengue in an endemic zone of Delhi, India. *PLoS One*, *11*(12), e0166768.

Naqqash, M. N., Gökçe, A., Bakhsh, A., & Salim, M. (2016). Insecticide resistance and its molecular basis in urban insect pests. *Parasitology Research*, *115*(4), 1363-1373.

Nkya, T. E., Poupardin, R., Laporte, F., Akhouayri, I., Mosha, F., Magesa, S., ... David, J. P. (2014). Impact of agriculture on the selection of insecticide resistance in the malaria vector *Anopheles gambiae*: a multigenerational study in controlled conditions. *Parasites and Vectors*, *7*(1), 480.

Nobuchi, H. (1979). The symptoms of a dengue-like illness recorded in a Chinese medical encyclopedia. *Kanpo Rinsho*, *26*, 422-425.

Nugroho, B., Edrada, R., Wray, V., Witte, L., Bringmann, G., Gehling, M., & Proksch, P. (1999). An insecticidal rocaglamide derivatives and related compounds from *Aglaia odorata* (Meliaceae). *Phytochemistry*, *51*(3), 367-376.

NVBDCP [National Vector Borne Disease Control Programme]. (2020). Dengue/DHF Situation In India.

Nyasembe, V. O., & Torto, B. (2014). Volatile phytochemicals as mosquito semiochemicals. *Phytochemistry Letters*, *8*, 196-201.

Ocampo, C. B., Salazar-Terreros, M. J., Mina, N. J., McAllister, J., & Brogdon, W. (2011). Insecticide resistance status of *Aedes aegypti* in 10 localities in Colombia. *Acta Tropica*, *118*(1), 37-44.

Ogunlesi, M., Okiei, W., Ofor, E., & Osibote, A. E. (2009). Analysis of the essential oil from the dried leaves of *Euphorbia hirta* Linn (Euphorbiaceae), a potential medication for asthma. *African Journal of Biotechnology*, *8*(24).

Oladipupo, S. O., Callaghan, A., Holloway, G. J., & Gbaye, O. A. (2019). Variation in the susceptibility of *Anopheles gambiae* to botanicals across a metropolitan region of Nigeria. *PLoS One, 14*(1), e0210440.

Oliveira, J. H. M., Talyuli, O. A., Goncalves, R. L., Paiva-Silva, G. O., Sorgine, M. H. F., Alvarenga, P. H., & Oliveira, P. L. (2017). Catalase protects *Aedes aegypti* from oxidative stress and increases midgut infection prevalence of dengue but not zika. *PLoS Neglected Tropical Diseases*, *11*(4), e0005525.

Oliveira, P. V., Ferreira, J. C., Moura, F. S., Lima, G. S., de Oliveira, F. M., Oliveira, P. E. S., ... Lemos, R. P. L. (2010). Larvicidal activity of 94 extracts from ten plant species of northeastern of Brazil against *Aedes aegypti* L. (Diptera: Culicidae). *Parasitology Research, 107*(2), 403-407.

Oliver, S. V., & Brooke, B. D. (2016). The role of oxidative stress in the longevity and insecticide resistance phenotype of the major malaria vectors *Anopheles arabiensis* and *Anopheles funestus*. *PLoS One, 11*(3), e0151049.

Oluwayelu, D., Adebiyi, A., & Tomori, O. (2018). Endemic and emerging arboviral diseases of livestock in Nigeria: a review. *Parasites and Vectors*, *11*(1), 337.

Pagare, S., Bhatia, M., Tripathi, N., Pagare, S., & Bansal, Y. (2015). Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy, 9*(3), 293-304.

Pall. Fl. ross. (1789) 1(2):107, t. 95. ("1788").

Pandey, M., Rastogi, S., & Rawat, A. (2013). Indian traditional ayurvedic system of medicine and nutritional supplementation. *Evidence-Based Complementary and Alternative Medicine*, *2013*.

Panneerselvam, C., Murugan, K., Kovendan, K., Kumar, P. M., & Subramaniam, J. (2013). Mosquito larvicidal and pupicidal activity of *Euphorbia hirta*

Linn. (Family: Euphorbiaceae) and *Bacillus sphaericus* against *Anopheles stephensi* Liston. (Diptera: Culicidae). *Asian Pacific Journal of Tropical Medicine, 6*(2), 102-109.

Park, H. M., & Park, I. K. (2012). Larvicidal activity of *Amyris balsamifera*, *Daucus carota* and *Pogostemon cablin* essential oils and their components against *Culex pipiens* pallens. *Journal of Asia-Pacific Entomology, 15*(4), 631-634.

Pavela, R. (2007). Lethal and sublethal effects of thyme oil (*Thymus vulgaris* L.) on the house fly (*Musca domestica* Lin.). *Journal of Essential Oil Bearing Plants, 10*(5), 346-356.

Pavela, R. (2011). Insecticidal properties of phenols on *Culex quinquefasciatus* Say and *Musca domestica* L. *Parasitology Research, 109*(6), 1547-1553.

Pavela, R., Maggi, F., Iannarelli, R., & Benelli, G. (2019). Plant extracts for developing mosquito larvicides: From laboratory to the field, with insights on the modes of action. *Acta Tropica, 193*, 236-271.

Pelah, D., Abramovich, Z., Markus, A., & Wiesman, Z. (2002). The use of commercial saponin from *Quillaja saponaria* bark as a natural larvicidal agent against *Aedes aegypti* and *Culex pipiens*. *Journal of Ethnopharmacology, 81*(3), 407-409.

Perez, C., Marina, C., Bond, J., Rojas, J., Valle, J., & Williams, T. (2007). Spinosad, a naturally derived insecticide, for control of *Aedes aegypti* (Diptera: Culicidae): efficacy, persistence, and elicited oviposition response. *Journal of Medical Entomology, 44*(4), 631-638.

Peters, A. L., & Noorden, C. J. V. (2009). Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women. *Journal of Histochemistry and Cytochemistry, 57*(11), 1003-1011.

Phy, D. O. P. (1999). *Eco'-Friendly Natural Insecticides* (PhD Thesis). Cochin University of Science And Technology.

Pierce, A. R., & Laird, S. A. (2003). In search of comprehensive standards for non-timber forest products in the botanicals trade. *International Forestry Review, 5*(2), 138-147.

Pilaquinga, F., Morejón, B., Ganchala, D., Morey, J., Piña, N., Debut, A., & Neira, M. (2019). Green synthesis of silver nanoparticles using *Solanum mammosum* L. (Solanaceae) fruit extract and their larvicidal activity against *Aedes aegypti* L. (Diptera: Culicidae). *PLoS One, 14*(10).

Pohlit, A. M., Rezende, A. R., Baldin, E. L. L., Lopes, N. P., & de Andrade Neto, V. F. (2011). Plant extracts, isolated phytochemicals, and plant-derived agents which are lethal to arthropod vectors of human tropical diseases–a review. *Planta Medica, 77*(06), 618-630.

Ponlawat, A., Scott, J. G., & Harrington, L. C. (2005). Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* across Thailand. *Journal of Medical Entomology, 42*(5), 821-825.

Poupardin, R., Riaz, M. A., Jones, C. M., Chandor-Proust, A., Reynaud, S., & David, J. P. (2012). Do pollutants affect insecticide-driven gene selection in mosquitoes? Experimental evidence from transcriptomics. *Aquatic Toxicology, 114*, 49-57.

Preethi, E. A., Raveen, R., Arivoli, S., Samuel, T., & Madhanagopal, R. (2014). Larvicidal efficacy of *Jasminum* sp. (Oleaceae) flower extracts against the dengue and chikungunya vector *Aedes aegypti* L. (Diptera: Culicidae). *Medicinal Chemistry, 4*, 672-675.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry, 269*(2), 337-341.

Procópio, T. F., Fernandes, K. M., Pontual, E. V., Ximenes, R. M., de Oliveira, A. R. C., de Santana Souza, C., ... Martins, G. F. (2015). Schinus terebinthifolius leaf extract causes midgut damage, interfering with survival and development of *Aedes aegypti* larvae. *PLoS One, 10*(5), e0126612.

Pulice, G., Pelaz, S., & Matías-Hernández, L. (2016). Molecular farming in *Artemisia annua*, a promising approach to improve anti-malarial drug production. *Frontiers in Plant Science, 7*, 329.

Puthur, S., Anoopkumar, A., Rebello, S., & Aneesh, E. M. (2018). *Hypericum japonicum*: a double-headed sword to combat vector control and cancer. *Applied Biochemistry and Biotechnology*, *186*(1), 1-11.

Puthur, S., Anoopkumar, A., Rebello, S., & Aneesh, E. M. (2019). Synergistic control of storage pest rice weevil using *Hypericum japonicum* and deltamethrin combinations: a key to combat pesticide resistance. *Environmental Sustainability*, *2*, 411-417.

Qi, G., Lan, N., Ma, X., Yu, Z., & Zhao, X. (2011). Controlling *Myzus persicae* with recombinant endophytic fungi *Chaetomium globosum* expressing *Pinellia ternata* agglutinin: using recombinant endophytic fungi to control aphids. *Journal of Applied Microbiology*, *110*(5), 1314-1322.

Queiroz, E. F., Wolfender, J. L., & Hostettmann, K. (2009). Modern approaches in the search for new lead antiparasitic compounds from higher plants. *Current Drug Targets, 10*(3), 202-211.

Rafińska, K., Pomastowski, P., Rudnicka, J., Krakowska, A., Maruśka, A., Narkute, M., & Buszewski, B. (2019). Effect of solvent and extraction technique on composition and biological activity of *Lepidium sativum* extracts. *Food Chemistry, 289*, 16-25.

Ragavendran, C., Manigandan, V., Kamaraj, C., Balasubramani, G., Prakash, J. S., Perumal, P., & Natarajan, D. (2019). Larvicidal, histopathological,

antibacterial activity of indigenous fungus *Penicillium* sp. against *Aedes aegypti* L. and *Culex quinquefasciatus* (Say) (Diptera: Culicidae) and its acetylcholinesterase inhibition and toxicity assessment of zebrafish (Danio rerio). *Frontiers in Microbiology, 10*, 427.

Rahman, M. M. (2013). Insecticide substitutes for DDT to control mosquitoes may be causes of several diseases. *Environmental Science and Pollution Research, 20*(4), 2064-2069.

Rahuman, A. A., Gopalakrishnan, G., Venkatesan, P., & Geetha, K. (2008a). Larvicidal activity of some Euphorbiaceae plant extracts against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). *Parasitology Research, 102*(5), 867-873.

Rahuman, A. A., Venkatesan, P., Geetha, K., Gopalakrishnan, G., Bagavan, A., & Kamaraj, C. (2008b). Mosquito larvicidal activity of gluanol acetate, a tetracyclic triterpenes derived from *Ficus racemosa* Linn. *Parasitology Research, 103*(2), 333.

Rai, P., Bharati, M., Subba, A., & Saha, D. (2019). Insecticide resistance mapping in the vector of lymphatic filariasis, *Culex quinquefasciatus* Say from Northern region of West Bengal, India. *PLoS One, 14*(5), e0217706.

Rajagopal, R. (1977). Malathion resistance in *Anopheles culicifacies* in Gujarat. *Indian Journal of Medical Research, 66*(1), 27-28.

Ramakrishnan, S., Gelfand, H., Bose, P., Sehgal, P., & Mukherjee, R. (1964). The epidemic of acute haemorrhagic fever, Calcutta, 1963: Epidemiological inquiry. *The Indian Journal of Medical Research, 52*, 633-650.

Ranson, H., & Hemingway, J. (2005). Mosquito glutathione transferases. *Methods in Enzymology, 401*, 226-241.

Ranson, H., & Lissenden, N. (2016). Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitology, 32*(3), 187-196.

Ranson, H., Burhani, J., Lumjuan, N., & Black IV, W. C. (2010). Insecticide resistance in dengue vectors. TropIKA. net [online], 1(1).

Ranson, H., Jensen, B., Vulule, J., Wang, X., Hemingway, J., & Collins, F. (2000). Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Molecular Biology*, *9*(5), 491-497.

Rao, B. (1958). The national malaria control programme in India and the possibilities of eradication of malaria in India and the tropics. *Bull Nat Soc Mal Mosq Dis*, *6*, 5-6.

Rather, I. A., Parray, H. A., Lone, J. B., Paek, W. K., Lim, J., Bajpai, V. K., & Park, Y. H. (2017). Prevention and control strategies to counter dengue virus infection. *Frontiers in Cellular and Infection Microbiology*, *7*, 336.

Rattan, R. S. (2010). Mechanism of action of insecticidal secondary metabolites of plant origin. *Crop Protection*, *29*(9), 913-920.

Ravi, V. (2006). Re-emergence of chikungunya virus in India. *Indian Journal of Medical Microbiology*, *24*(2), 83.

Rawlins, S. C. (1998). Spatial distribution of insecticide resistance in Caribbean populations of *Aedes aegypti* and its significance. *Revista Panamericana de Salud Pública*, *4*, 243-251.

Regnault-Roger, C., Philogène, B., & Vincent, C. (2002). Biopesticides d'origines végétales. *Paris*, *337*.

Reiskind, M., & Lounibos, L. (2009). Effects of intraspecific larval competition on adult longevity in the mosquitoes *Aedes aegypti* and *Aedes albopictus*. *Medical and Veterinary Entomology*, *23*(1), 62-68.

Retz. (1979). Mauritius Inst. Bull. 8(4):85.

Rey Vega, G. (2011). *Determinación de los grados de resistencia al insecticida temefos en poblaciones de Aedes aegypti linnaeus 1762,(diptera:*

culicidae) y su implicación en la eficacia del insecticida en los departamentos de Cauca, La Guajira, Cundinamarca y Atlántico (PhD Thesis). National University of Colombia.

Rey, D., Cuany, A., Pautou, M. P., & Meyran, J. C. (1999). Differential sensitivity of mosquito taxa to vegetable tannins. *Journal of Chemical Ecology*, *25*(3), 537-548.

Rico-Hesse, R., Harrison, L. M., Salas, R. A., Tovar, D., Nisalak, A., Ramos, C., ... da Rosa, A. T. (1997). Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology*, *230*(2), 244-251.

Roark, R. (1947). Some promising insecticidal plants. *Economic Botany, 1*(4), 437-445.

Roberts, J. R., & Reigart, J. R. (2013). *Recognition and management of pesticide poisonings*. United States Environmental Protection Agency, Washington, DC.

Rodríguez, M. M., Bisset, J., Ruiz, M., & Soca, A. (2002). Cross-resistance to pyrethroid and organophosphorus insecticides induced by selection with temephos in *Aedes aegypti* (Diptera: Culicidae) from Cuba. *Journal of Medical Entomology, 39*(6), 882-888.

Roiz, D., Wilson, A. L., Scott, T. W., Fonseca, D. M., Jourdain, F., Müller, P., ... Corbel, V. (2018). Integrated Aedes management for the control of *Aedes*-borne diseases. *PLoS Neglected Tropical Diseases, 12*(12), e0006845.

Rose, R. I. (2001). Pesticides and public health: integrated methods of mosquito management. *Emerging Infectious Diseases, 7*(1), 17.

Rosen, L. (1987). Mechanism of vertical transmission of the dengue virus in mosquitoes. *Comptes rendus de l'Académie des Sciences. Série III, Sciences de la vie, 304*(13), 347.

Royle. (1835) Ill. bot. Himal. Mts. 2(2): t. 28, fig. 2. 1834; 1(5):151.

Roxb. Wall. (1840.) Calc. Gard. Rep. 26

Rozendaal, J. A. (1997). *Vector control: methods for use by individuals and communities*: World Health Organization.

R, Wight. (1834) Contr. bot. India 24.

Russell, P. F., West, L. S., Manwell, R. D., & Macdonald, G. (1963). *Practical Malariology*. London, Oxford University Press.

Samidurai, K. (2012). Mosquito larvicidal and ovicidal properties of *Pemphis acidula* Frost. (Lythraceae) against *Culex tritaeniorhynchus* Giles and *Anopheles subpictus* Grassi (Diptera: Culicidae). *Asian Pacific Journal of Tropical Biomedicine, 2*(3), S1862-S1866.

Sánchez-Fortún, S., & Barahona, M. (2005). Comparative study on the environmental risk induced by several pyrethroids in estuarine and freshwater invertebrate organisms. *Chemosphere, 59*(4), 553-559.

Santhosh, S. B., Ragavendran, C., & Natarajan, D. (2015). Spectral and HRTEM analyses of *Annona muricata* leaf extract mediated silver nanoparticles and its larvicidal efficacy against three mosquito vectors *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*. *Journal of Photochemistry and Photobiology B: Biology, 153*, 184-190.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines, 8*(1), 1-10.

Saxena, R., Dixit, O., & Sukumaran, P. (1992). Laboratory assessment of indigenous plant extracts for anti-juvenile hormone activity in *Culex quinquefasciatus*. *The Indian Journal of Medical Research, 95*, 204-206

Schneider, D., Boppré, M., Zweig, J., Horsley, S. B., Bell, T. W., Meinwald, J., ... Diehl, E. W. (1982). Scent organ development in Creatonotos moths: regulation by pyrrolizidine alkaloids. *Science, 215*(4537), 1264-1265.

Scott, J. G. (1999). Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology, 29*(9), 757-777.

Sengar, N., Joshi, A., Prasad, S. K., & Hemalatha, S. (2015). Anti-inflammatory, analgesic and anti-pyretic activities of standardized root extract of *Jasminum sambac*. *Journal of Ethnopharmacology, 160*, 140-148.

Shaalan, E. A. S., Canyon, D., Younes, M. W. F., Abdel-Wahab, H., & Mansour, A. H. (2005). A review of botanical phytochemicals with mosquitocidal potential. *Environment International, 31*(8), 1149-1166.

Sharififar, F., Dehghn-Nudeh, G., & Mirtajaldini, M. (2009). Major flavonoids with antioxidant activity from *Teucrium polium* L. *Food Chemistry, 112*(4), 885-888.

Sharma, G. (1984). Review of malaria and its control in India. V. P. Sharma (Ed). *Proceedings of the Indo-UK workshop on malaria education*. Delhi, India, pp. 13-40.

Sharma, P. & Samant, S. S. (2014). Diversity, distribution and indigenous uses of medicinal plants in Parbati valley of Kullu district in Himachal Pradesh, Northwestern Himalaya. *Asian Journal of Advanced Basic Sciences, 2*(1), 77-98.

Sharma, V. (2001). Health hazards of mosquito repellents and safe alternatives. *Current Science, 80*(3), 341-343.

Sharon, G., Segal, D., Zilber-Rosenberg, I., & Rosenberg, E. (2011). Symbiotic bacteria are responsible for diet-induced mating preference in *Drosophila melanogaster*, providing support for the hologenome concept of evolution. *Gut Microbes, 2*(3), 190-192.

Shetty, S., Udupa, S., & Udupa, L. (2008). Evaluation of antioxidant and wound healing effects of alcoholic and aqueous extract of *Ocimum sanctum* Linn in rats. *Evidence-Based Complementary and Alternative Medicine, 5*(1), 95-101.

Siegwart, M., Graillot, B., Blachere Lopez, C., Besse, S., Bardin, M., Nicot, P. C., & Lopez-Ferber, M. (2015). Resistance to bio-insecticides or how to enhance their sustainability: a review. *Frontiers in Plant Science, 6*, 381.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology: Translation and Integration, 82*(2), 291-295.

Silva, E. C., Cavalcanti, B. C., Amorim, R. C., Lucena, J. F., Quadros, D. S., Tadei, W. P., … Moraes, M. O. (2009). Biological activity of neosergeolide and isobrucein B (and two semi-synthetic derivatives) isolated from the Amazonian medicinal plant *Picrolemma sprucei* (Simaroubaceae). *Memorias do Instituto Oswaldo Cruz, 104*(1), 48-56.

Silva, H. H. G. d., Silva, I. G. d., Santos, R. M. G. d., Rodrigues Filho, E., & Elias, C. N. (2004). Larvicidal activity of tannins isolated of *Magonia pubescens* St. Hil. (Sapindaceae) against *Aedes aegypti* (Diptera, Culicidae). *Revista da Sociedade Brasileira de Medicina Tropical, 37*(5), 396-399.

Simon-Oke, I. A., & Akeju, A. V. (2017). Laboratory evaluation of extract from peels and seeds of some *Citrus* species against *Anopheles* mosquitoes (Diptera: Culicidae). *International Journal of Mosquito Research, 4*(5), 48-54

Singh, R., Dhiman, R., & Mittal, P. (2006). Mosquito larvicidal properties of *Momordica charantia* Linn (family: Cucurbitaceae). *Journal of Vector Borne Diseases, 43*(2), 88.

Siraichi, J. T. G., Felipe, D. F., Brambilla, L. Z. S., Gatto, M. J., Terra, V. n. A., Cecchini, A. L., ... Cortez, D. A. G. (2013). Antioxidant capacity of the leaf extract obtained from *Arrabidaea chica* cultivated in Southern Brazil. *PLoS One, 8*(8), e72733.

Smith, J. B. (1904). *Report of the New Jersey state agricultural experiment station upon the mosquitoes occurring within the state, their habits, life history, & C.* Trenton, N. J., MacCrellish & Quigley, State Printers.

Sohal, R. S., Sohal, B. H., & Orr, W. C. (1995). Mitochondrial superoxide and hydrogen peroxide generation, protein oxidative damage, and longevity in different species of flies. *Free Radical Biology and Medicine, 19*(4), 499-504.

Soper, F. L. (1963). The elimination of urban yellow fever in the Americas through the eradication of *Aedes aegypti*. *American Journal of Public Health and the Nations Health, 53*(1), 7-16.

Spencer, J. P. (2010). The impact of fruit flavonoids on memory and cognition. *British Journal of Nutrition, 104*(S3), S40-S47.

Sreelatha, T., Hymavathi, A., Murthy, J. M., Rani, P., Rao, J. M., & Babu, K. S. (2010). Bioactivity-guided isolation of mosquitocidal constituents from the rhizomes of *Plumbago capensis* Thunb. *Bioorganic and Medicinal Chemistry Letters, 20*(9), 2974-2977.

Stanaway, J. D., Shepard, D. S., Undurraga, E. A., Halasa, Y. A., Coffeng, L. E., Brady, O. J., ... Castañeda-Orjuela, C. A. (2016). The global burden of dengue: an analysis from the global burden of disease study 2013. *The Lancet Infectious Diseases, 16*(6), 712-723.

Stein, G. J. (1970). *Malaria eradication in Thailand*: US Operations Mission to Thailand (USOM), US Agency for International Development.

Stevenson, B. J., Bibby, J., Pignatelli, P., Muangnoicharoen, S., O'Neill, P. M., Lian, L. Y., . . . Hemingway, J. (2011). Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. *Insect Biochemistry and Molecular Biology, 41*(7), 492-502.

Strode, C., Wondji, C. S., David, J. P., Hawkes, N. J., Lumjuan, N., Nelson, D. R., ... Ranson, H. (2008). Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. *Insect Biochemistry and Molecular Biology, 38*(1), 113-123.

Su, X. Z., & Miller, L. H. (2015). The discovery of artemisinin and the Nobel Prize in Physiology or Medicine. *Science China Life Sciences, 58*(11), 1175-1179.

Su, X., Guo, Y., Deng, J., Xu, J., Zhou, G., Zhou, T., ... Wang, X. (2019). Fast emerging insecticide resistance in *Aedes albopictus* in Guangzhou, China: Alarm to the dengue epidemic. *PLoS Neglected Tropical Diseases, 13*(9), e0007665.

Sukumar, K., Perich, M. J., & Boobar, L. (1991). Botanical derivatives in mosquito control: a review. *Journal of the American Mosquito Control Association, 7*(2), 210-237.

Suryawanshi, R., Patil, C., Borase, H., Narkhede, C., & Patil, S. (2015). Screening of Rubiaceae and Apocynaceae extracts for mosquito larvicidal potential. *Natural Product Research, 29*(4), 353-358.

Suwanchaichinda, C., & Brattsten, L. B. (2002). Induction of microsomal cytochrome P450s by tire-leachate compounds, habitat components of *Aedes albopictus* mosquito larvae. *Archives of Insect Biochemistry and Physiology, 49*(2), 71-79.

Sw. (1788). Nov. Gen. Sp. Pl. Prodr. 47.

Talontsi, F. M., Matasyoh, J. C., Ngoumfo, R. M., & Chepkorir, R. (2011). Mosquito larvicidal activity of alkaloids from *Zanthoxylum lemairei* against the malaria vector *Anopheles gambiae*. *Pesticide Biochemistry and Physiology, 99*(1), 82-85.

Tasanor, O., Brem, B., Leitsch, D., Binder, M., Duchêne, M., Greger, H., & Wernsdorfer, W. H. (2007). Development of a pharmacodynamic

screening model with *Entamoeba histolytica*. *Wiener Klinische Wochenschrift, 119*(3), 88-95.

Tavares, W. S., Costa, M. A., Cruz, I., Silveira, R. D., Serrao, J. E., & Zanuncio, J. C. (2010). Selective effects of natural and synthetic insecticides on mortality of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and its predator *Eriopis connexa* (Coleoptera: Coccinellidae). *Journal of Environmental Science and Health Part B, 45*(6), 557-561.

Tawatsin, A., Asavadachanukorn, P., Thavara, U., Wongsinkongman, P., Bansidhi, J., Boonruad, T., ... Mulla, M. S. (2006). Repellency of essential oils extracted from plants in Thailand against four mosquito vectors (Diptera: Culicidae) and oviposition deterrent effects against *Aedes aegypti* (Diptera: Culicidae). *Southeast Asian Journal of Tropical Medicine and Public Health, 37*(5), 915.

Tennyson, S., Ravindran, K. J., & Arivoli, S. (2012). Screening of twenty five plant extracts for larvicidal activity against *Culex quinquefasciatus* Say (Diptera: Culicidae). *Asian Pacific Journal of Tropical Biomedicine, 2*(2), S1130-S1134.

Thanispong, K., Sathantriphop, S., & Chareonviriyaphap, T. (2008). Insecticide resistance of *Aedes aegypti* and *Culex quinquefasciatus* in Thailand. *Journal of Pesticide Science, 33*(4), 351-356.

Tholl, D., Boland, W., Hansel, A., Loreto, F., Röse, U. S., & Schnitzler, J. P. (2006). Practical approaches to plant volatile analysis. *The Plant Journal, 45*(4), 540-560.

Thomas, P. E., Ryan, D., & Levin, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry, 75*(1), 168-176.

Thongyuan, S., & Kittayapong, P. (2017). First evidence of dengue infection in domestic dogs living in different ecological settings in Thailand. *PLoS One, 12*(8), e0180013.

Thunberg, C. P. (1978) . Bull. Mus. Natl. Hist. Nat., Sér. 3, Bot. 35: 13.

Traboulsi, A. F., El-Haj, S., Tueni, M., Taoubi, K., Nader, N. A., & Mrad, A. (2005). Repellency and toxicity of aromatic plant extracts against the mosquito *Culex pipiens molestus* (Diptera: Culicidae). *Pest Management Science, 61*(6), 597-604.

Uniyal, A., Tikar, S. N., Mendki, M. J., Singh, R., Shukla, S. V., Agrawal, O. P., ... Sukumaran, D. (2016). Behavioral response of *Aedes aegypti* mosquito towards essential oils using olfactometer. *Journal of Arthropod-borne Diseases, 10*(3), 370.

Valzania, L., Coon, K. L., Vogel, K. J., Brown, M. R., & Strand, M. R. (2018). Hypoxia-induced transcription factor signaling is essential for larval growth of the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences, 115*(3), 457-465.

Valzania, L., Martinson, V. G., Harrison, R. E., Boyd, B. M., Coon, K. L., Brown, M. R., & Strand, M. R. (2018). Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. *PLoS Neglected Tropical Diseases, 12*(7), e0006638.

van den Berg, H., Zaim, M., Yadav, R. S., Soares, A., Ameneshewa, B., Mnzava, A., ... Ejov, M. (2012). Global trends in the use of insecticides to control vector-borne diseases. *Environmental Health Perspectives, 120*(4), 577-582.

Verma, R., Sahu, R., & Holla, V. (2014). Neurological manifestations of dengue infection: a review. *Journal of the Neurological Sciences, 346*(1-2), 26-34.

Verpoorte, R., van der Heijden, R., & Memelink, J. (2000). Engineering the plant cell factory for secondary metabolite production. *Transgenic Research, 9*(4-5), 323-343.

Villanes, A., Griffiths, E., Rappa, M., & Healey, C. G. (2018). Dengue fever surveillance in India using text mining in public media. *The American Journal of Tropical Medicine and Hygiene, 98*(1), 181-191.

Vingtdeux, V., Dreses-Werringloer, U., Zhao, H., Davies, P., & Marambaud, P. (2008). Therapeutic potential of resveratrol in Alzheimer's disease. *BMC Neuroscience, 9*(S2), S6.

Vontas, J., Blass, C., Koutsos, A., David, J. P., Kafatos, F., Louis, C., ... Ranson, H. (2005). Gene expression in insecticide resistant and susceptible *Anopheles gambiae* strains constitutively or after insecticide exposure. *Insect Molecular Biology, 14*(5), 509-521.

Vontas, J., Kioulos, E., Pavlidi, N., Morou, E., Della Torre, A., & Ranson, H. (2012). Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. *Pesticide Biochemistry and Physiology, 104*(2), 126-131.

Vulule, J., Beach, R., Atieli, F., McAllister, J., Brogdon, W., Roberts, J., ... Hawley, W. (1999). Elevated oxidase and esterase levels associated with permethrin tolerance in *Anopheles gambiae* from Kenyan villages using permethrin-impregnated nets. *Medical and Veterinary Entomology, 13*(3), 239-244.

Wakeel, A., Jan, S. A., Ullah, I., Shinwari, Z. K., & Xu, M. (2019). Solvent polarity mediates phytochemical yield and antioxidant capacity of *Isatis tinctoria*. *Peer J, 7*, e7857.

Wang, J. (2000). Resistance and response to selection to deltamethrin in *Anopheles sinensis* from Zhejiang, China. *Journal of the American Mosquito Control Association, 16*(1), 9-12.

Wang, X., & Hai, C. (2016). Novel insights into redox system and the mechanism of redox regulation. *Molecular Biology Reports, 43*(7), 607-628.

Watson, M. (1921). *The prevention of malaria in the Federated Malay States: a record of twenty years' progress*: EP Dutton & Company.

Weeratunga, P., Rodrigo, C., Fernando, S. D., & Rajapakse, S. (2017). Control methods for *Aedes albopictus* and *Aedes aegypti*. *Cochrane Database of Systematic Reviews, 2017*(8).

White, G. (1973). The insect-repellent value of *Ocimum* spp. (Labiatae): traditional anti-mosquito plants. *East African Medical Journal, 50*(5), 248-252.

White, R. S. (1945). House Spraying with DDT and with pyrethrum extract compared: first results. *Journal of the Malaria Institute of India, 6*(1), 83-93.

WHO [World Health Organization]. (1981). Criteria and meaning of tests for determining the susceptibility or resistance of insects to insecticides. WHO/VBC/81.6, Annex of twenty-second report of the WHO expert committee on insecticide-resistance of vectors and reservoirs of disease to pesticides, Tech. Rep. Ser., 585.

WHO [World Health Organization]. (2010). Spinosad DT in drinking-water: use for vector control in drinking-water sources and containers. Geneva: World Health Organization.

WHO [World Health Organization]. (2014). Dengue and severe dengue: World Health Organization. Regional Office for the Eastern Mediterranean.

WHO [World Health Organization]. (2015). Guidelines for the treatment of malaria: World Health Organization.

WHO [World Health Organization]. (2016). Test procedures for insecticide resistance monitoring in malaria vector mosquitoes.

Wiegand, I., Hilpert, K., & Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols, 3*(2), 163.

Wight, R. (1834). Contributions to the Botany of India. Parbury, Allen.

Wight. (1839) Icon. Pl. Ind. Orient. 1: t. 109.

Wilke, A. B. B., & Marrelli, M. T. (2015). Paratransgenesis: a promising new strategy for mosquito vector control. *Parasites and Vectors, 8*(1), 342.

Wink, M. (2000). Interference of alkaloids with neuroreceptors and ion channels. *Studies in natural products chemistry, 21*(B), 3-122.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry, 64*(1), 3-19.

Xu, Q., Liu, H., Zhang, L., & Liu, N. (2005). Resistance in the mosquito, *Culex quinquefasciatus*, and possible mechanisms for resistance. *Pest Management Science: formerly Pesticide Science, 61*(11), 1096-1102.

Yadav, K. K., Bora, A., Datta, S., Chandel, K., Gogoi, H. K., Prasad, G., & Veer, V. (2015). Molecular characterization of midgut microbiota of *Aedes albopictu*s and *Aedes aegypti* from Arunachal Pradesh, India. *Parasites and Vectors, 8*(1), 641.

Yadav, R., Srivastava, V., Chandra, R., & Singh, A. (2002). Larvicidal activity of latex and stem bark of *Euphorbia tirucalli* plant on the mosquito *Culex quinquefasciatus*. *The Journal of Communicable Diseases, 34*(4), 264-269.

Yazdanparast, R., Bahramikia, S., & Ardestani, A. (2008). Nasturtium officinale reduces oxidative stress and enhances antioxidant capacity in hypercholesterolaemic rats. *Chemico-Biological Interactions, 172*(3), 176-184.

Youmsi, R. D. F., Fokou, P. V. T., Menkem, E. Z. o., Bakarnga-Via, I., Keumoe, R., Nana, V., & Boyom, F. F. (2017). Ethnobotanical survey of medicinal

plants used as insects repellents in six malaria endemic localities of Cameroon. *Journal of Ethnobiology and Ethnomedicine, 13*(1), 33.

Yu, B., Sun, J., & Yang, X. (2012). Assembly of naturally occurring glycosides, evolved tactics, and glycosylation methods. *Accounts of Chemical Research, 45*(8), 1227-1236.

Yu, S. (2015). *Classification of insecticides. The toxicology and biochemistry of insects*. Boca Raton, Florida, USA, CRC Press.

Yuriev, E., Agostino, M., & Ramsland, P. A. (2011). Challenges and advances in computational docking: 2009 in review. *Journal of Molecular Recognition, 24*(2), 149-164.

Zenk, M. H., & Juenger, M. (2007). Evolution and current status of the phytochemistry of nitrogenous compounds. *Phytochemistry, 68*(22-24), 2757-2772.

Zhu, L., & Tian, Y. J. (2011). Chemical composition and larvicidal effects of essential oil of *Blumea martiniana* against *Anopheles anthropophagus*. *Asian Pacific Journal of Tropical Medicine, 4*(5), 371-374.

Zikankuba, V. L., Mwanyika, G., Ntwenya, J. E., & James, A. (2019). Pesticide regulations and their malpractice implications on food and environment safety. *Cogent Food and Agriculture, 5*(1), 1601544.

Zulak, K. G., Liscombe, D. K., Ashihara, H., & Facchini, P. J. (2006). Alkaloids. In A. Crozier, M. N. Clifford & H. Ashihara (Eds.), *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet* (pp. 102-136). Wiley-Blackwell.

PUBLICATIONS

Publications (2017-2021)

Sl. No.	Authors	Title	Publisher
1	**Anoopkumar, A. N.,** Aneesh, E. M.	Environmental epidemiology and neurological manifestations of dengue serotypes with special inference on molecular trends, virus detection, and pathogenicity.	Environment, Development and Sustainability, 1-23. Springer Nature Switzerland **IF: 2.19**
2	**Anoopkumar, A. N.,** Aneesh, E. M., & Sudhikumar, A. V.	Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases.	International Journal of Tropical Insect Science, 40, 573–585 (2020). Springer Nature Switzerland **IF: 0.748**
3	**Anoopkumar, A. N**., Rebello, S., Sudhikumar, A. V., Puthur, S., & Aneesh, E. M.	A novel intervention on the inhibiting effects of *Catunaregam spinosa* induced free radical formation and DNA damage in *Aedes aegypti* (Diptera: Culicidae): a verdict for new perspectives on microorganism targeted vector control approach.	International Journal of Tropical Insect Science, 2020 May 23. (2020). SpringerNature Switzerland **IF: 0.748**
4	Rebello, Sharrel, **A. N. Anoopkumar**, Embalil Mathachan Aneesh, Raveendran Sindhu, Parameswaran Binod, Sang Hyoun Kim, and Ashok Pandey.	Hazardous minerals mining: Challenges and solutions.	Journal of Hazardous Materials 402: 123474 (2020) Elsevier B.V. ScienceDirect Amsterdam, **IF: 9.03**
5	**Anoopkumar, A.N**., Puthur, S., Rebello, S. and Aneesh, E.M.	Molecular characterization of *Aedes, Culex, Anopheles*, and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis.	Biologia, 74(9), 1125-1138. (2019). Springer Switzerland **IF: 0.875**

6	**Anoopkumar, A. N**., Rebello, S., Devassy, E., Raj, K. K., Puthur, S., Aneesh, E. M., ... & Pandey, A.	Phytoextraction of Heavy Metals. Methods for Bioremediation of Water and Wastewater Pollution, 267.	Springer, Singapore Online ISBN 978-3-030-48985-4 **BOOK CHAPTER**
7	**Anoopkumar, A.N,** Sreedev Puthur, P. Varghese, S. Rebello, and E. M. Aneesh.	Life cycle, bio-ecology and DNA barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse).	The Journal of Communicable Diseases 49 (2017). Indian Society of Malaria and Other Communicable Diseases. **IF: 0.128 (SJR)**
8	**Anoopkumar A.N**., Sreedev P., Aneesh E.M. and Sharrel Rebello	Screening of a Few traditionally used Medicinal Plants for their Larvicidal Efficacy against *Aedes aegypti* Linn (Diptera: Culicidae), a Dengue Fever Vector.	SOJ Microbiol Infect Dis 5(4): 1-5. (2017) Symbiosis Group LLC. USA **IF: 2.0 (RG)**
9	Sharrel Rebello, **Anoopkumar A.N**, Sindhu Raveendran, Binod Parameswaran, Ashok Pandey, Embalil Mathachan Aneesh	Comparative Life Cycle Analysis of synthetic detergents and biosurfactants – an overview. In book: Refining biomass residues for sustainable	Technology, Advances, Life Cycle Assessment, and Economics. Academic press.2020, Pages 511-521.Elsevier **BOOK CHAPTER**
10	Rebello, S., **Anoopkumar, A. N**., Aneesh, E. M., Sindhu, R., Binod, P., & Pandey, A.	Sustainability and life cycle assessments of lignocellulosic and algal pretreatments.	BioresourceTechnology, 301, 122678. Elsevier B.V. ScienceDirect **IF: 7.53**
11	Rebello, Sharrel, Divya Balakrishnan, **A.N. Anoopkumar**,Raveendran Sindhu, Parameswaran Binod, Ashok Pandey, and Embalil Mathachan Aneesh.	Industrial Enzymes as Feed Supplements—Advantages to Nutrition and Global Environment	Springer, Singapore Online ISBN: 978-981-13-3263-0 **BOOK CHAPTER**

12	Puthur, S., **Anoopkumar, A.N.**, Rebello, S. and Aneesh, E.M., 2019.	Synergistic control of storage pest rice weevil using *Hypericum japonicum* and deltamethrin combinations: a key to combat pesticide resistance.	Environmental Sustainability 2, 411–417 (2019). Springer Switzerland
13	Puthur, S., Raj, K. K., Anoopkumar, A. N., Rebello, S., & Aneesh, E. M. (2020).	*Acorus calamus* mediated green synthesis of ZnONPs: A novel nano antioxidant to future perspective.	Advanced Powder Technology. 2020 Nov 21. Elsevier B.V. ScienceDirect Amsterdam, **IF:4.21**
14	Rebello, Sharrel, **A. N. Anoopkumar**, Sreedev Puthur, Raveendran Sindhu, Parameswaran Binod, Ashok Pandey, and Embalil Mathachan Aneesh.	Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife.	Bioresource Technology Reports. Volume 3, September 2018, Pages 1-6. Elsevier B.V. **IF: 0758 (SJR)**
15	Puthur S., **Anoopkumar A.N**., Rebello S., Aneesh E.M.	*Hypericum japonicum*: a Double-Headed Sword to Combat Vector Control and Cancer.	Applied biochemistry and biotechnology, 2018 Sep;186(1):1-11 Springer Switzerland **IF: 2.27**

Environment, Development and Sustainability
https://doi.org/10.1007/s10668-020-01161-7

REVIEW

Environmental epidemiology and neurological manifestations of dengue serotypes with special inference on molecular trends, virus detection, and pathogenicity

A. N. Anoopkumar[1] · **Embalil Mathachan Aneesh**[1]

Received: 8 January 2020 / Accepted: 15 December 2020

Abstract
The emergence of new viruses is a matter of significant concern in the current decade. It has dazed the indigenous healthcare systems in various parts of the world. Consequently, the resources for fighting the rapid spread of the DENV are ineffectual, insufficient, and incompetent. Several environmental factors have been allied with dengue fever transmission. High documentation of neuropathogenesis in dengue-infected individuals in recent years reflects the significance of our study, and the information discussed in this review can be used to enhance clinical awareness along with advances in diagnostic approaches. The replication of the DENV genome is usually engaged in a membrane-linked replication complex; the virion budding and morphogenesis have been found to take place in the modified endoplasmic reticulum membranes. Most of these non-structural proteins are believed to be responsible for RNA replication and polyprotein processing. The nucleic acid-based strategies have provided high sensitivity and progressively replacing conventional techniques. Advanced technologies like PCR have offered timely serotyping of dengue viruses, which illustrates the early warning of dengue epidemics. The molecular methods help to characterize the key factors responsible for the rapid spread of viruses and thereby update the vector control programmes targeted at extenuating their adverse impacts on public health. The vast diversity of dengue virus strains worldwide with special inference on their clinical manifestations and genetic characteristics was also analyzed in this study. As dengue threatens one third of the world's population, practical applications of advanced molecular strategies from an environmental and phylogenetic perspective are critical for disease control.

Keywords DENV · Neuropathogenesis · RNA replication · PCR · Serotyping · Phylogenetic perspective

✉ Embalil Mathachan Aneesh
aneeshembalil@stjosephs.edu.in

A. N. Anoopkumar
anoopkumar.247@gmail.com

[1] Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Joseph's College (Autonomous), Irinjalakuda, Thrissur, Kerala, India

Published online: 21 January 2021

1 Introduction

Unapparent infection of dengue virus (DENV) from the genus *Flavivirus* (family-Flaviviridae) results in an extensive spectrum of clinical presentation, from mild febrile illness to the severe dengue shock syndrome (DSS) including dengue hemorrhagic Fever (DHF) (Murray et al. 2013; Anoopkumar et al. 2017a). The reservoirs of DENV (*Flavivirus*) in sylvatic cycles are characterized by nonhuman primates, and the virus transmission is accompanied by arboviral mosquito vector species such as *Aedes furcifer* and *Aedes luteocephalus* (Diallo et al. 2003). The anthropophilic mosquito vectors, typically *Aedes aegypti* and *Aedes albopictus*, transmit DENV to the human population since humans are the principal reservoirs in the human cycle (Vijayan 2010; Kamgang et al. 2012; Puthur et al. 2018; Anoopkumar et al. 2020a). Conventional dengue fever control programmes principally fail to reflect the impact of environmental circumstances on the life-history traits and ecosystem, together with vector populations' innate immunity.

The prevalence of endemic and epidemic dengue increased extensively in the Americas in 1977 (Hayes and Gubler 1992). Alarmingly, the aforementioned acute vector-borne viral infections have been placing a prominent impact on the health of the human population, those lives in tropical and subtropical areas of the world (Anoopkumar et al. 2019). Triggering DHF by thrombocytopenia, plasma leakage, hemorrhage, together with coagulation abnormalities, has validated the influence of mosquito-borne diseases on the human population (Martina et al. 2009). The dengue virus serotypes maintained two significant transmission cycles, such as sylvatic and human cycles. The former cycle involves arboreal *Aedes* mosquitoes and nonhuman primates, while the human cycle involves peridomestic *Aedes albopictus* and domestic *Aedes aegypti.*

The future of dengue in context with the environmental epidemiology and molecular biology, if precisely projected, would help national and international agencies, including the public health officials and governments, to take preemptive actions in saving human populations from the dengue crisis. There exist many knowledge gaps that need to be filled in this area. First, incorporating the information of the vast genetic diversity of dengue viruses all over the world into projections would provide an evolutionary basis for the dengue epidemics. Second, certain significant environmental factors may have a prominent role in dengue transmission as we all know. Identifying such significant factors in relation to molecular strategies is warranted. Third, it is of immense significance to explore phylogenetic aspects of dengue viruses along with neurological manifestations and detection strategies since studies based on these aspects have been scanty in the current scenario. Most of the studies have focused on either climate change or molecular detection strategies, phylogenetic aspects, pathogenicity or environmental aspects or neurological manifestations, or clinical trials. Some of the recent studies Hashan et al. (2020) have focused on the meta-analysis that reveals the close link between the various factors and elements, including the blood group, mast cell mediators (Sherif et al. 2020), and allergic symptoms with dengue fever infection Kien et al. 2020). A study by Mata et al. 2020 has discussed the diagnostic accuracy of rapid immunochromatographic tests (IgG ICT or IgA, NS1, IgM) in suspected cases of dengue infection using a combination of advanced strategies such as RT-PCR, ELISA NS1, and IgM-IgG. While searching the review of the literature for this article, the authors failed to find even a single article in recent years that constitutively discuss all the aforesaid aspects.

To fill such gaps, a comprehensive literature search was conducted in June 2017 (updated in November 2020) using the well-established electronic databases and relevant

research Web sites including Scopus, Web of Science, Science Direct, PROSPERO, PubMed, ClinicalTrials.gov, ProQuest, and WHO ICTRP. We limited the search to peer-reviewed articles written in English. The following keywords were used to retrieve the articles from online: "dengue," "climate," "dengue disease transmission," "diagnostic tests," "virus," "DENV," "environmental factors," "mosquito vectors," "*Aedes aegypti*," "environmental risk factors," "neurological," "clinical," "PCR," "pathogenicity," "detection," "phylogeny," and "molecular."

Here we also intend to discuss how the environmental conditions including stress lead to the susceptibility of *Aedes aegypti* to DENV. Analysis of this aspect is recognized as the potential to update the precise commencement and implementation of effective vector control programmes. The diagnosis of dengue fever infection is accomplished by either recognizing virus or antibody (IgM and IgG) formed in the blood at the time of infection. The basic approaches routinely used by most laboratories include the isolation and characterization of viruses, recognition of DENV-specific antibodies (IgM and IgG) together with the recognition of genomic sequences by PCR amplification techniques (Kouri 1996). The PCR-based techniques, including nested RT-PCR, have been used for the serum analysis to detect the DENV nucleic acid from the susceptible individual (Gurukumar et al. 2009). The plaque reduction neutralization assay (PRNT) also permits detection of the dengue virus infection by analyzing the dengue specific neutralizing antibodies. The present review provides a detailed insight into dengue epidemics with special inference on the environmental influence, clinical manifestations, molecular trends, strategies, and pathogenicity.

2 Environmental epidemiology

The global circumstances, including migration and traveling, followed by improper environmental management (Anoopkumar et al. 2020b, c; Rebello et al. 2020a, b), enhance the rapid spread of mosquito vectors. As a result, approximately 390 million people are infected by all dengue serotypes every year and of which 96 million incidents are symptomatic (Ly et al. 2018). The literature concerning the influence of environmental factors on dengue fever epidemics confounded that environmental epidemiology has a significant link with the susceptibility to different arboviruses in *A. aegypti* mosquito vectors (Muturi and Alto 2011). This was further convoluted by the annexation of the literature regarding other arboviruses, proposing that the environmental factors induced stress and decreased their susceptibility against flaviviruses in *A. aegypti* (Kang et al. 2018; Puthur et al. 2019). It is important to record that the age of dengue virus-infected individuals after 2010 was tremendously older than that of the dengue-infected individuals before 2010. One of the possible reasons for this severe situation is that most of the people of younger age groups have spent their daytime in air-conditioned settings and are not as likely to be exposed with vector mosquitoes (Lin et al. 2012). The significant environmental factors responsible for the spread of dengue fever, have been studied in Mexico, Taiwan, and Puerto Rico (Pino et al. 1993). Among the environmental factors, rainfall, temperature, and humidity have been noted as the prominent climate parameters correlated with dengue fever transmission.

The dengue fever transmitting mosquitoes exhibit shorter periods of development in their life cycle when the temperature increases due to climate change and other reasons. However, this eventually leads to augmented population growth within a short period of time. Simultaneously, this also leads to increased feeding rate and availability of habitat of vector mosquitoes along with the longevity of the virus. A study by Teurlai et al. 2015

reported that increasing temperature at 3 °C may double the dengue epidemics rate. The augmented temperature may allow the mosquito vectors to survive and attain maturity much earlier than at lower temperatures. In agreement with the aforesaid patterns, climatic drivers of dengue fever in Mexico, Bangladesh, Cambodia, Vietnam, Nepal, Thailand, Philippine, Myanmar, Sri Lanka, and India have been reported from several previous studies (Anwar et al. 2019; Colón et al. 2011; Tuladhar et al. 2019). According to the fact sheet by WHO, it was found that the disease is now endemic in more than 100 countries (WHO 2020). A recent study by Lee et al. (2019) also revealed a perilous situation in Japan, which signals that some of the non-dengue-endemic countries are severely exposed to the threat of sporadic behaviours of dengue. Such drastic circumstances justify the significance of our investigation since research based on these aspects can support the appropriate prevention activities.

Guzmán and Kouri (2002) reported that waste management, climate change, and inadequate water supply are recognized as the key determinants of dengue incidents (Guzmán and Kouri 2002). The various environmental perceptions, including geographical changes and unsustainable use of land in accordance with the development, can affect climate change. This, in turn, may lead to severe disease incidents interlinked with the environment (Kesetyaningsih et al. 2018; Rebello et al. 2019a). Kesetyaningsih et al. 2018 also validated the correlation between the climate factors and dengue incidents in endemic provinces of Taiwan using regression analysis. The link between dengue incidents and environmental factors with the possible impact of the distance factor revealed that the average distance travelled by the mosquito per day extended the need for the analysis of the dengue epidemics in spatial pattern preferences.

3 Neurological manifestations

3.1 Neurological manifestations of dengue serotypes and viral infection

The clinical manifestation of dengue virus infections from asymptomatic to alarming circumstances, when improperly managed, leads to death. The symptomatic cases of dengue viral infection include dengue hemorrhagic fever (DHF), dengue fever (DF), undifferentiated febrile illness (UF), expanded dengue syndrome (EDS), and unusual dengue (UD) followed by dengue shock syndrome (DSS) (WHO 2011). Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have triggered the plasma leakage into the peritoneal and pleural cavities, thereby leading to ascites and pleural effusion. The three important phases of dengue illness are (1) the febrile phase, (2) the leakage phase, and (3) the convalescence phase (Fig. 1). In the first stage of the febrile phase, symptomatic treatment can be accompanied. The ultrasonography, chest radiology-based techniques (chest film—right lateral decubitus approach), and serum albumin techniques (≤ 3.5 gm%) are usually used to detect this stage of infection. The second stage of plasma leakage began at the end of the febrile phase. Disastrously, a lack of proper medical treatment will increase the risk of severe plasma leakage. Patients with pleural effusion and massive ascites may necessitate diuretic throughout this convalescence phase. Administration of potassium supplements is the prominent way to overcome this phase in dengue patients since the loss of potassium in the urine and diuresis condition may increase the threat. The convalescence phase may prolong for 2–4 weeks together with fatigue in adults.

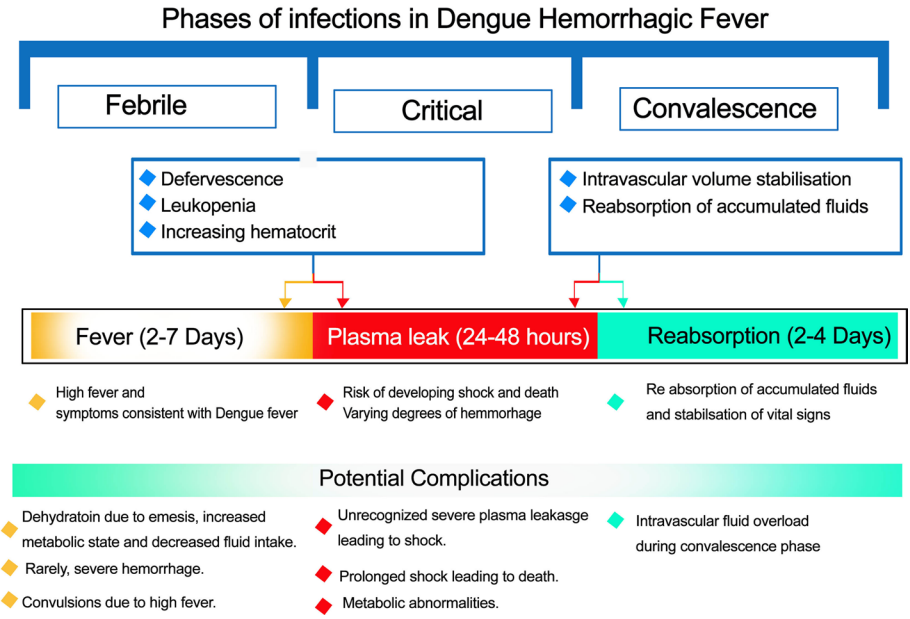


Fig. 1 Three significant phases of dengue fever infection triggered by any one of five distinct dengue serotypes (DENV-1, 2, 3, 4, and 5)

The diagnosis of undifferentiated febrile illness (UF) is completely based on virology or serology since it was not possible to diagnose it clinically (Kalayanarooj and Nimmannitya 2004). Following the febrile period, the infected individuals have experienced the onset of fever together with arthralgia, myalgia, nausea, anorexia, headaches, sore throat, macular skin rash and is followed by vomiting (Ligon 2005). Many studies have predicted and explained the risk factors responsible for the progression of the disease from dengue fever (DF) to dengue hemorrhagic fever (DHF) (Endy et al. 2004; Wichmann et al. 2004). These factors include population genetics, viremia titers, proinflammatory cytokines, and the immune status of susceptible individuals (Clyde et al. 2006). Nearly 400 million individuals are infected by dengue viral infections every year and with approximately 96 million people exhibiting clinical relevance, of which 2.5% of all infected individuals die. Presently, the neurological manifestations of viral infections caused by the aetiological agent DENV have been radically reported, though their actual frequency rate persists unclearly (Li et al. 2017). Manifestations of neurological complications described in dengue fever infections are sleeplessness, headache, dizziness, and altered sensorium, followed by somnolence.

Neuropathogenesis is a significant term that is usually related to the invasion of the aetiological agent, flavivirus, directly into the central nervous system (CNS), metabolic alterations, and autoimmune reactions. The DENV is generally reflected as a non-neurotropic flavivirus (Cam et al. 2001). Conversely, recent studies report the correlation of dengue viral infection with neurological complications induced by DENV. More than two decades back, the aforesaid virus was detected in the cerebrospinal fluid (CSF) (Thisyakorn et al. 1999). The neurological involvement of the dengue virus was first conveyed in 1976, and their incidence rate progressively increased from 0.5 to 20% (Saini et al. 2017). Raised hematocrit, rash, liver dysfunction, thrombocytopenia together with high body temperature

are the distinct risk factors of neurological impediments. The DENV-2 and DENV-3 are the two specific serotypes usually associated with neurological complications. Both of these two serotypes are detected from the pontine, cerebellar, basal gangliar, parietal, and frontal lobes region of the brain of dengue-infected patients. One of the major factors responsible for the rapid increase in dengue epidemics is the prominent alterations of dengue virus serotypes. For instance, a DENV-3 virus subtype was described in 1998 from India (Messer et al. 2003).

3.2 Clinical spectrum of dengue virus infection with special inference on neurological complications

3.2.1 Encephalitis and encephalopathy

Headache, altered consciousness, seizures, and fever are common symptoms found in dengue-infected patients. Encephalopathy is reflected as the most common neurological complication of dengue infection and is exclusively accompanied by dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The reason for the origin and development of DHF/DSS is not well understood. However, increased vascular permeability is renowned as the principal hallmark for the aforementioned infections. Presently, no specific and direct treatments exist to equipoise and reduce the increased vascular permeability that happens during the infection. The lack of better understanding of the processes leads to the dengue virus (DV) prompted vascular leakage (Martina et al. 2009). It was characterized by cognitive impairment, reduced sensitivity, behaviour disorders, collected with agoraphobia, anxiety, acute mania, emotional liability, psychosis, and depression (Solbrig and Perng 2015; Anoopkumar et al. 2017b). Verma et al. (2013) had described that dengue encephalitis leads to *Epilepsia partialis* continua (Verma and Varatharaj 2011). The most preferred method for neuroimaging modality is magnetic resonance imaging (MRI). Several encephalitis causing flaviviruses incline to involve specific structures on the brain resulting in distinguishing image patterns. These structures could definitely aid in the clinical diagnosis of Encephalitis induced by DENV. The MRI has been seen as normal in the initial stage of infection, or it may appear disseminated as a focal abnormality, oedema, and hemorrhage. However, MRI results bear hyperintense regions of the globus pallidus in some of the dengue encephalitis-affected individuals (Misra et al. 2006).

3.2.2 Meningitis, myelitis, and acute disseminated encephalomyelitis and other neurological manifestations

The dengue virus infection is rarely characterized by meningitis. Myelitis is typically flaccid type in the acute phase of viral infection, whereas it is spastic in the delayed phase of infection. Several studies on manifestations of dengue fever infection revealed that the spinal cord has significantly involved in dengue infection, expressing post-infectious myelopathy (Mamdouh et al. 2013). Most probably, this myelitis is instigated by the direct invasion of the virus. Very rarely, dengue virus infection leads to acute disseminated encephalomyelitis (ADEM) (Brito et al. 2007). The ADEM's probable mechanism is the autoimmune responses against myelin or self-antigens through molecular mimicry (Kunishige et al. 2004). Guillain–Barré syndrome (GBS) is characterized by a quickly rising paralysis. Only a few cases have been reported regarding the syndrome. The precise mechanism of dengue allied Guillain–Barré syndrome remains imprecise. Yet myalgia is a very common term in

dengue virus infection. Muscle weakness and myositis are conspicuously uncommon for the same. Several studies have indicated very high CPK levels, and myalgia are regarded as the distinct form of myositis (Paliwal et al. 2011). Dengue infection resulting in hypokalemic paralysis is also reported in extreme cases. Approximately 35 severe cases of hypokalemic paralysis have been reported from scientific research articles from India. Moreover, in addition to Guillain–Barré syndrome and myositis, hypokalemic paralysis could also be predisposed for the acute pure motor quadriparesis in dengue-infected people. Potassium supplementation is accepted as a significant approach for the management of hypokalemic paralysis (Jha and Ansari 2010). Weeratunga et al. (2014) described three dengue-infected individuals, and after two weeks of diagnosis, they were noticed with severe symptoms of cerebellar syndrome. The manifestations of cerebellar syndrome comprise dysarthria, bilateral limb, and gait ataxia together with horizontal and bilateral vertical nystagmus.

Current studies in dengue virus infection also revealed that overproduction of cytokine in dengue-infected individuals results in endothelial cell damage (Seet and Lim 2007), including hemorrhagic and ischemic strokes (Liou et al. 2008). Current studies have conveyed the dengue-infected patients showing uncommon neurological anomalies that include: abducens nerve palsy, brachial neuritis, peripheral facial palsy, phrenic nerve palsy, long thoracic nerve palsy, and abducens nerve palsy (Patey et al. 1993). There has been substantial progress in the rate of dengue infection associated with ophthalmic complications. Maculopathy has been reported as the most frequent neurological anomalies associated with dengue virus infection, whereas optic neuropathy, cranial nerve palsy, and retina vasculopathy are regarded as the less commonly faced complications (Yip et al. 2012).

3.3 The dengue serotypes and their epidemiological pattern and genome structure of viruses

The first report on probable dengue fever infection was described in a Chinese medical encyclopedia (265–420 AD) (Rush 1951). Historical aspects of dengue fever infection throughout the world revealed that the most significant outbreaks have happened in Panama and French West Indies in the 1660s, in Mexico, Peru, Colombia, Caribbean, and Philadelphia of USA in the 1770s followed by Argentina, Brazil, and Venezuela in the 1800s–1900s (Istúriz et al. 2000). The dengue virus infection is primarily regarded as a self-limiting viral disease caused by four distinct types of serotypes (DENV-1, DENV-2, DENV-3, DENV-4) (Flaviviridae), together with a new fifth serotype termed as DENV-5 reported in 2013. The virus is primarily transmitted to the human population through *Aedes aegypti* and *Aedes albopictus* mosquito bite (Mustafa et al. 2015). As an arboviral disease transmission, the epidermis of the skin is the initial site of virus inoculation and primary host immune responses (Rathore and St. John 2018). The dengue virus-carrying female mosquitoes use their mouthparts, proboscis, and probe to collect blood from the capillary, thereby leading to the invagination of the virus into the dermis and epidermis. The virus elements have then been injected into the inside during each probing incident. This DENV uses the lymph system as a channel for their further transmission (Fig. 2) (Barban et al. 2018). The macrophage lineage cells have also exhibited a dual role in transmission since it was noted that macrophages' diminution results in reduced infection. The DENV particle transmitted by the mosquitoes persuades a degranulation response by macrophages (St John et al. 2013). These cytotoxic cells (NKT, NK, CD8 þ T) have been recruited into the infected

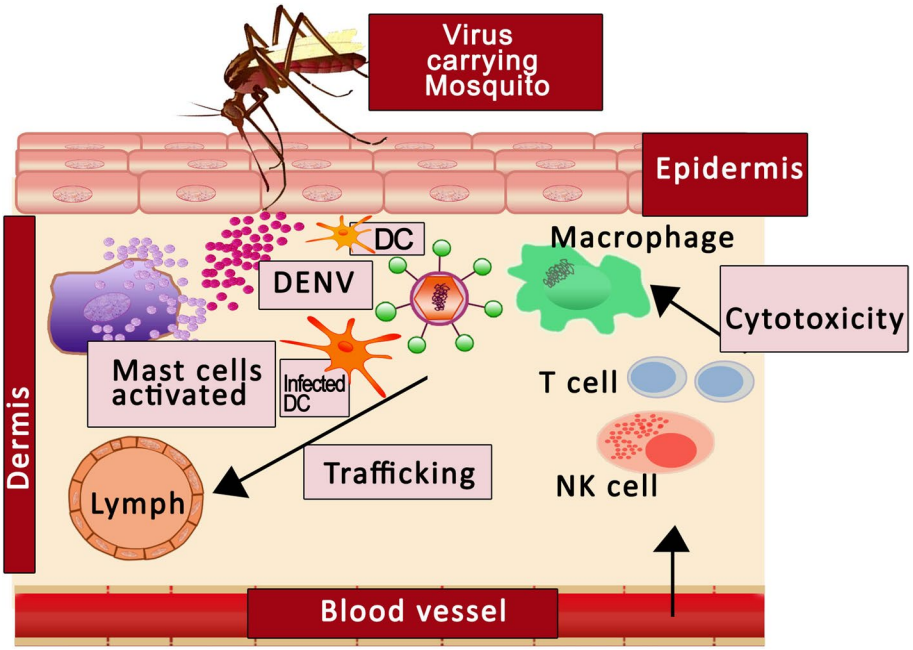
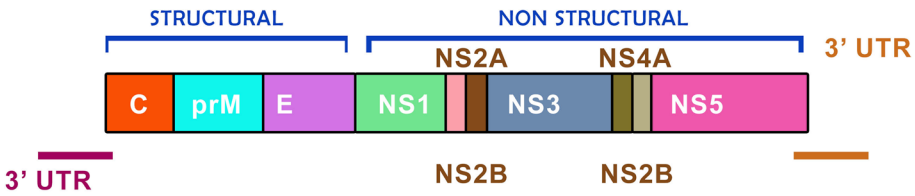


Fig. 2 Mosquito vector bite induces infection by transmitting the DENV followed by generating toxic effects to cellular constituents including defence mechanisms

regions in a macrophage-dependent manner. The NK cells always have the capability to destroy the virus-infected DCs through antibody relied mechanisms. During the severe dengue infection, activated CD8 T has exhibited a skin-homing phenotype mechanism, including the expression of CCR5 and CXCR3 together with lymphocyte-associated antigen. Studies based on these aspects showed the migration of DCs, monocytes, and macrophages (Schmid et al. 2016).

Dengue viruses comprise single-stranded genomic RNA (ssRNA) (Kuno et al. 1990) (approximately11 kb length) including a single open reading frame (ORF) for encoding large polyproteins, a capsid and three structural proteins followed by a glycoprotein-E and seven non-structural proteins such as NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 3) (Lindenbach 2007). Of which, NS5 is recognized as the largest one, approximately having 105 kDa with two domains including N-7, 2_-*O*-methyltransferase and guanylyl transferase (Issur et al. 2009) on the N-terminal for capping of the genome. The non-structural NS1 (47 kDa) has well-known significance in viral replication since all kinds of flaviviruses secrete it during the initial stages of infection (Young et al. 2000). The replication of the RNA genome has been fulfilled by RNA-dependent RNA polymerase (POL) activity of the C-terminal domain (Tan et al. 1996). The binding of DENV to host cell receptors has resulted in endocytosis, and the infected person is viremic for up to 8 days after the onset of severe symptoms. Enzyme-linked immunosorbent assay (ELISA) with specific NS1 glycoprotein monoclonal antibody (mAb) has been principally used to detect NS1 glycoprotein from dengue-infected individuals since the technique offers rapid results (Alcon et al. 2002).



Protein organisation and the full length DENV genome. The open reading frames of DENV encodes 3 stratural proteins: Caspid (C), Precursor membrane (prM), and envelope (E), with 7 nonstrutural (NS). The UTR indicates the untranslated region.

Fig. 3 Structure of DENV

4 Molecular trends and virus detection and pathogenicity

4.1 Molecular diagnostic tools for dengue virus infection

The isolation and characterization can make the diagnosis of dengue virus infection in laboratories of the virus, identification of dengue virus-specific antibodies, and characterization of the genomic sequence using nucleic acid amplification technology assay (Fig. 4) (Kouri 1996). The current diagnostic methods, including RT-PCR and ELISA (enzyme-linked immunosorbent assay), necessitate well-developed laboratories and trained experts (Rebello et al. 2019b). Furthermore, the transport of frozen sample serum from remote areas to laboratories has been renowned as a challenging problem in most tropical regions with poor laboratory circumstances. Numerous attempts have been made, and convenient, affordable, disposable, and easily accessible techniques have been offered for the detection of dengue infections (Loureiro et al. 2017). The isolation of viruses through cell lines from plasma samples, including acute-phase serum, has remained as the "gold standard approach," although it has exhibited the drawback that more than 7 days is necessary to accomplish the process. In general, some of the available strategies like the HI-test have become less popular in the current scenario and were replaced by new inventions since the HI test possessed inherent disadvantages (Shu and Huang 2004).

The serological tests for the dengue virus-specific antibody detection were completely based on the advent of the immune response from the host after the beginning of fever within 5–6 days. Likewise, the isolation of the virus from the infected serum sample usually requires at least seven days for incubation together with the screening of the virus. The nucleic acid-based methods usually offer specific detection of dengue virus serotypes within a short time and are progressively substituting the culture and serological techniques (Fig. 5). The aforementioned techniques include real-time RT-PCR (Lo et al. 2007; Rebello et al. 2018), nested RT-PCR (Lanciotti et al. 1992), nucleic acid sequence-based amplification (Parida et al. 2005; Anoopkumar et al. 2020d), Taqman assay (Kong et al. 2006), and loop-mediated isothermal amplification (Parida et al. 2005).

The primary step for detecting and identifying different serotypes of dengue virus from the infected individual using the molecular approach is the extraction and isolation of viral RNA from the patient serum using a specific and easily accessible purification kit for viral RNA. The M-MLV reverse transcriptase and dengue serotype-specific primers permit the viral RNA to transcribe into complementary DNA

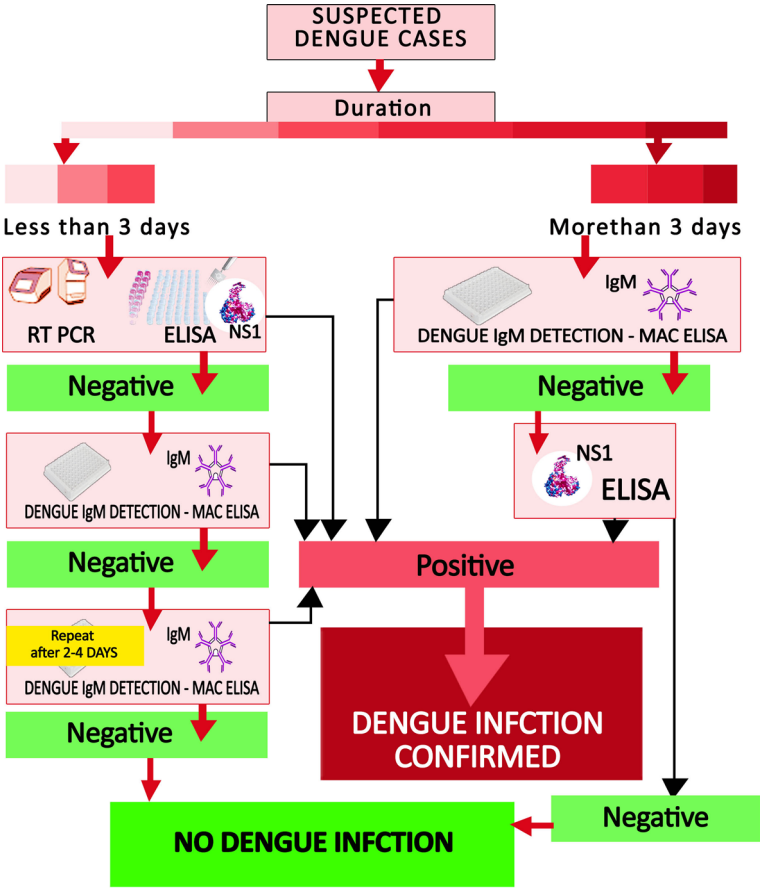


Fig. 4 Mode of laboratory diagnosis of dengue virus infection in suspected individuals

(cDNA). The transcribed complementary DNA (cDNA) was then amplified using polymerase chain reaction (PCR) (Chutinimitkul et al. 2005), specifically by using the SYBR Green dye since the SYBR Green expose strong affinity towards dsDNA minor grooves. The dsDNA products amplified during the PCR process have been detected using the compounds' fluorescence during binding. Hence, this method is employed to assess the early level of viral RNA (Shu et al. 2003). The RT-LAMP assay has been used as a sensitive approach to diagnose and distinguish the dengue virus serotypes since it offers high sensitivity and can amplify 10^9 copies per hour (Lau et al. 2015). The noticeable color change of hydroxylnaphthol blue to sky blue from violet color has been recognized as the principal indicator of the genome amplification process. In addition to this, the diagnosis of dengue virus infection was also accompanied by using specific serological tests. IgG and IgM, followed by NS1 antigen, have been used for the aforementioned serological tests (Lau et al. 2015).

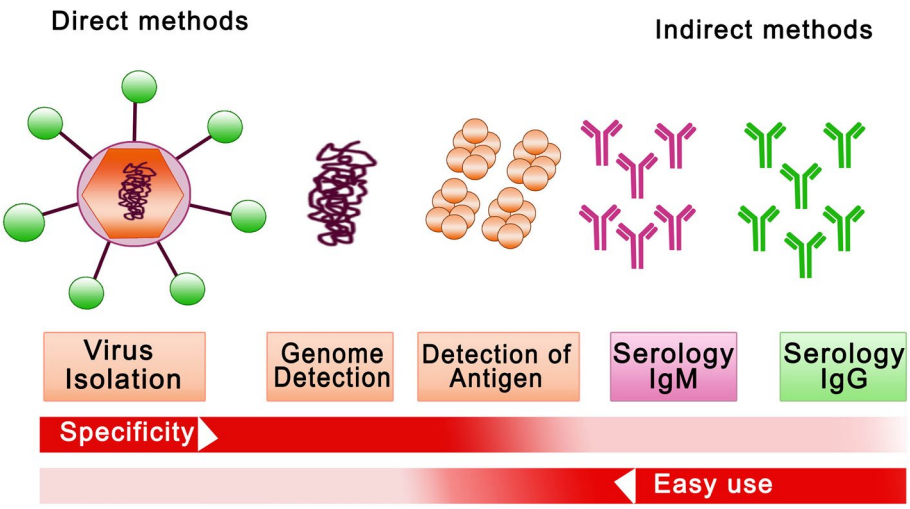


Fig. 5 Various dengue diagnostic techniques and their clinical applications

4.2 Advances in the dengue virus serotyping

In 2014, a distinctive DENV strain was successively isolated from an individual who had displayed specific symptoms reliable with the dengue virus infection. The Bayesian evolutionary phylogenetic examination revealed that Brun 2014 characterizes a novel, greatly divergent genotype (DENV-1), usually designated as VI serotype. It has been mentioned previously that the dengue virus serotype I has diverged from different strains of human beings (100–200 years ago) in Asia and the Americas (Lau et al. 2015). The genetically diverged strains' innovation specifies that the different dengue virus serotypes were changed promptly since it is characterized as an RNA virus (single-stranded). The definitive laboratory diagnosis of dengue virus has relied on the detection of virus-encoded antigens, virus-impelled antibodies, infective virus serotype, and viral genomic RNA (Kao et al. 2005). It has been shown that PCR-based diagnosis approaches are more sensitive than other frequently employed techniques, including viral culture, and it necessitates less time to complete each stage of the detection, as for the reason that the PCR-based approaches are capable of detecting the dengue virus serotypes (DENV1–DEN-4) in the acute phase of the ailment. The viral culture-based approaches usually require more than seven days for diagnosis, and sometimes, the low sensitivity of isolation has been reported.

The virus culture-based diagnostic approaches typically accessed the inoculation of plasma, serum, and buffy coat. In fatal cases, the autopsy tissues have been taken from the liver, spleen, lymph nodes, and thymus for the isolation of the dengue virus. The identification of dengue virus serotype is accompanied by immunofluorescence with specific monoclonal antibodies, and this method is partially restricted in endemic countries since it necessitates spacious laboratory, sophisticated safety measures together with labour-intensive, adequate professional trainers and much more time than other advanced approaches (Rosen and Gubler 1974). During the beginning of the 1980s, various methods including indirect immunofluorescent antibody tests (IFAT), complement fixation tests, hemagglutination inhibition (HI), dot-blot assays, plaque reduction neutralization tests, Western blotting test, and IgM and IgG antibody-capture ELISA have been used for the determination

of dengue viruses. The cross-reactivity (Henchal et al. 1991) of antibodies against certain flaviviruses (E protein) has been primarily considered as a challenging factor for the precise, specific, and rapid diagnosis of dengue fever infection in certain regions of the world where the extensive outbreak of Japanese encephalitis, yellow fever, and Zika virus infection has been observed. For instance, in Brazil, the characterization of dengue viruses using serological methods has been specifically problematic and complicated in recent epidemics (Muller et al. 2017).

The burden of DENV infection together with socioeconomic impacts remains unabated since the antigenically distinct different serotypes of virus extend into new regions and generated the endemic and hyperendemic provinces all over the world (Roth et al. 2014). The dengue virus infection by distinct serotypes has been shown to generate protective monotypic immunity including the formation of dengue-specific immunoglobulin G (IgG) and dengue immunoglobulin M (IgM). The two pathologic mechanisms have been used by this immunoglobulin G (IgG) and dengue immunoglobulin M (IgM) antibodies to target the severe DENV infection enhancement and virus neutralization. The susceptible persons mount an explicit immune response together with disease severity after the dengue virus infection, and the persons infected with secondary infections are more probable to evolve serious symptoms compared to the persons who are exposed to primary infections (Halstead 2007).

The ELISA-based IgM method developed by Bundo and Igarashi (1985) has been recognized as an ideal diagnostic approach for the surveillance of the dengue infection since it can productively distinguish Japanese encephalitis from dengue virus infection; however, it shows several disadvantages including the specificity. Another drawback of the serological detection approaches is that it is difficult to recognize the serotype of the virus since the secondary infection shows the "original antigenic sin" (Halstead 1988) phenomenon. This has increased the importance of PCR-based approaches in virus detection as a highly sensitive approach that uses plasma or human serum for the diagnosis of dengue virus infection. Besides, the two-step nested RT-PCR (Harris et al. 1998) and one-step multiplex RT-PCR (Morita et al. 1991) is well known for serotyping as well as detection of dengue virus.

Yong et al. 2007 compared the real-time SYBR Green RT-PCR technique with multiplex RT-PCR assay for their sensitivity and specificity towards DENV1–DENV-4. They concluded that the multiplex RT-PCR offers precision and accuracy together with speed with special reference to the epidemiological surveillance. Moreover, the TaqMan one-step RT-PCR is another kind of RT-PCR, which can have the ability to increase the sequence specificity of one-step SYBR Green I-based RT-PCR assay. The specificity, high amplification efficiency, and sensitivity made the RT-PCR and RT-LAMP assays convenient methods for detecting viral nucleic acid (Sahni et al. 2013). The serotype-specific RT-PCR has been employed as a suitable technique for the determination and characterization of DENV-2 in previous studies (Lanciotti et al. 1992). The immunochromatography assay for NS1 antigen has been employed as a very effective, sensitive, and less time-consuming (15 min for one test) diagnostic approach for the detection of viral antigens. Epidemiological perceptions (Gupta et al. 2015), together with mosquito vector control, are the accessible measures for the control of dengue fever infections.

The epidemiological perception of the dengue virus (DENV-1 to DENV-5) uses genomic evidence on the infecting virus strains. So, this review article will help the public health system create a comprehensive virological picture in terms of clinical aspects of dengue outbreaks from various regions all over the world. For understanding dengue outbreaks, we use genomic analysis as a powerful tool in virology studies. The nucleotide sequences of different strains of DENV from various endemic provinces all over the world

were retrieved using the NCBI database (last accessed 27-04-2019) and analyzed using the neighbor-joining method. The vast diversity of dengue virus strains over the endemic regions (Table 1) of the worlds with special inference on their genetic characteristics has been displayed in Fig. 6. Genetic recombination and spatiotemporal elements in evolutionary aspects of DENV-2 are significant in present and future studies since it possesses six different genotypes including American/Asian, Cosmopolitan, Asian I, Asian II, Sylvatic and American (Twiddy et al. 2002). The comparative studies concerning the genomic aspects to infer the genetic characterization of DENV-2 have been found to be predominantly imperative in the current studies since the spatiotemporal and evolutionary factors might strongly influence the emergence of diversifying lineages (Waman et al. 2016). Suleman et al. (2016) have reported that the detection approach based on NS1 antigen offers high sensitivity than the IgM-ELISA and RT-PCR during the early stage and can be used as a greatly suitable diagnostic tool (Suleman et al. 2016). The IgM and IgG serology with NS1 antigen capture have provided better positive results than negative results.

The updated commercial approaches with immunochromatographic strip method together with NS1 antigen-capture ELISA have been intended to be highly explicit without perceptible cross-reactivity (Muller et al. 2017) towards NS1 species. Tesfaye Gelanew and Elizabeth Hunsperger recently developed a serotype-specific monoclonal antibodies approach using a small ubiquitin-like modifier (SUMO*) cloning vector against the DENV-4 (Gelanew and Hunsperger 2018). This recent approach is specific to DENV-4, and it can be used to detect and characterize dengue virus serotype for the infected patients worldwide. The accuracy, sensitivity, and serotype specificity of this method have been assessed using (1) the supernatant collected from the DENV-1 to DENV-4 infected cell cultures, (2) rNS1s of DENV-1 to DENV-4, (3) rNS1s of West Nile virus (WNV) and yellow fever virus (YFV). The next-generation sequencing strategy and indirect immunofluorescent assay (IFA) can be used to isolate the whole genome of DENV serotype 3 (DENV-3) from the cerebrospinal fluid (CSF) of the dengue-infected patient (Dhenni et al. 2018). Recent studies indicate that the RT-PCR-based approaches offer accuracy and precision than conventional strategies for the rapid diagnosis of dengue virus infection together with its serotyping.

The recent developments with special reference to DNA sequencing have improved the way we think regarding mosquito-borne diseases' molecular diagnostics. Yamagishi et al. (2017) reported a novel, well-developed method using a nano-pore-type portable DNA sequencer to detect and characterize the tropical disease pathogens, especially from the serum samples of dengue-infected individuals. They combined MinION sequencer and LAMP to identify and characterize the dengue virus serotypes from DENV1–DENV4. This method will become an indispensable approach in providing substantial evidence for the etiological analysis concerning the provincial diversification of pathogens that can cause dengue infection. In addition, this method will definitely boost the impact on clinical practices over developing nations in the future.

4.2.1 An overview on comparative analysis of diagnostic approaches

The virus isolation strategies may comparatively expose certain following limitations such as (1) the specimen should be collected during the period of viremia, (2) appropriate handling and fast delivery of the collected specimen to the laboratory are necessitated since DENV virus particle is more prone to heat and (3) sensitivity depends on the timing of collection, storage, and transport of specimen. For short-term storage up to 24 h, the

Table 1 Diverse dengue virus strains reported from the various regions over the globe

S. no	Strain	Accession	Protein ID	Note	Date and year	Country
1	DENV1	GenBank: KY672931.1	ARO78342.1	Genotype: I	20-FEB-2019	China
2		GenBank: MH279620.1	QBO23646.1	Contains prM/E/NS1 protein	24-MAR-2019	French Guiana
3		GenBank: KY672932.1	ARO78343.1	Genotype: I	20-FEB-2019	China
4		GenBank: KY672934.1	ARO78345.1	Genotype: I	20-FEB-2019	China
5		GenBank: KY672933.1	ARO78344.1	Genotype: I	20-FEB-2019	China
6		GenBank: KY672935.1	ARO78346.1	Genotype: I	20-FEB-2019	China
7		GenBank: KY672941.1	ARO78352.1	Genotype: I	20-FEB-2019	China
8		GenBank: KY672943.1	ARO78354.1	Genotype: I	20-FEB-2019	China
9		GenBank: KY672944.1	ARO78355.1	Genotype: III	20-FEB-2019	China
10		GenBank: MK024375.1	QBA36582.1	Product = non-structural protein NS5	10-FEB-2019	Italy
11		GenBank: MH051271.1	AZL40855.1	Capsid-premembrane junction region; CprM	16-DEC-2018	India
12		GenBank: MH051269.1	AZL40853.1	Capsid-premembrane junction region; CprM	16-DEC-2018	India
13	DENV 2	GenBank: KY672945.1	ARO78356.1	Genotype: Asian I	20-FEB-2019	China
14		GenBank: KY672946.1	ARO78357.1	Genotype: Asian I	20-FEB-2019	China
15		GenBank: KY672947.1	ARO78358.1	genotype: Asian I	20-FEB-2019	China
16		GenBank: MH781013.1	QBQ87236.1	genotype: Asian/American	03-APR-2019	Mexico
17		GenBank: MH781014.1	QBQ87237.1	Asian/American	03-APR-2019	Mexico
18		GenBank: MK328810.1	QBA83314.1	Anchored capsid protein C	12-FEB-2019	India
19		GenBank: MG721054.1	AYJ72757.1	Sequencing from serum (without culture)	01-JAN-2019	India
20		GenBank: MH822939.1	AYJ72763.1	Strain = cosmopolitan	31-DEC-2018	India
21		GenBank: MK328814.1	QBA83318.1	Anchored capsid protein C	12-FEB-2019	India
22		GenBank: MH822942.1	AYJ72766.1	Strain = cosmopolitan	31-DEC-2018	India
23		GenBank: MH822945.1	AYJ72769.1	Strain = cosmopolitan	31-DEC-2018	India
24		GenBank: JQ045686.1	AFD28530.1	Polyprotein	19-MAR-2012	Singapore
25	DENV3	GenBank: MK040425.1	QBT58293.1	Polyprotein	10-APR-2019	Colombia
26		GenBank: MH734370.1	QBR34790.1	Polyprotein	06-APR-2019	India
27		GenBank: MH734371.1	QBR34791.1	Polyprotein	06-APR-2019	India

Table 1 (continued)

S. no	Strain	Accession	Protein ID	Note	Date and year	Country
28		GenBank: KY673721.1	AVQ93632.1	Truncated polyprotein	25-MAR-2019	China
29		GenBank: MH622984.1	QBM00845.1	Polyprotein	19-MAR-2019	India
30		GenBank: MH623018.1	QBM00879.1	Polyprotein	19-MAR-2019	India
31		GenBank: MF682966.1	ASX95464.1	Polyprotein	27-OCT-2018	China
32		GenBank: MF598865.1	ASX95585.1	Envelope protein	31-JAN-2019	China
33		GenBank: MG450891.1	AXP19796.1	Envelope protein	10-SEP-2018	Malaysia
34		GenBank: MG450908.1	AXP19813.1	Envelope protein	10-SEP-2018	Malaysia
35		GenBank: MG895175.1	AXL67665.1	Envelope glycoprotein	17-AUG-2018	Taiwan
36		GenBank: MG895181.1	AXL67671.1	Envelope glycoprotein	17-AUG-2018	Taiwan
37		GenBank: MH544649.1	AXG22238.1	Genotype: II	31-JUL-2018	Colombia
38	DENV4	GenBank: MK514144.1	QBT58299.1	Polyprotein	10-APR-2019	Haiti
39		GenBank: MK426996.1	QBH74432.1	Anchored capsid protein C	06-MAR-2019	India
40		GenBank: MK427000.1	QBH74436.1	Anchored capsid protein C	06-MAR-2019	India
41		GenBank: MK450490.1	QBH74389.1	Membrane glycoprotein Precursor M	06-MAR-2019	Thailand
42		GenBank: MK040399.1	QBF29234.1	Envelope glycoprotein	25-FEB-2019	Puerto Rico
43		GenBank: MK268745.1	QBA83297.1	Polyprotein	12-FEB-2019	Philippines
44		GenBank: MK238003.1	QBA57634.1	polyprotein	11-FEB-2019	Viet Nam: DakLak
45		GenBank: MG450914.1	AXP19819.1	Envelope protein	10-SEP-2018	Malaysia
46		GenBank: KT750003.1	AOQ25527.1	Envelope protein	02-NOV-2017	Indonesia

The genetic information of the DENV was retrieved from the nucleotide database NCBI in 2019

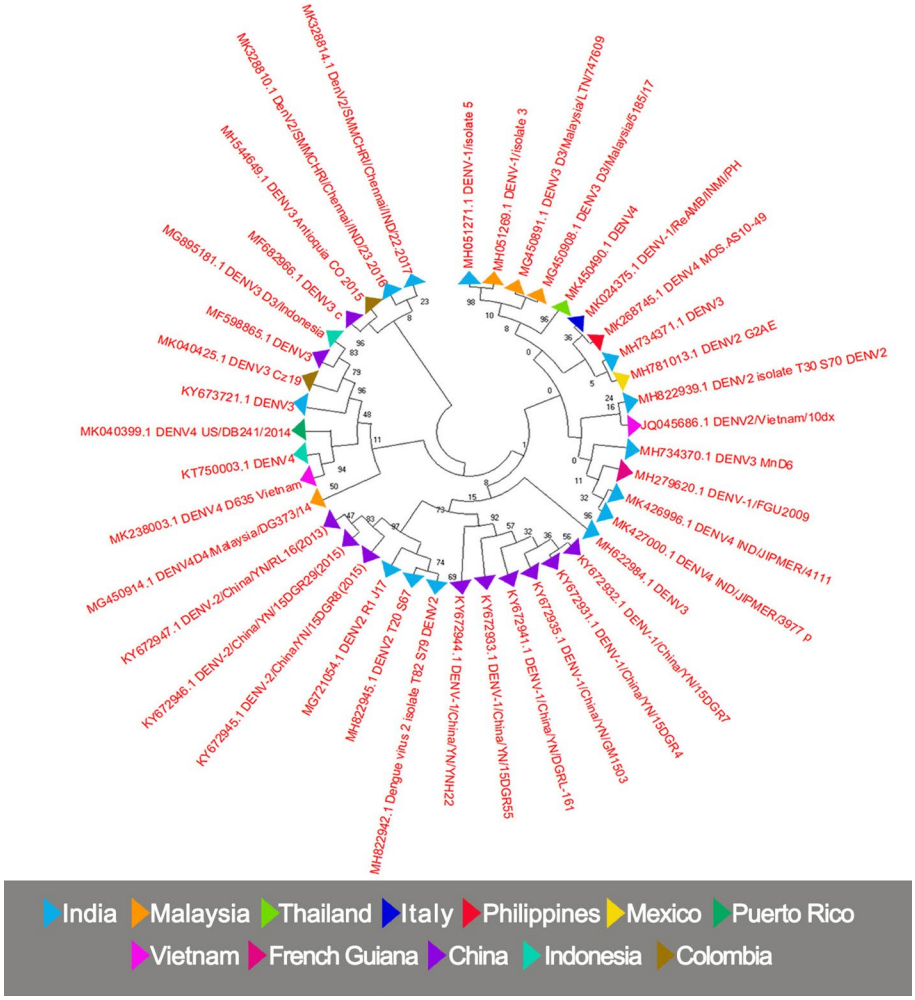


Fig. 6 Geographical distribution of dengue virus serotypes over the epidemic provinces of world. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 67.05552493 is shown. There were a total of 10,738 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

specimens should be kept at a particular temperature range (between +4 and +8 °C). The specimens should be frozen in a deep freezer (at − 70 °C) if long-term storage is required. Compared to the former method, the sensitivity of nucleic acid-based strategies varies from 80 to 100%. However, it also depends on the approach used to detect or amplify the PCR products, the region of the genome targeted by the primers, and the method used for subtyping. The nucleic acid-based strategies may also necessitate well-experienced professionals since any contamination may develop false-positive results. One of the major benefits of RT-PCR is the ability to analyze the viral titer in a sample that may be considered as a platform to investigate the pathogenicity.

Conventional strategies can also be used to verify the presence of a target nucleotide sequence in a given sample with the help of Southern blot, colorimetric enzyme-linked

immunosorbent assay, and agarose gel electrophoresis. In spite of being easy to perform, the conventional approaches expose limited inference in comparison to the versatile PCR-based strategies since the former increases the chance of risk in contamination, thereby leading to false-positive results. Several recent studies have reported the benefits of fully automatic real-time RT-PCR assays for dengue detection. Likewise, RT-LAMP is an excellent tool for dengue virus detection in terms of its sensitivity and specificity (87.5–100%). The ELISA-based methods may usually illustrate lower specificity. However, such situations can be managed by using the serum samples from both convalescent and acute phases. The hemagglutination inhibition (HI) methods have also exhibited a high degree of sensitivity. However, advanced developments in the dengue detection strategies have forced the scientific community to pull back such conventional strategies. The HI test may sometimes fail to distinguish dengue from closely related yellow fever and Japanese encephalitis flaviviruses. Certain immunochromatographic tests have also verified its prominent role in dengue detection. The major advantage of this method is that the result can be analyzed by virtue of color change within a short time that is visible to the naked eye. Most of all, the strategies have many advantages and disadvantages; hence challenges in developing specific and highly sensitive diagnostic methods should be addressed. More possibilities for development lie in uniting the current detecting strategies that could augment both specificity and sensitivity.

5 Conclusion

The insect-specific flavivirus detection rate in arthropod disease vectors, especially in mosquitoes, has rapidly increased through the availability of novel advanced sequencing technologies pooled with metagenomic concepts. Apart from new virus detection, molecular-based studies have become a strong approach for characterizing the viral populations of epidemiological significance. Dengue viral infections have emerged as the most common arboviral disease, representing the world's alacritous spreading vector-borne diseases. The enhanced rate of international travel and sophisticated global connectivity has escalated the risk of establishing and extending many mosquito-borne diseases. The incidence of dengue infections has been growing steadily in the tropics and subtropics of the world. For effective vector control actions and to diminish the incidence of dengue virus transmission, struggles should be focussed on the risk factors, specifically, the environmental factors. Despite the vector control strategies and the various molecular levels-based measures provided by the relevant agencies, most of the tropics and subtropics regions are still facing aggravating dengue crisis in the last few years. Given that the infection induces diverse clinical symptoms, precise and accurate laboratory diagnosis is vital for patient management.

Early detection of dengue fever in presumptive cases is challenging due to low levels of viremia. Delays in diagnosing dengue fever may hinder the scientific community and health professionals' ability to identify the outbreaks quickly. Early diagnosis using molecular procedures with high specificity contributes to proper dengue management, thereby reducing the increasing epidemic. The serological strategies (convalescent-phase and paired acute specimens) have been recognized as a base for dengue infection diagnosis, but the fact is that it confirms the infection only after the infected individual recover. Recently, advanced molecular strategies like RT-PCR could expedite and direct vector control interventions to combat dengue infections. The use of this greatly sensitive molecular strategy would permit rapid and epidemiological assessments that may turn into enhanced patients

care along with appropriate community interventions. There has been a rallying cry for the increased need to further improve the conventional strategies of vector control programmes and develop innovative and inventive approaches to prevent the occurrence and spread of mosquito-borne arboviruses.

Acknowledgements The authors would like to thank Principal, St. Joseph's College, Irinjalakuda, for the laboratory facilities provided. The author Embalil Mathachan Aneesh thanks University Grants Commission, Government of India (UGC Research Award—F30-6/20-16(SA-II)), and DBT (BT/IN/Indo-US/Foldscope/39/2015) for funding.

Funding The study was financed by UGC Research Award (F30-6/20-16(SA-II) and DBT Foldscope project (BT/IN/Indo-US/Foldscope/39/2015).

Compliance with ethical standards

Conflict of interest A. N. Anoopkumar and Embalil Mathachan Aneesh declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Alcon, S., Talarmin, A., Debruyne, M., Falconar, A., Deubel, V., & Flamand, M. (2002). Enzyme-linked immunosorbent assay specific to Dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. *Journal of Clinical Microbiology, 40*(2), 376–381. https://doi.org/10.1128/JCM.40.02.376-381.2002.

Anoopkumar, A. N., Aneesh, E. M., & Sudhikumar, A. V. (2020a). Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: A microbes based double-edged weapon to fight against Arboviral diseases. *International Journal of Tropical Insect Science*. https://doi.org/10.1007/s42690-020-00104-z.

Anoopkumar, A. N., Puthur, S., Rebello, S., & Aneesh, E. M. (2017a). Screening of a few traditionally used medicinal plants for their larvicidal efficacy against *Aedes aegypti* (Linn Diptera: Culicidae), a dengue fever vector. *SOJ Microbiology and Infectious Diseases*. https://doi.org/10.15226/sojmid/5/4/00181.

Anoopkumar, A. N., Puthur, S., Rebello, S., & Aneesh, E. M. (2019). Molecular characterization of *Aedes, Culex, Anopheles*, and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis. *Biologia*. https://doi.org/10.2478/s11756-019-00231-0.

Anoopkumar, A. N., Puthur, S., Varghese, P., Rebello, S., & Aneesh, E. M. (2017b). Life cycle, bio-ecology and DNA barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). *The Journal of Communicable Diseases*. https://doi.org/10.24321/0019.5138.201719.

Anoopkumar, A. N., Rebello, S., Aneesh, E. M., Sindhu, R., Binod, P., Pandey, A., & Gnansounou, E. (2020b). *Use of different enzymes in biorefinery systems. Biorefinery production technologies for chemicals and energy* (pp. 357–368). New York: Wiley.

Anoopkumar, A. N., Rebello, S., Devassy, E., Raj, K. K., Puthur, S., Aneesh, E. M., & Pandey, A. (2020c). Phytoextraction of heavy metals. In M. I. A. Inamuddin, E. Lichtfouse, & A. M. Asiri (Eds.), *Methods for bioremediation of water and wastewater pollution* (pp. 267–276). Cham: Springer.

Anoopkumar, A. N., Rebello, S., Sudhikumar, A. V., Puthur, S., & Aneesh, E. M. (2020d). A novel intervention on the inhibiting effects of *Catunaregam spinosa* induced free radical formation and DNA damage in Aedes aegypti (Diptera: Culicidae): A verdict for new perspectives on microorganism targeted vector control approach. *International Journal of Tropical Insect Science, 40,* 989–1002. https://doi.org/10.1007/s42690-020-00157-0.

Anwar, A., Khan, N., Ayub, M., Nawaz, F., Shah, A., & Flahault, A. (2019). Modeling and predicting dengue incidence in highly vulnerable countries using panel data approach. *International Journal of Environmental Research and Public Health, 16*(13), 2296.

Barban, V., Mantel, N., De Montfort, A., Pagnon, A., Pradezynski, F., Lang, J., et al. (2018). Improvement of the dengue virus (DENV) nonhuman primate model via a reverse translational approach based on dengue vaccine clinical efficacy data against DENV-2 and-4. *Journal of Virology, 92*(12), e00440-e1418. https://doi.org/10.1128/JVI.00440-18.

Brito, C. A., Sobreira, S., Cordeiro, M. T., & Lucena-Silva, N. (2007). Acute disseminated encephalomyelitis in classic dengue. *Revista da Sociedade Brasileira de Medicina Tropical, 40*(2), 236–238. https://doi.org/10.1590/s0037-86822007000200019.

Bundo, K., & Igarashi, A. (1985). Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. *Journal of Virological Methods, 11*(1), 15–22. https://doi.org/10.1016/0166-0934(85)90120-x.

Cam, B., Fonsmark, L., Hue, N., Phuong, N., Poulsen, A., & Heegaard, E. (2001). Prospective case-control study of encephalopathy in children with dengue hemorrhagic fever. *The American Journal of Tropical Medicine and Hygiene, 65*(6), 848–851. https://doi.org/10.4269/ajtmh.2001.65.848.

Chutinimitkul, S., Payungporn, S., Theamboonlers, A., & Poovorawan, Y. (2005). Dengue typing assay based on real-time PCR using SYBR green I. *Journal of Virological Methods, 129*(1), 8–15. https://doi.org/10.1016/j.jviromet.2005.05.006.

Clyde, K., Kyle, J. L., & Harris, E. (2006). Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. *Journal of Virology, 80*(23), 11418–11431. https://doi.org/10.1128/JVI.01257-06.

Colón-González, F. J., Lake, I. R., & Bentham, G. (2011). Climate variability and dengue fever in warm and humid Mexico. *The American Journal of Tropical Medicine and Hygiene, 84*(5), 757–763.

Dhenni, R., Karyanti, M. R., Putri, N. D., Yohan, B., Yudhaputri, F. A., Ma'roef, C. N., et al. (2018). Isolation and complete genome analysis of neurotropic dengue virus serotype 3 from the cerebrospinal fluid of an encephalitis patient. *PLoS Neglected Tropical Diseases, 12*(1), e0006198. https://doi.org/10.1371/journal.pntd.0006198.

Diallo, M., Ba, Y., Sall, A. A., Diop, O. M., Ndione, J. A., Mondo, M., et al. (2003). Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999–2000: Entomologic findings and epidemiologic considerations. *Emerging Infectious Diseases, 9*(3), 362. https://doi.org/10.3201/eid0903.020219.

Endy, T. P., Nisalak, A., Chunsuttitwat, S., Vaughn, D. W., Green, S., Ennis, F. A., et al. (2004). Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. *The Journal of Infectious Diseases, 189*(6), 990–1000. https://doi.org/10.1086/382280.

Gelanew, T., & Hunsperger, E. (2018). Development and characterization of serotype-specific monoclonal antibodies against the dengue virus-4 (DENV-4) nonstructural protein (NS1). *Virology Journal, 15*(1), 30. https://doi.org/10.1186/s12985-018-0925-7.

Gupta, B. P., Singh, S., Kurmi, R., Malla, R., Sreekumar, E., & Manandhar, K. D. (2015). Re-emergence of dengue virus serotype 2 strains in the 2013 outbreak in Nepal. *The Indian Journal of Medical Research, 142*(Suppl 1), S1. https://doi.org/10.4103/0971-5916.176564.

Gurukumar, K., Priyadarshini, D., Patil, J., Bhagat, A., Singh, A., Shah, P., et al. (2009). Development of real time PCR for detection and quantitation of Dengue viruses. *Virology Journal, 6*(1), 10. https://doi.org/10.1186/1743-422X-6-10.

Guzmán, M. G., & Kouri, G. (2002). Dengue: An update. *The Lancet Infectious Diseases, 2*(1), 33–42. https://doi.org/10.1016/S1473-3099(01)00171-2.

Halstead, S. B. (1988). Pathogenesis of dengue: Challenges to molecular biology. *Science, 239*(4839), 476–481. https://doi.org/10.1126/science.3277268.

Halstead, S. B. (2007). Dengue. *The Lancet, 370*(9599), 1644–1652. https://doi.org/10.1016/S0140-6736(07)61687-0.

Harris, E., Roberts, T. G., Smith, L., Selle, J., Kramer, L. D., Valle, S., et al. (1998). Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *Journal of Clinical Microbiology, 36*(9), 2634–2639.

Hashan, M. R., Ghozy, S., El-Qushayri, A. E., Pial, R. H., Hossain, M. A., et al. (2020). Association of dengue disease severity and blood group: A systematic review and meta-analysis. *Reviews in Medical Virology*. https://doi.org/10.1002/rmv.2147.

Hayes, E. B., & Gubler, D. J. (1992). Dengue and dengue hemorrhagic fever. *The Pediatric Infectious Disease Journal, 11*(4), 311–317.

Henchal, E. A., Polo, S. L., Vorndam, V., Yaemsiri, C., Innis, B. L., & Hoke, C. H. (1991). Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridization. *The American Journal of Tropical Medicine and Hygiene, 45*(4), 418–428. https://doi.org/10.4269/ajtmh.1991.45.418.

Issur, M., Geiss, B. J., Bougie, I., Picard-Jean, F., Despins, S., Mayette, J., et al. (2009). The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure. *RNA (New York, N.Y.), 15*(12), 2340–2350. https://doi.org/10.1261/rna.1609709.

Istúriz, R. E., Gubler, D. J., & del Castillo, J. B. (2000). Dengue and dengue hemorrhagic fever in Latin America and the Caribbean. *Infectious Disease Clinics, 14*(1), 121–140. https://doi.org/10.1016/s0891-5520(05)70221-x.

Jha, S., & Ansari, M. (2010). Dengue infection causing acute hypokalemic quadriparesis. *Neurology India, 58*(4), 592. https://doi.org/10.4103/0028-3886.68657.

Kalayanarooj, S., & Nimmannitya, S. (2004). *Guidelines for dengue hemorrhagic fever case management* (p. 458). Bangkok: Bangkok Medical Publisher. Dengue/DHF/DSS, Bangkok, Thailand.

Kamgang, B., Nchoutpouen, E., Simard, F., & Paupy, C. (2012). Notes on the blood-feeding behavior of *Aedes albopictus* (Diptera: Culicidae) in Cameroon. *Parasites and Vectors, 5*(1), 57. https://doi.org/10.1186/1756-3305-5-57.

Kang, D. S., Barron, M. S., Lovin, D. D., Cunningham, J. M., Eng, M. W., Chadee, D. D., et al. (2018). A transcriptomic survey of the impact of environmental stress on response to dengue virus in the mosquito *Aedes aegypti*. *PLoS Neglected Tropical Diseases, 12*(6), e0006568. https://doi.org/10.1371/journal.pntd.0006568.

Kao, C.-L., King, C.-C., Chao, D.-Y., Wu, H.-L., & Chang, G. (2005). Laboratory diagnosis of dengue virus infection: Current and future perspectives in clinical diagnosis and public health. *Journal of Microbiology, Immunology, and Infection, 38*(1), 5–16.

Kesetyaningsih, T. W., Andarini, S., Sudarto, S., & Pramoedyo, H. (2018). Determination of environmental factors affecting dengue incidence in Sleman District, Yogyakarta Indonesia. *African Journal of Infectious Diseases, 12*(1S), 13–25. https://doi.org/10.2101/Ajid.12v1S.3.

Kien, N. D., El-Qushayri, A. E., Ahmed, A. M., Safi, A., Mageed, S. A., Mehyar, S. M., et al. (2020). Association of allergic symptoms with dengue infection and severity: A systematic review and meta-analysis. *Virologica Sinica, 35*(1), 83–92.

Kong, Y. Y., Thay, C. H., Tin, T. C., & Devi, S. (2006). Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. *Journal of Virological Methods, 138*(1–2), 123–130. https://doi.org/10.1016/j.jviromet.2006.08.003.

Kouri, G. (1996). Advances in dengue diagnosis. *Clinical and Diagnostic Laboratory Immunology, 3,* 621–627. https://doi.org/10.1128/CDLI.11.4.642-650.2004.

Kunishige, M., Mitsui, T., Tan, B., Leong, H., Takasaki, T., Kurane, I., et al. (2004). Preferential gray matter involvement in dengue myelitis. *Neurology, 63*(10), 1980–1981. https://doi.org/10.1212/01.WNL.0000144194.29643.D0.

Kuno, G., Vorndam, A., Gubler, D., & Gomez, I. (1990). Study of anti-dengue NS1 antibody by Western blot. *Journal of Medical Virology, 32*(2), 102–108. https://doi.org/10.1002/jmv.1890320207.

Lanciotti, R. S., Calisher, C. H., Gubler, D. J., Chang, G.-J., & Vorndam, A. V. (1992). Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology, 30*(3), 545–551.

Lau, Y.-L., Lai, M.-Y., Teoh, B.-T., Abd-Jamil, J., Johari, J., Sam, S.-S., et al. (2015). Colorimetric detection of dengue by single tube reverse-transcription-loop-mediated isothermal amplification. *PLoS ONE, 10*(9), e0138694. https://doi.org/10.1371/journal.pone.0138694.

Lee, J.-S., & Farlow, A. (2019). The threat of climate change to non-dengue-endemic countries: Increasing risk of dengue transmission potential using climate and non-climate datasets. *BMC Public Health, 19*(1), 934.

Li, G.-H., Ning, Z.-J., Liu, Y.-M., & Li, X.-H. (2017). Neurological manifestations of dengue infection. *Frontiers in Cellular and Infection Microbiology, 7,* 449. https://doi.org/10.3389/fcimb.2017.00449.

Ligon, B. L. (2005). Dengue fever and dengue hemorrhagic fever: A review of the history transmission treatment and prevention. In *Seminars in pediatric infectious diseases* (Vol. 16, pp. 60–65, Vol. 1). https://doi.org/https://doi.org/10.1053/j.spid.2004.09.013.

Lin, C.-H., Schiøler, K. L., Jepsen, M. R., Ho, C.-K., Li, S.-H., & Konradsen, F. (2012). Dengue outbreaks in high-income area, Kaohsiung City, Taiwan, 2003–2009. *Emerging Infectious Diseases, 18*(10), 1603. https://doi.org/10.3201/eid1810.111929.

Lindenbach, B. D. (2007). The viruses and their replication. In D. M. Knipe & P. M. Howley (Eds.), *Fields virology* (5th ed., pp. 1101–1152). Philadelphia: Lippincott-Raven Publishers.

Liou, L.-M., Lan, S.-H., & Lai, C.-L. (2008). Dengue fever with ischemic stroke: A case report. *The Neurologist, 14*(1), 40–42. https://doi.org/10.1097/NRL.0b013e3180d0a391.

Lo, C. L., Yip, S. P., Cheng, P. K., To, T. S., Lim, W. W., & Leung, P. H. (2007). One-step rapid reverse transcription–PCR assay for detecting and typing dengue viruses with GC tail and induced fluorescence

resonance energy transfer techniques for melting temperature and color multiplexing. *Clinical Chemistry, 53*(4), 594–599. https://doi.org/10.1373/clinchem.2006.077446.

Loureiro, F. C., Neff, H., Melcher, E. U., Roque, R. A., de Figueiredo, R. M., Thirstrup, C., et al. (2017). Simplified immunoassay for rapid Dengue serotype diagnosis, revealing insensitivity to non-specific binding interference. *Sensing and Bio-Sensing Research, 13,* 96–103. https://doi.org/10.1016/j.sbsr.2016.10.002.

Ly, M. H. P., Moi, M. L., Vu, T. B. H., Tun, M. M. N., Saunders, T., Nguyen, C. N., et al. (2018). Dengue virus infection-enhancement activity in neutralizing antibodies of healthy adults before dengue season as determined by using FcγR-expressing cells. *BMC Infectious Diseases, 18*(1), 31. https://doi.org/10.1186/s12879-017-2894-7.

Mamdouh, K. H., Mroog, K. M., Hani, N. H., & Nabil, E. M. (2013). Atypical dengue meningitis in Makkah, Saudi Arabia with slow resolving, prominent migraine like headache, phobia, and arrhythmia. *Journal of Global Infectious Diseases, 5*(4), 183. https://doi.org/10.4103/0974-777X.122021.

Martina, B. E., Koraka, P., & Osterhaus, A. D. (2009). Dengue virus pathogenesis: An integrated view. *Clinical Microbiology Reviews, 22*(4), 564–581. https://doi.org/10.1128/CMR.00035-09.

Mata, V. E., Ferreira, C. A., de Andrade, S., Passos, R. L., Hökerberg, Y. H. M., Fukuoka, L. V. B., et al. (2020). Rapid immunochromatographic tests for the diagnosis of dengue: A systematic review and meta-analysis. *Cadernos de Saúde Pública, 36,* e00225618.

Messer, W. B., Gubler, D. J., Harris, E., Sivananthan, K., & De Silva, A. M. (2003). Emergence and global spread of a dengue serotype 3, subtype III virus. *Emerging Infectious Diseases, 9*(7), 800. https://doi.org/10.3201/eid0907.030038.

Misra, U., Kalita, J., Syam, U., & Dhole, T. (2006). Neurological manifestations of dengue virus infection. *Journal of the Neurological Sciences, 244*(1–2), 117–122. https://doi.org/10.3389/fcimb.2017.00449.

Morita, K., Tanaka, M., & Igarashi, A. (1991). Rapid identification of dengue virus serotypes by using polymerase chain reaction. *Journal of Clinical Microbiology, 29*(10), 2107–2110.

Muller, D. A., Depelsenaire, A. C., & Young, P. R. (2017). Clinical and laboratory diagnosis of dengue virus infection. *The Journal of Infectious Diseases, 215*(Supply_2), S89–S95. https://doi.org/10.1093/infdis/jiw649.

Murray, N. E. A., Quam, M. B., & Wilder-Smith, A. (2013). Epidemiology of dengue: Past, present and future prospects. *Clinical Epidemiology, 5,* 299. https://doi.org/10.2147/CLEP.S34440.

Mustafa, M., Rasotgi, V., Jain, S., & Gupta, V. (2015). Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. *Medical Journal Armed Forces India, 71*(1), 67–70. https://doi.org/10.1016/j.mjafi.2014.09.011.

Muturi, E. J., & Alto, B. W. (2011). Larval environmental temperature and insecticide exposure alter *Aedes aegypti* competence for arboviruses. *Vector-Borne and Zoonotic Diseases, 11*(8), 1157–1163. https://doi.org/10.1089/vbz.2010.0209.

Paliwal, V. K., Garg, R. K., Juyal, R., Husain, N., Verma, R., Sharma, P. K., et al. (2011). Acute dengue virus myositis: A report of seven patients of varying clinical severity including two cases with severe fulminant myositis. *Journal of the Neurological Sciences, 300*(1–2), 14–18. https://doi.org/10.1016/j.jns.2010.10.022.

Parida, M., Horioke, K., Ishida, H., Dash, P. K., Saxena, P., Jana, A. M., et al. (2005). Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay. *Journal of Clinical Microbiology, 43*(6), 2895–2903. https://doi.org/10.1128/JCM.43.6.2895-2903.2005.

Patey, O., Ollivaud, L., Breuil, J., & Lafaix, C. (1993). Unusual neurologic manifestations occurring during dengue fever infection. *The American Journal of Tropical Medicine and Hygiene, 48*(6), 793–802. https://doi.org/10.4269/ajtmh.1993.48.793.

Pino, L., Ma, J., Ale, F., Paredes, E. D. P. R., Kuno, G., & Gubler, D. (1993). Epidemic dengue 4 in the Yucatan, Mexico. *Revista do instituto de medicina tropical de Sao Paulo, 35,* 449–455. https://doi.org/10.1590/S0036-46651993000500011.

Puthur, S., Anoopkumar, A. N., Rebello, S., & Aneesh, E. M. (2018). *Hypericum japonicum*: A double-headed sword to combat vector control and cancer. *Applied Biochemistry and Biotechnology*. https://doi.org/10.1007/s12010-018-2713-7.

Puthur, S., Anoopkumar, A. N., Rebello, S., & Aneesh, E. M. (2019). Synergistic control of storage pest rice weevil using Hypericum japonicum and deltamethrin combinations: A key to combat pesticide resistance. *Environmental Sustainability*. https://doi.org/10.1007/s42398-019-00086-w.

RathoreSt. John, A. P. A. L. (2018). Immune responses to dengue virus in the skin. *Open Biology, 8*(8), 180087. https://doi.org/10.1098/rsob.180087.

Rebello, S., Anoopkumar, A. N., Aneesh, E. M., Sindhu, R., Binod, P., Kim, S. H., & Pandey, A. (2020b). Hazardous minerals mining: Challenges and solutions. *Journal of Hazardous Materials, 402,* 123474.

Rebello, S., Anoopkumar, A., Aneesh, E. M., Sindhu, R., Binod, P., & Pandey, A. (2019a). Sustainability and life cycle assessments of lignocellulosic and algal pretreatments. *Bioresource Technology*. https://doi.org/10.1016/j.biortech.2019.122678.

Rebello, S., Anoopkumar, A. N., Puthur, S., Sindhu, R., Binod, P., Pandey, A., et al. (2018). Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. *Bioresource Technology Reports, 3,* 1–6. https://doi.org/10.1016/j.biteb.2018.05.007.

Rebello, S., Anoopkumar, A. N., Sindhu, R., Binod, P., Pandey, A., & Aneesh, E. M. (2020a). Comparative life-cycle analysis of synthetic detergents and biosurfactants—An overview. In R. P. Kumar, E. Gnansounou, J. K. Raman, & G. Baskar (Eds.), *Refining biomass residues for sustainable energy and bioproducts* (pp. 511–521). London: Academic Press.

Rebello, S., Balakrishnan, D., Anoopkumar, A.N, Sindhu, R., Binod, P., Pandey, A., et al. (2019b). Industrial enzymes as feed supplements—advantages to nutrition and global environment. In B. Parameswaran, S. Varjani, & S. Raveendran (Eds.), *Green bio-processes* (pp. 293–304). Berlin: Springer.

Rosen, L., & Gubler, D. (1974). The use of mosquitoes to detect and propagate dengue viruses. *The American Journal of Tropical Medicine and Hygiene, 23*(6), 1153–1160. https://doi.org/10.4269/ajtmh.1974.23.1153.

Roth, A., Mercier, A., Lepers, C., Hoy, D., Duituturaga, S., Benyon, E., et al. (2014). Concurrent outbreaks of dengue, chikungunya and Zika virus infections—An unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012–2014. *Euro Surveillance: Bulletin Europeen sur les Maladies Transmissibles=European Communicable Disease Bulletin, 19*(41), 20929.

Rush, B. (1951). An account of the bilious remitting fever: As it appeared in philadelphia, in the summer and autumn of the year 1780. *The American Journal of Medicine, 11*(5), 546–550. https://doi.org/10.1016/0002-9343(51)90035-6.

Sahni, A. K., Grover, N., Sharma, A., Khan, I. D., & Kishore, J. (2013). Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for diagnosis of dengue. *Medical Journal Armed Forces India, 69*(3), 246–253. https://doi.org/10.1016/j.mjafi.2012.07.017.

Saini, L., Chakrabarty, B., Pastel, H., Israni, A., Kumar, A., & Gulati, S. (2017). Dengue fever triggering hemiconvulsion hemiplegia epilepsy in a child. *Neurology India, 65*(3), 636. https://doi.org/10.4103/neuroindia.NI_1367_15.

Schmid, M. A., Glasner, D. R., Shah, S., Michlmayr, D., Kramer, L. D., & Harris, E. (2016). Mosquito saliva increases endothelial permeability in the skin, immune cell migration, and dengue pathogenesis during antibody-dependent enhancement. *PLoS Pathogens, 12*(6), e1005676. https://doi.org/10.1371/journal.ppat.1005676.

Seet, R.C.-S., & Lim, E.C.-H. (2007). Dysarthria-clumsy hand syndrome associated with dengue type-2 infection. *Journal of Neurology, 254*(8), 1129–1130. https://doi.org/10.1371/journal.ppat.1005676.

Sherif, N. A., Zayan, A. H., Elkady, A. H., Ghozy, S., Ahmed, A. R., Omran, E. S., et al. (2020). Mast cell mediators in relation to dengue severity: A systematic review and meta-analysis. *Reviews in Medical Virology, 30*(1), e2084.

Shu, P.-Y., Chang, S.-F., Kuo, Y.-C., Yueh, Y.-Y., Chien, L.-J., Sue, C.-L., et al. (2003). Development of group-and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus. *Journal of Clinical Microbiology, 41*(6), 2408–2416. https://doi.org/10.1128/jcm.41.6.2408-2416.2003.

Shu, P.-Y., & Huang, J.-H. (2004). Current advances in dengue diagnosis. *Clinical and Diagnostic Laboratory Immunology, 11*(4), 642–650. https://doi.org/10.1128/CDLI.11.4.642-650.2004.

Solbrig, M. V., & Perng, G.-C. (2015). Current neurological observations and complications of dengue virus infection. *Current Neurology and Neuroscience Reports, 15*(6), 29. https://doi.org/10.3389/fcimb.2017.00449.

St John, A. L., Rathore, A. P., Raghavan, B., Ng, M.-L., & Abraham, S. N. (2013). Contributions of mast cells and vasoactive products, leukotrienes and chymase, to dengue virus-induced vascular leakage. *Elife, 2,* e00481. https://doi.org/10.7554/eLife.00481.

Suleman, M., Faryal, R., Alam, M. M., Sharif, S., Shaukat, S., Aamir, U. B., et al. (2016). NS1 antigen: A new beam of light in the early diagnosis of dengue infection. *Asian Pacific Journal of Tropical Medicine, 9*(12), 1212–1214. https://doi.org/10.1016/j.apjtm.2016.10.007.

Tan, B.-H., Fu, J., Sugrue, R. J., Yap, E.-H., Chan, Y.-C., & Tan, Y. (1996). Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coliexhibits* RNA-dependent RNA polymerase activity. *Virology, 216*(2), 317–325. https://doi.org/10.1006/viro.1996.0067.

Teurlai, M., Menkès, C. E., Cavarero, V., Degallier, N., Descloux, E., Grangeon, J.-P., et al. (2015). Socio-economic and climate factors associated with dengue fever spatial heterogeneity: A worked example in New Caledonia. *PLoS Neglected Tropical Diseases, 9*(12), e0004211.

Thisyakorn, U., Thisyakorn, C., Limpitikul, W., & Nisalak, A. (1999). Dengue infection with central nervous system manifestations. *The Southeast Asian Journal of Tropical Medicine and Public Health, 30*(3), 504–506.

Tuladhar, R., Singh, A., Varma, A., & Choudhary, D. K. (2019). Climatic factors influencing dengue incidence in an epidemic area of Nepal. *BMC Research Notes, 12*(1), 1–7.

Twiddy, S. S., Farrar, J. J., Chau, N. V., Wills, B., Gould, E. A., Gritsun, T., et al. (2002). Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. *Virology, 298*(1), 63–72. https://doi.org/10.1006/viro.2002.1447.

Verma, R., Lalla, R., & Patil, T. B. (2013). Levetiracetam induced encephalopathy in a patient with normal renal function: An unusual clinical encounter. *Annals of Indian Academy of Neurology*, *16*(4), 727.

Verma, R., & Varatharaj, A. (2011). Epilepsia partialis continua as a manifestation of dengue encephalitis. *Epilepsy and Behavior, 20*(2), 395–397. https://doi.org/10.1016/j.yebeh.2010.12.003.

Vijayan, V. (2010). Laboratory selection of carbofuran tolerant line of Culex quinquefasciatus Say, the filarial vector at Mysore. *Journal of Communicable Diseases, 42*(3), 201–207.

Waman, V. P., Kolekar, P., Ramtirthkar, M. R., Kale, M. M., & Kulkarni-Kale, U. (2016). Analysis of genotype diversity and evolution of Dengue virus serotype 2 using complete genomes. *PeerJ, 4,* e2326. https://doi.org/10.7717/peerj.2326.

Weeratunga, P. N., Caldera, H. M. C., Gooneratne, I. K., Gamage, R., Perera, W. S. P., Ranasinghe, G. V., et al. (2014). Spontaneously resolving cerebellar syndrome as a sequelae of dengue viral infection: A case series from Sri Lanka. *Practical Neurology, 14*(3), 176–178. https://doi.org/10.1136/practneurol-2013-000571.

WHO. (2011). Comprehensive guideline for prevention and control of dengue and dengue haemorrhagic fever.

WHO. (2020). Dengue and severe dengue. Key facts. 23 June 2020. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue#text=The%20disease%20is%20now%20endemic,the%20global%20burden%20of%20disease. Last Accessed on 14 November, 2020.

Wichmann, O., Hongsiriwon, S., Bowonwatanuwong, C., Chotivanich, K., Sukthana, Y., & Pukrittayakamee, S. (2004). Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi Thailand. *Tropical Medicine and International Health, 9*(9), 1022–1029. https://doi.org/10.1111/j.1365-3156.2004.01295.x.

Yamagishi, J., Runtuwene, L. R., Hayashida, K., Mongan, A. E., Thi, L. A. N., Thuy, L. N., et al. (2017). Serotyping dengue virus with isothermal amplification and a portable sequencer. *Scientific Reports*, *7*(1), 1–10.

Yip, V.C.-H., Sanjay, S., & Koh, Y. T. (2012). Ophthalmic complications of dengue fever: A systematic review. *Ophthalmology and Therapy, 1*(1), 2. https://doi.org/10.1007/s40123-012-0002-z.

Yong, Y., Thayan, R., Chong, H., Tan, C., & Sekaran, S. (2007). Rapid detection and serotyping of dengue virus by multiplex RT-PCR and real-time SYBR green RT-PCR. *Singapore Medical Journal, 48*(7), 662.

Young, P. R., Hilditch, P. A., Bletchly, C., & Halloran, W. (2000). An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. *Journal of Clinical Microbiology, 38*(3), 1053–1057.

Terms and Conditions

Springer Nature journal content, brought to you courtesy of Springer Nature Customer Service Center GmbH ("Springer Nature").

Springer Nature supports a reasonable amount of sharing of research papers by authors, subscribers and authorised users ("Users"), for small-scale personal, non-commercial use provided that all copyright, trade and service marks and other proprietary notices are maintained. By accessing, sharing, receiving or otherwise using the Springer Nature journal content you agree to these terms of use ("Terms"). For these purposes, Springer Nature considers academic use (by researchers and students) to be non-commercial.

These Terms are supplementary and will apply in addition to any applicable website terms and conditions, a relevant site licence or a personal subscription. These Terms will prevail over any conflict or ambiguity with regards to the relevant terms, a site licence or a personal subscription (to the extent of the conflict or ambiguity only). For Creative Commons-licensed articles, the terms of the Creative Commons license used will apply.

We collect and use personal data to provide access to the Springer Nature journal content. We may also use these personal data internally within ResearchGate and Springer Nature and as agreed share it, in an anonymised way, for purposes of tracking, analysis and reporting. We will not otherwise disclose your personal data outside the ResearchGate or the Springer Nature group of companies unless we have your permission as detailed in the Privacy Policy.

While Users may use the Springer Nature journal content for small scale, personal non-commercial use, it is important to note that Users may not:

1. use such content for the purpose of providing other users with access on a regular or large scale basis or as a means to circumvent access control;
2. use such content where to do so would be considered a criminal or statutory offence in any jurisdiction, or gives rise to civil liability, or is otherwise unlawful;
3. falsely or misleadingly imply or suggest endorsement, approval , sponsorship, or association unless explicitly agreed to by Springer Nature in writing;
4. use bots or other automated methods to access the content or redirect messages
5. override any security feature or exclusionary protocol; or
6. share the content in order to create substitute for Springer Nature products or services or a systematic database of Springer Nature journal content.

In line with the restriction against commercial use, Springer Nature does not permit the creation of a product or service that creates revenue, royalties, rent or income from our content or its inclusion as part of a paid for service or for other commercial gain. Springer Nature journal content cannot be used for inter-library loans and librarians may not upload Springer Nature journal content on a large scale into their, or any other, institutional repository.

These terms of use are reviewed regularly and may be amended at any time. Springer Nature is not obligated to publish any information or content on this website and may remove it or features or functionality at our sole discretion, at any time with or without notice. Springer Nature may revoke this licence to you at any time and remove access to any copies of the Springer Nature journal content which have been saved.

To the fullest extent permitted by law, Springer Nature makes no warranties, representations or guarantees to Users, either express or implied with respect to the Springer nature journal content and all parties disclaim and waive any implied warranties or warranties imposed by law, including merchantability or fitness for any particular purpose.

Please note that these rights do not automatically extend to content, data or other material published by Springer Nature that may be licensed from third parties.

If you would like to use or distribute our Springer Nature journal content to a wider audience or on a regular basis or in any other manner not expressly permitted by these Terms, please contact Springer Nature at

onlineservice@springernature.com

Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in Aedes aegypti: *a microbes based double-edged weapon to fight against Arboviral diseases*

A. N. Anoopkumar, Embalil Mathachan Aneesh & Ambalaparambil Vasu Sudhikumar

International Journal of Tropical Insect Science

e-ISSN 1742-7592

Int J Trop Insect Sci
DOI 10.1007/s42690-020-00104-z

Springer

International Journal of Tropical Insect Science
https://doi.org/10.1007/s42690-020-00104-z

ORIGINAL RESEARCH ARTICLE

Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases

A. N. Anoopkumar[1,2] · Embalil Mathachan Aneesh[2] · Ambalaparambil Vasu Sudhikumar[1]

Received: 30 June 2019 / Accepted: 6 January 2020

Abstract
The excessive accumulation of Reactive oxygen species (ROS) has been linked to myriad of toxic effects in several signaling pathways including growth regulatory pathways and apoptosis in insects. The current upsurge in mosquito research into the underlying mechanism of phytochemicals induced reactive oxygen species accumulation and larvicidal potential prompts the attention to exploring the role of oxidative stress and microbial consortia inhabited in the mosquitoes. In addition, understanding the impacts of mosquito microbiomes annihilation is vital for disentangling their underlying effects on arboviral diseases transmission. No investigation has been conducted in this aspect using isolated bioactive compounds from *Jasminum brevilobum*, *Aglaia edulis* and *Pogostemon auricularius* against *Aedes aegypti*. This study aimed to investigate the mode of action of bioactive compounds with special inference on ROS production and microbial inhabitants. Isolation and characterization of bioactive compounds were performed using the Thin-layer chromatography, Column chromatography, Gas chromatography-mass spectrometry and Fourier-transform infrared spectroscopy. R software and SPSS were used for statistical analyses. The isolated bioactive compounds induced excessive production of reactive oxygen species thereby leading to oxidative stress and death. All the isolated compounds exhibited a sturdy inhibitory effect against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli, Klebsiella pneumonia*, and *Proteus mirabilis*, with strong MIC values ranging from 0.08–1.65 mg/ml. Also, they showed promising larvicidal efficacy against *Aedes aegypti* larvae.

Keywords *Jasminum brevilobum* · *Aglaia edulis* · *Pogostemon auricularius* · Vector control · Oxidative stress · Mosquito control

Introduction

Insecticides exhibit prominent role in controlling mosquito vectors, principally when an outbreak of mosquito-borne disease has happened. To resist the outbreak of dengue fever infection, several synthetic insecticides has been primarily employed for reducing the *Aedes aegypti* population, the principal vector of dengue fever infection (Thongwat et al. 2017). According to the report provided by the World Health Organization (WHO) in 2018, about 200 million people are suffering from severe mosquito-borne disease, which provides the current status of an epidemic over the world (WHO 2018). The arboviral diseases including Chikungunya, Zika, and Dengue, have currently occupied a principal spot in the worldwide discussions regarding the increasing epidemics and the elevated level of associated syndromes including microcephaly, instigating the World Health Organization to announce vector-borne diseases as a public health crisis (Gulland 2016; Oliveira Melo et al. 2016; Anoopkumar et al. 2019). Concerning the adverse effects from the mosquito-borne disease, more specific and precise data on epidemiological aspects are available, and the number of viral infections elevated

✉ Embalil Mathachan Aneesh
aneeshembalil@stjosephs.edu.in

A. N. Anoopkumar
anoopkumar.247@gmail.com

Ambalaparambil Vasu Sudhikumar
avsudhi@rediffmail.com

[1] Department of Zoology, Christ College (Autonomus) Irinjalakuda (Affiliated to University of Calicut), Irinjalakuda, Thrissur, Kerala, India

[2] Department of Zoology, Communicable Disease Research Laboratory (CDRL), St. Joseph's College (Autonomous), Irinjalakuda, Thrissur, Kerala, India

Published online: 23 January 2020

nearly half a million in every year (Bhatt et al. 2013; Anoopkumar et al. 2017a).

The inappropriate and frequent use of synthetic insecticides has developed diverse adverse effects such as severe toxic effects to non-target organisms and undesirable effects to mammals together with environmental pollution (WHO 1986; Gallicchio et al. 1987; Yang et al. 2002; Aneesh and Vijayan 2010; Puthur et al. 2018). Therefore, specific consideration has been paid to research on several medicinal plants for defining their larvicidal efficacy against *Aedes aegypti* (Anoopkumar et al. 2017a). The plant-based products are reputed to have high toxicity and are species-specific compared to chemical insecticides for vectors of epidemiological significance (Sarwar et al. 2009). Like several research programmes on biopesticide discovery, mosquito vector control programmes has also used botanical sources of bioactive compounds for optimization (Cantrell et al. 2012; Aivazi and Vijayan 2009). The defensive elements of medicinal plants are renowned as inherent silent tools for self protection, and have non residual effects on the environment; therefore, the vector control strategies based on these aspects gain much more significance than any other conventional synthetic insecticides employed approaches in recent years (Poole et al. 1990; Puthur et al. 2019).

Due to the presence of various bioactive elements and its significant role in generating oxidative stress in mosquito vectors, the traditionally used medicinal plants have taken a significant place in the regions where human population is threatened by severe infectious diseases (Joseph et al. 2004; Kunwar and Priyadarsini 2011). An inevitable form of specific metabolism in insects has led to the development of oxidative stress by the action of too much amount of free radicals or reactive oxygen species (ROS) (Sies 1997). These free radicals have been recognized as highly unstable elements and they form strong reactions towards other molecules to accomplish stability, instigating chain reactions of ROS (Devasagayam et al. 2004). These reactions have led to the reduction of antioxidants resulting in a disparity between antioxidant defense processes and ROS level (Tilak et al. 2004). This intense condition developed by the free radicals causes severe pathological conditions including programmed cell death (Devasagayam et al. 2004).

In addition, an excessive amount of ROS has suppressed or induced certain genes and signaling pathways that prompted toxicological impact on the organisms (Mittler et al. 2004). The studies that focussed on bioactive compound induced oxidative stress development in *Aedes aegypti* larvae are scanty. This study was intended i) to isolate the bioactive compounds from three traditionally used medicinal plants against *Aedes aegypti* fourth instar larvae, ii) to investigate the plant extracts and bioactive compounds mediated free radical formation and oxidative stress, iii) to evaluate the toxicity of bioactive compounds towards the microbial consortia that symbiotically influences the life cycles of *Aedes* mosquito vectors.

Materials and methods

Plant material collection and extraction

Fresh leaves of *Jasminum brevilobum*, *Aglaia edulis,* and *Pogostemon auricularius* were collected from Thirunelly (11°53'N 76°0'E) (belongs to Wayanad district of Kerala), India, a part of Western Ghats. The shade dried (25 ± 2 °C temperature) leaves were milled into fine powder by means of an electric grinder. Soxhlet extraction apparatus was employed for the extraction of *J. brevilobum*, *A. edulis* and *P. auricularius* with acetone, methanol, hexane, petroleum ether, and water as the solvents. The final concentrated extract was stored at −20 °C for further experiments.

Experimental insects

Early fourth instar *Aedes aegypti* larvae were used for the executed experiments. The larvae used in this study were from the laboratory colony reared at Communicable Disease Research Laboratory, Department of Zoology, St. Josephs's College, Irinjalakuda, Kerala, India. The *Ae. aegypti* mosquito eggs were placed in the plastic trays containing dechlorinated water (28 L × 39 W× 14 D cm). After egg hatching, the larvae were transferred into another plastic tray (28 L × 39 W× 14 D cm). They were fed on dog biscuit and yeast in the ratio of 3:1 in the following environmental conditions; 27 ± 2 °C temperature, 55–60% relative humidity followed by 12 L:12 D photoperiod cycle.

Fractionation and isolation of bioactive compounds from the leaf extracts of the three plants

The compounds were obtained by repeated chromatographic purification (Column chromatography and Thin layer chromatography (TLC) until a single spot was obtained for each sample in TLC. The analytical grade acetone, hexane, and methanol were used for TLC analyses. TLC plate and Silica gel (70–30 mesh) for TLC was bought from Merck Co. (Germany). The acetone extracts of *J. brevilobum*, *A. edulis,* and *P. auricularius* were used for thin layer chromatographic analysis. Thin layer chromatography for *J. brevilobum* acetone extract was done by using hexane and acetone as the mobile phase in the ratio 11:5. *A. edulis* acetone extract was subjected for TLC analysis with methanol (M), Acetone (A) and hexane (H) as the mobile phase in the ratio 4:5:1. The bioactive element of *P. auricularius* extract was separated using methanol, acetone, and hexane in the ratio 4:5:4. The bioactive fractions of acetone extract of the three medicinal plants were separated using column chromatography. Silica gel for column chromatography having 60–120 M was packed in column (40 × 330 mm) (Merck KGaA, Darmstadt,

Germany). Analytical grade acetone, methanol, and hexane were used for the mobile phase.

Twenty grams of the acetone extract of *J. brevilobum* was loaded on to the column packed with silica with hexane and acetone as mobile phase in the ratio 11:5. Sample names were given to each fraction from C1 to C12. All the isolated fractions were subjected for mosquito larvicidal bioassay protocol provided by WHO (WHO 2005). The bioactive fraction C7 of *J. brevilobum* was again separated using TLC and Column chromatography with the following conditions; Hexane (90 ml): Acetone (40 ml). Seven sub fractions were isolated from C7 and they were provided with the sample names from CC1-CC7. These seven fractions were subjected to bioassay and the process of separation of CC7 was repeated using the same condition since it showed potent activity. Then the bioactive fraction CC7 was purified and stored at −20 °C for further analysis.

Twenty grams of *A. edulis* acetone extract was loaded on the column using methanol, hexane, and acetone in the ratio 6:1:5; and 19 fractions were separated. Each fraction was provided with a sample name from E1 to E19. All the collected fractions were subjected to bioassay. The bioactive fraction E11 was again subjected for TLC and Column chromatography using the following conditions; Methanol (70 ml): Hexane (15 ml): Acetone (1 ml). Five sub-fractions (EE1-EE5) were isolated from E11. The bioactive EE5 fraction was purified and stored at −20 °C for further analysis.

The *P. auricularius* acetone extract was subjected for bioactive element separation using methanol, hexane, and acetone as mobile phase in the ratio 4:5:4. The isolated 18 fractions were provided with sample names from P1 to P18. These fractions were subjected to larvicidal bioassay. Each isolated eluent was analyzed using TLC. The bioactive P8 (from P1-P18) fraction was again subjected for column chromatography with hexane and acetone in the ratio 8:3. Fourteen sub fractions were isolated from P8 and they were provided with sample names from PP1 to PP14. These sub-fractions (PP1- PP14) were subjected for larvicidal bioassay and the bioactive fraction PP4 was stored at −20 °C for further analysis.

Identification of purified bioactive compounds using GC-MS and FTIR

The isolated samples such as CC7, EE5, and PP4 were subjected for GC-MS (MSQP2010 (Shimadzu, Kyoto, Japan). One ml of each of the bioactive fractions (CC7, EE5, and PP4) were injected separately for GC-MS analysis with the following conditions; 30 m × 0.25 mm, film thickness 0.25 μm DB-5MS column, initial temperature 50 °C (2 min) together with rate of 20 °C/min to 130 °C; 12 °C/min to a 180 °C; and a final temperature to 280 °C at 3 °C/min. The final temperature condition was maintained for 15 min. As a carrier gas, helium was used at 1 ml/min flow rate. The 250 °C temperature was maintained for ion source and injection port temperature. The machine was run in electron impact (EI) mode for 58 min with electron energy (70 ev). Selective ion mode (SIM) was employed for the confirmation of analytes (Abirami 2012). The peak areas of the samples were matched with those on the National Institute of Standards and Technology library database (NIST 08-S, available at https://chemdata.nist.gov/). Fourier-transform infrared spectroscopy (FTIR) (Thermo Fisher, U.S.), a rapid technology to identify and elucidate the structure of chemical compounds has been used in this study to fingerprint the bioactive compounds from the purified samples.

Toxicity of isolated plant extracts and compounds on microbial consortia

The toxicity of isolated bioactive fractions and compounds was determined against *Klebsiella pneumonia* (MTCC 661), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 443), and *Proteus mirabilis* (MTCC 442). Different concentrations of isolated bioactive fractions were loaded on 10-mm discs positioned over microbe plated agar plates. Controls were maintained and the plates were kept at 37 °C for 15 to 18 h in an incubator (ROTEK, India). The diameter of the zone of inhibition of the isolated bioactive fraction towards the microbial consortia was measured. Chloramphenicol was used as positive control and triplicates were maintained for all the toxicity studies. The data obtained have been expressed as mean ± standard deviation and the minimum inhibitory concentrations (MIC) were determined using the method recommended by Wiegand et al. 2008 and Bussmann et al. 2010.

Toxicity effects of plants extracts and its fraction on mosquito larvae

Toxicity effects of plants extracts and the isolated fractions were performed as per the guidelines of World Health Organization (WHO 2005). Twenty early fourth instar larvae of *A. aegypti* were transferred to 250 ml glass beakers containing various extracts and isolated fractions in different concentrations. Three replicates of treatment were maintained and control was included for all the executed experiments. For the larvicidal bioassay, 250 ppm, 100 ppm, 50 ppm, and 25 ppm of each of the acetone extracts of the three medicinal plants was taken. One ppm to 9 ppm of the isolated compounds was used for determining their larvicidal efficacy. The mortality rates were recorded after 24 h of continuous exposure.

Phytochemicals prompted free radical generation

The ability of isolated bioactive fractions and compounds to generate an excessive amount of free radicals resulted to oxidative stress that was verified using the ammonium molybdate method (Prieto et al. 1999) with slight modification. Twenty-

fourth instar larvae of *Aedes aegypti* treated (ranging from 1 to 10 ppm) with the isolated bioactive fractions (CC7, EE5, and PP4) was homogenized as separate samples. The control group represents the homogenized extract which contains the distilled water and *Ae. aegypti* fourth instar larvae. The amount of free radicals generated in the untreated and treated larvae was determined as per the protocol given by Prieto et al. 1999 with slight modifications. The phytochemicals prompted free radicals in the treated and untreated samples were determined using Ultraviolet-visible spectroscopy (Thermo Fisher, U.S.) at 695 nm.

Statistical analysis

All data were entered and systemized in Microsoft Excel 2010 spreadsheet. The entered data were exported into SPSS version 24.0.0 (Anoopkumar et al. 2017a; Pitarokili et al. 2011) and R software version 3.2.3 (Team RC 2013) for probit regression analysis (to get the LC_{50} and LC_{90} of crude extracts and their isolated bioactive fractions) and Chi-Square analysis. The data on corrected mortality rates were calculated as the mean percentage ± standard deviation (SD) for each bioassays and toxicity studies on microbial consortia. The graphs were plotted with SPSS and GraphPad Prism version 7.0 (Rao et al. 2013) for Windows operating systems.

Results

Phytochemistry

Bioassay-guided chromatographic fractionation of the *Jasminum brevilobum*, *Aglaia edulis,* and *Pogostemon auricularius* extracts yielded three bioactive compounds. They were compared and identified using the database of GC-MS library provided by NIST. The compounds were identified as Jatamansone (CC7; $C_{15}H_{26}O$) (Fig. 1), 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4; $C_{12}H_{24}O_3$) (Fig. 2), and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (EE5; $C_{16}H_{30}O_4$) (Fig. 3) from *J. brevilobum*, *P. auricularius*, and *A. edulis* respectively using GC-MS (Table 1). The structural characterization was verified using FTIR.

Toxicity of isolated plant extracts and compounds on microbial consortia

The toxic effect of plant extracts and the isolated compounds were assessed against five bacterial species. All the extracts and isolated compounds have predominantly verified strong inhibitory effect for *Staphylococcus aureus* (MTCC 3160) and *Proteus mirabilis* with MIC values ranging from 0.08–1.65 mg/ml. The acetone and water extracts of *A. edulis, J. brevilobum* and *P. auricularius* largely showed maximum toxicity towards the *S. aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 443), *Klebsiella pneumonia* (MTCC 661) and *P. mirabilis* (MTCC 442) with MIC values ranging from 0.08–1.06 mg/ml. The *J. brevilobum* and *P. auricularius* extracts were notably displayed strong inhibiting activity towards all the tested microbial consortia (MIC ranging from 0.42–1.65 mg/ml). However, the methanol and petroleum ether extracts of *A. edulis*, *J. brevilobum*, and *P. auricularius* has displayed a relatively considerable range of growth suppression rate towards the bacterial species with MIC values ranging from 0.4–1.65 mg/ml. The toxic effects of isolated bioactive compounds were also evaluated against the aforementioned bacterial species. 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate showed the maximum zone of inhibition towards *S. aureus* (22.65 ± 0.700, MIC = 0.04) and *P. mirabilis* (19.95 ± 1.530, MIC = 0.14) (Table 2). The jatamansone exhibited strong inhibiting activity towards *Staphylococcus aureus* (21.72 ± 0.571, MIC = 0.0565 mg/ml) and *E. coli* (20.42 ± 1.598, MIC = 0.0765 mg/ml). 3-hydroxy-2,2,4-trimethylpentyl isobutyrate isolated from *P. auricularius* verified sturdy growth inhibition activity with MIC values ranging from 0.08–0.46 mg/ml (Table 3). These results indicated that all the extracts and isolated compounds sustained significant toxicity to the bacterial species.

Toxicity effects of plants extracts and its fraction on mosquito larvae

The larvicidal efficacy of three compounds isolated is presented in Table 4. All the isolated compounds have showed potent larvicidal efficacy. The larvicidal potential of the three plants extracts and their subfractions against *Ae. aegypti* are shown in Table 4 and graphically in Fig. 4. The acetone extracts of *A. edulis* exhibited the highest larvicidal potential against *Ae. aegypti* larvae with LC_{50} value of 64.466 μg/ml (fiducial limit - 38.726 - 83.414) and LC_{90} value of 200.212 μg/ml (fiducial limit-146.789 - 415.844). The water extracts of three plants were also showed higher larvicidal activity. Amongst the compounds isolated 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate isolated from *A. edulis* showed a promising range of larvicidal efficacy with LC_{50} value of 1.724 μg/ml. The other compounds such as jatamansone and 3-hydroxy-2,2,4-trimethylpentyl isobutyrate isolated from *J. brevilobum* and *P. auricularius* respectively showed an adequate range of larvicidal efficacy.

Mode of action of phytochemicals prompted reactive oxygen species in the target insect body

We assessed the phytochemicals induced reactive oxygen species formation against the *Aedes aegypti* larvae (Table 5). The isolated phytocompounds prompted reactive oxygen species (Fig. 5) determined in the present study were considered as a double-edged sword since their excessive production has

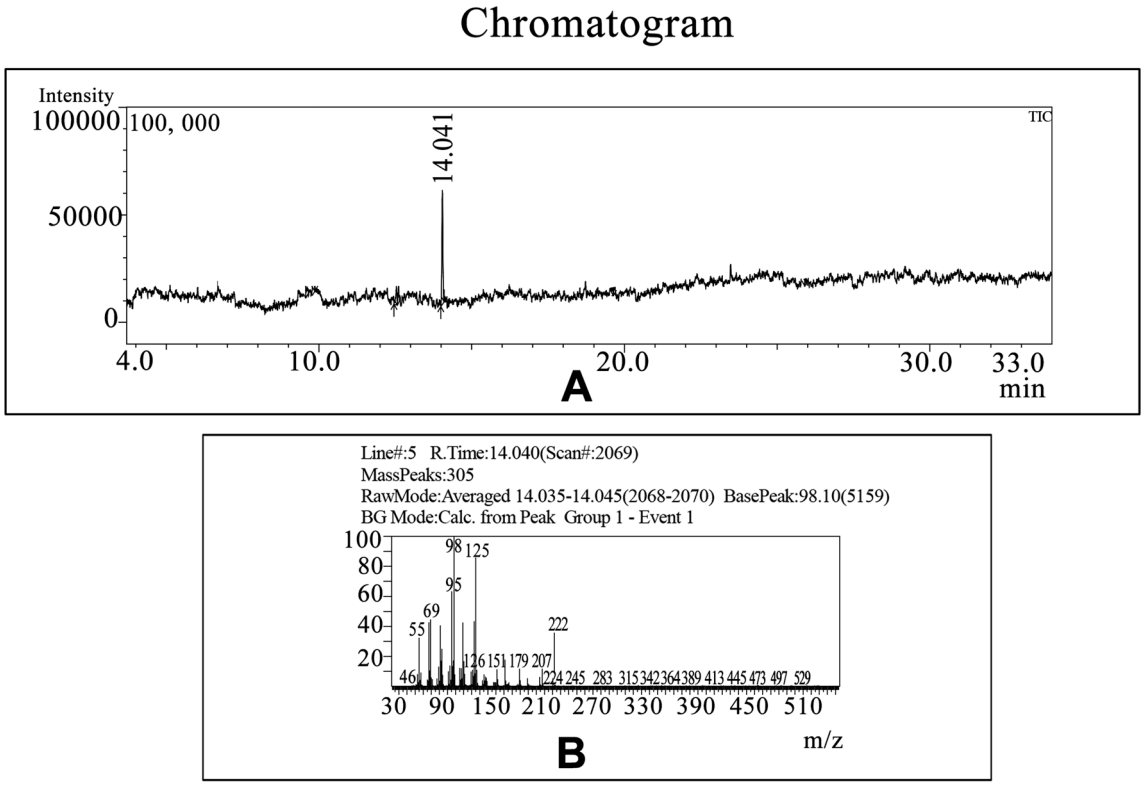


Fig. 1 Gas chromatography-mass spectrometer (GC-MS) chromatogram and mass spectra of the fraction and compound from J. brevilobum: **a** GC-MS chromatogram of the isolated bioactive fraction CC7; **b** Mass spectra of the identified bioactive compound, jatamansone

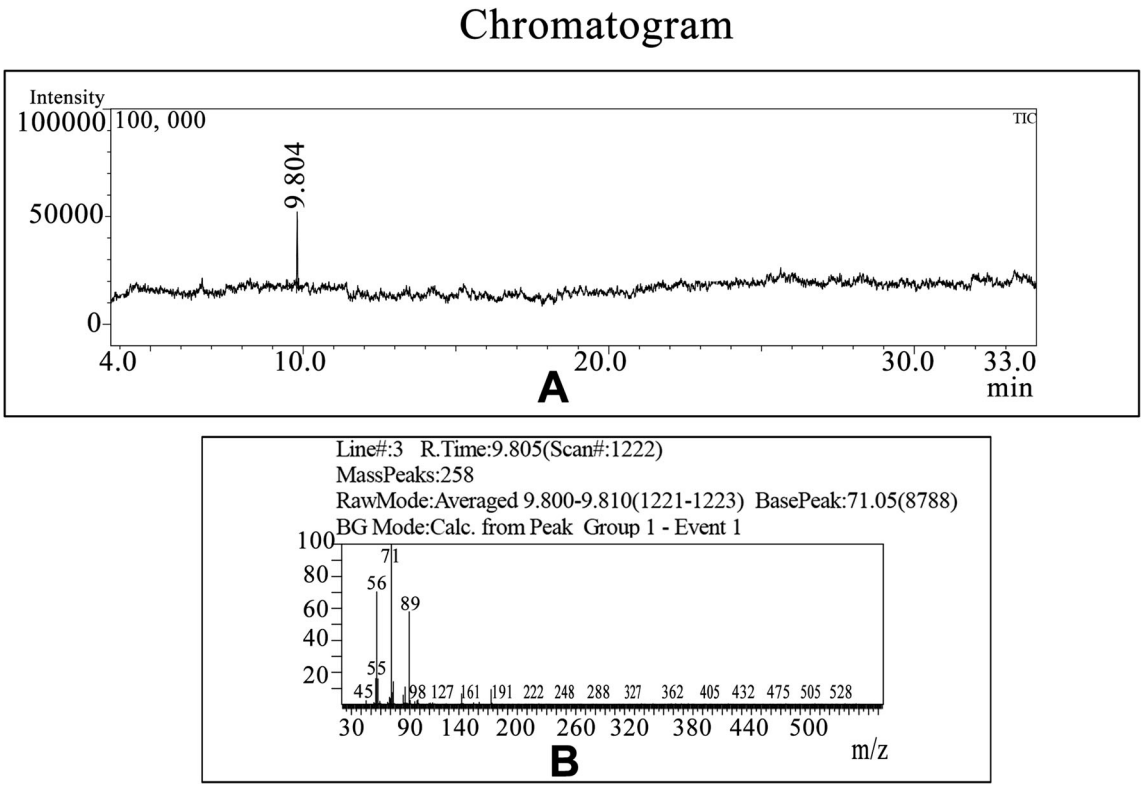


Fig. 2 Gas chromatography-mass spectrometer (GC-MS) chromatogram and mass spectra of the fraction and compound from *P. auricularius*: **a** GC-MS chromatogram of the isolated bioactive fraction PP4; **b** Mass spectra of the identified bioactive compound, 3-hydroxy-2,2,4-trimethylpentyl isobutyrate

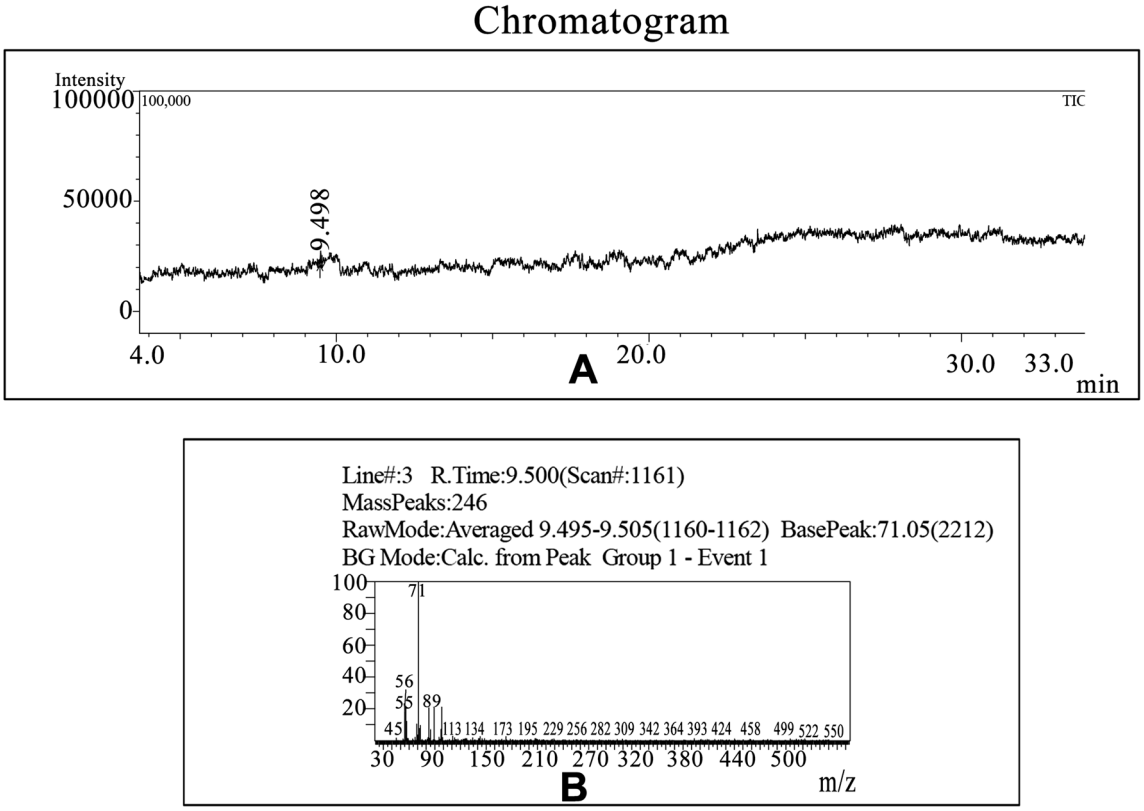


Fig. 3 Gas chromatography-mass spectrometer (GC-MS) chromatogram and mass spectra of the fraction and compound from *A. edulis*: **a** GC-MS chromatogram of the isolated bioactive fraction EE5; **b** Mass spectra of the identified bioactive compound, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate

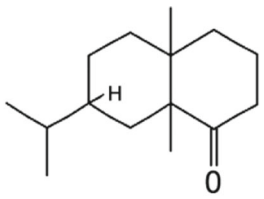
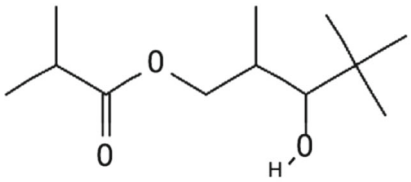
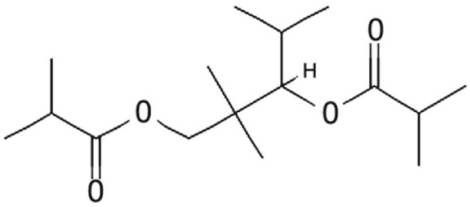
arbitrated oxidative stress (Table 6) eventually causing cell damage and death. The amount of free radicals generated in the acetone and water extracts of *A. edulis* significantly increased than the control group. Only a few reactive oxygen species was formed in the control group. The acetone, water, methanol, and petroleum ether extracts of *J. brevilobum* and *P. auricularius* was exhibited large amount of free radicals and this has drawn our concern to further investigate the compound-specific oxidative stress. We established that the 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, jatamansone, and 3-hydroxy-2,2,4-trimethylpentyl isobutyrate exposure towards the *Ae. aegypti* larvae caused excessive production of free radicals inside the body, that also seem to be interconnected with numerous physiological processes.

Discussion

Toxicity of isolated plant extracts and compounds on microbial consortia

In this study, we provided for evidence of phytochemical prompted reactive oxygen species formation in *Aedes aegypti* larvae together with the microorganism based vector control approach. The microbiota that resides in the insects has been shown to draw a significant role in the biology of mosquito vectors by exhibiting intricate give-and-take multifarious interactions (Rosenberg and Zilber-Rosenberg 2011). These inhabited microbiota has offers several beneficial impacts for their insect hosts by providing essential nutrients together with inducing resistance to parasitoids and pathogens (Valzania et al. 2018). Therefore, the use of microbes' oriented techniques to investigate their benefits in humans has dramatically increased in recent times (Dillon and Dillon 2004; Rebello et al. 2018; Rebello et al. 2019a; Rebello et al. 2019b). Recent report indicates that the larval stages of *Ae. aegypti* are strongly interrelated with the microorganisms exist in their body and the larvae cannot complete their life cycle process in the absence of these microorganisms (Valzania et al. 2018). The reported reason for the death of larvae in the absence of bacteria is the certain abnormalities observed in the bacteria-induced hypoxia signal (Lenaz et al. 2002; Coon et al. 2017; Valzania et al. 2018). The activation of hypoxia-induced transcription factors will not happen if the population of bacteria has been destroyed inside the body (Dillon and Dillon 2004; Puthur et al. 2019). Here we choose this loophole as a base to control the rapid proliferation of *Ae. aegypti*. Our study validated the elimination of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis* by the direct administration of phyto-extracts and purified compounds. In which *S. aureus, E. coli*, *K. pneumonia*, *Bacillus* sp.

Table 1 Bioactive fractions and compounds isolated from *Aglaia edulis*, *Jasminum brevilobum*, *Pogostemon auricularius*

Sl. No	Retention time	Area	Area%	Name of the compound	Molecular weight (g/mol)	Structure
1	14.041	123577	23.30	Jatamansone	222.37	 CC7
2	9.804	53442	46.28	3-hydroxy-2,2,4-trimethylpentyl isobutyrate	216.32	 PP4
3	9.498	12271	9.56	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	286.41	 EE5

exist inside the *Ae. aegypti* mosquito larvae are known for their host-parasite interaction. They provide supplements of growth factors through this interactions and most of the microbiota has drastically increased their vectorial capacity (Azambuja et al. 2005; Yadav et al. 2015; Jayakrishnan et al. 2018). Our results suggest all the extracts and purified compounds we tested can be used to eradicate the microbial inhabitants thereby providing a novel idea for microorganism based vector control strategy.

Mode of action of phytochemicals prompted reactive oxygen species in the target insect body

Studies on the mode of action of phytochemical prompted reactive oxygen species with special emphasis on mosquito vector control are scanty in the current scenario. Therefore, we examined the probable mode of action of isolated bioactive compounds induced oxidative stress with special reference to mosquito control. The three isolated compounds from the present study have prominently generated excessive amounts of free radicals inside the treated *Ae. aegypti* larvae thereby causing an imbalance in homeostasis. Only a few studies have reported the toxic effects of jatamansone (CC7), 3-hydroxy-2,2,4-trimethylpentyl isobutyrate (PP4), and 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (EE5) (Arora et al. 1958; Astill et al. 1972; Chen and Oxford 1965; Kodak 1971; Wright et al. 2017). The elevated level of reactive oxygen species has activated a diverse set of events linked with the abnormal functioning of cells like disruption of redox signaling pathways,

Table 2 Toxic effects of selected plant extracts and isolated compounds against microbial consortia at 50 μl. (Zone of inhibition measured in mm)

Plant	Solvent used for extraction	Zone of inhibition (mm)				
		Gram positive		Gram Negative		
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*
Aglaia edulis	Acetone	20.04 ± 1.440	18.00 ± 0.535	16.73 ± 0.679	16.99 ± 0.432	18.22 ± 0.997
	Water	18.42 ± 0.212	17.56 ± 0.336	15.84 ± 0.545	15.42 ± 0.650	17.44 ± 0.887
	Methanol	14.32 ± 0.156	16.56 ± 0.210	14.52 ± 0.469	16.40 ± 0.333	16.22 ± 0.998
	Petroleum ether	12.25 ± 0.351	13.41 ± 0.210	10.16 ± 0.548	15.07 ± 0.432	14.43 ± 0.564
Jasminum brevilobum	Acetone	17.41 ± 0.364	17.74 ± 1.365	14.53 ± 0.578	16.46 ± 0.333	17.03 ± 0.350
	Water	14.40 ± 0.169	15.60 ± 0.335	10.32 ± 0.465	15.48 ± 0.241	16.44 ± 0.560
	Methanol	11.62 ± 0.374	13.42 ± 0.460	12.36 ± 0.659	14.67 ± 0.550	15.03 ± 0.833
	Petroleum ether	9.18 ± 0.444	10.55 ± 0.865	12.44 ± 0.643	13.57 ± 0.420	14.00 ± 0.660
Pogostemon auricularius	Acetone	15.00 ± 0.555	17.56 ± 0.433	16.37 ± 0.769	16.68 ± 0.222	16.38 ± 0.157
	Water	12.10 ± 0.250	13.02 ± 0.137	14.30 ± 0.580	13.00 ± 0.100	14.34 ± 0.336
	Methanol	10.74 ± 0.300	13.78 ± 0.555	12.05 ± 0.560	12.05 ± 0.258	13.32 ± 0.025
	Petroleum ether	8.05 ± 0.246	12.37 ± 0.778	10.22 ± 0.464	10.46 ± 0.466	13.04 ± 0.360
Isolated compounds						
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		22.65 ± 0.700	20.56 ± 0.478	18.68 ± 0.579	18.55 ± 0.507	19.95 ± 1.530
Jatamansone		21.72 ± 0.571	19.55 ± 0.689	20.42 ± 1.598	19.75 ± 0.458	20.03 ± 0.444
3-hydroxy-2,2,4-trimethylpentyl isobutyrate		17.91 ± 0.456	18.55 ± 0.100	18.59 ± 0.760	17.98 ± 0.123	19.57 ± 0.268

Table 3 Minimum inhibitory concentrations of selected plant extracts and isolated compounds against microbial inhabitants

Plant	Solvent used for extraction	Minimum Inhibitory Concentration				
		Gram positive		Gram Negative		
		Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*
Aglaia edulis	Acetone	0.08	0.26	0.58	0.51	0.29
	Water	0.29	0.60	0.58	0.61	0.33
	Methanol	0.44	0.72	0.89	0.71	0.80
	Petroleum ether	1.03	0.91	1.26	0.66	0.92
Jasminum brevilobum	Acetone	0.44	0.42	0.89	0.54	0.49
	Water	0.92	0.62	1.24	0.66	0.51
	Methanol	1.17	1.04	1.09	0.95	0.60
	Petroleum ether	1.56	1.36	1.08	1.00	0.92
Pogostemon auricularius	Acetone	0.66	0.44	0.54	0.53	0.54
	Water	1.03	1.02	0.94	1.06	0.96
	Methanol	1.30	1.02	1.08	1.05	1.03
	Petroleum ether	1.65	1.08	1.36	1.42	1.16
Isolated compounds						
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate		0.04	0.06	0.26	0.28	0.14
Jatamansone		0.05	0.15	0.07	0.14	0.09
3-hydroxy-2,2,4-trimethylpentyl isobutyrate		0.08	0.23	0.25	0.46	0.17

Microdilution method was used for MIC determination. 3 biological replicates were maintained for all executed experiments

Table 4 Larvicidal efficacy of isolated compounds and extracts from *Aglaia edulis, Jasminum brevilobum,* and *Pogostemon auricularius*

Plant	Solvent used	LC_{50}	LC_{90}	Chi-Square	df[b]
Aglaia edulis	Acetone	64.466 (38.726–83.414)	200.212 (146.789–415.844)	2.416	2
	Water	120.933 (92.051–165.471)	418.761 (258.302–1652.422)	3.364	2
	Methanol	101.192 (65.915–140.734)	455.782 (256.354–3601.863)	.038	2
	Petroleum ether	107.799 (73.553–152.393)	475.489 (265.172–3781.023)	.883	2
Jasminum brevilobum	Acetone	75.769 (51.872–95.311)	221.528 (162.587–446.465)	3.223	2
	Water	111.552 (80.820–152.680)	426.236 (255.090–2079.013)	.647	2
	Methanol	123.084 (85.546–198.167)	616.541 (305.356–11,602.368)	1.538	2
	Petroleum ether	111.55 (280.820–152.680)	426.236 (255.090–2079.013)	.647	2
Pogostemon auricularius	Acetone	86.700 (66.409–105.635)	218.202 (166.375–377.585)	3.534	2
	Water	111.274 (73.894–166.061)	551.322 (284.491–8123.569)	0.416	2
	Methanol	118.518 (85.761–170.459)	488.096 (275.216–3351.831)	0.456	2
	Petroleum ether	145.828 (114.751–210.745)	466.020 (284.962–1851.210)	1.703	2
Isolated bioactive compounds					
CC7		2.213 (1.292–3.032)	13.549 (8.445–39.290)	1.101	4
EE5		1.724 (0.834–2.479)	11.775 (7.335–35.706)	4.760	4
PP4		3.356 (2.331–4.518)	19.667 (11.460–68.386)	1.597	4

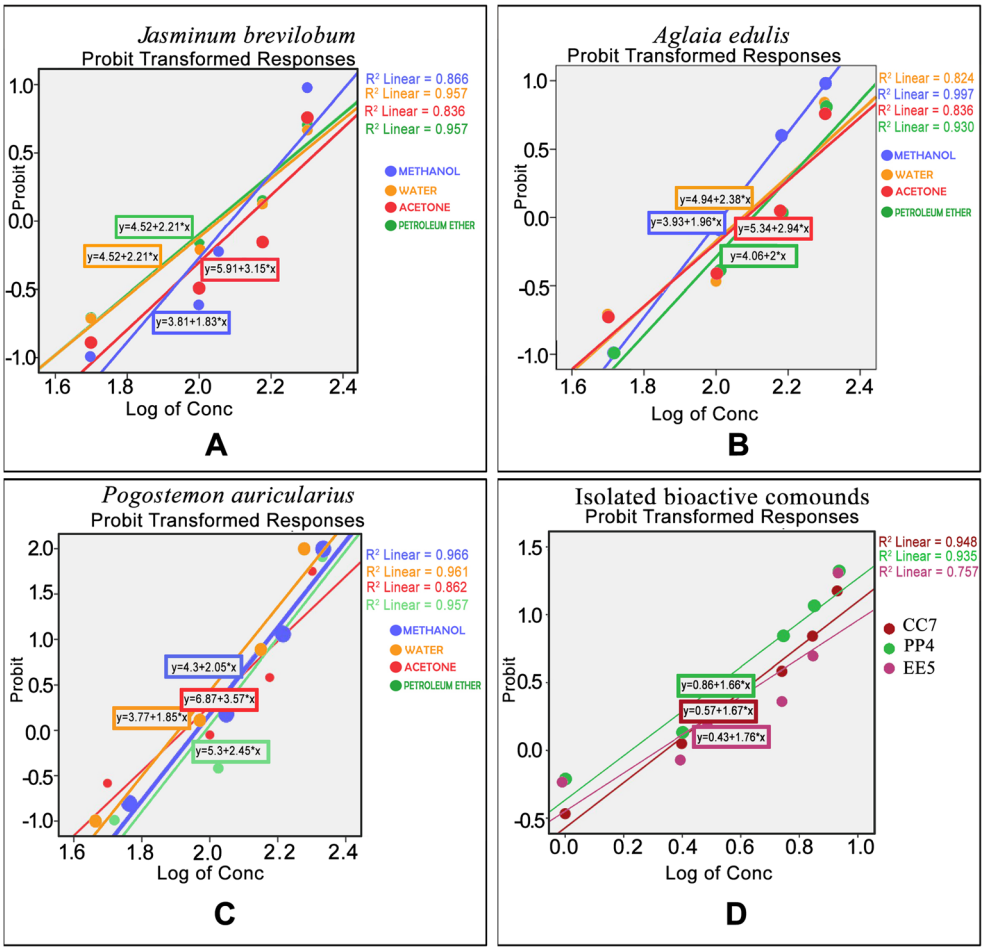


Fig. 4 Probit regression analysis – Larvicidal potential of **a** *J. brevilobum*, **b** *A. edulis*, **c** *P. auricularius* **d** Isolated bioactive compounds

Table 5 Phytochemicals prompted reactive oxygen species in the *Aedes aegypti larvae*

Sample/ Solvent used for extraction of the plant sample	Extract Dose(µg/mL)	Absorbance Mean ± SD		
		Aglaia edulis	*Jasminum brevilobum*	*Pogostemon auricularius*
Aedes aegypti Larvae + Distilled water (µg/mL)	125	0.006 ± 0.004	0.005 ± 0.002	0.006 ± 0.000
	250	0.017 ± 0.004	0.018 ± 0.002	0.018 ± 0.000
	500	0.023 ± 0.004	0.023 ± 0.000	0.020 ± 0.000
	1000	0.041 ± 0.004	0.040 ± 0.004	0.041 ± 0.000
Acetone extract (µg/mL)	125	0.224 ± 0.004	0.171 ± 0.000	0.100 ± 0.000
	250	0.395 ± 0.006	0.265 ± 0.000	0.221 ± 0.002
	500	0.459 ± 0.004	0.366 ± 0.000	0.299 ± 0.004
	1000	0.599 ± 0.000	0.461 ± 0.000	0.315 ± 0.000
Water extract (µg/mL)	125	0.141 ± 0.001	0.123 ± 0.000	0.103 ± 0.000
	250	0.296 ± 0.000	0.198 ± 0.002	0.200 ± 0.000
	500	0.378 ± 0.000	0.247 ± 0.002	0.299 ± 0.000
	1000	0.495 ± 0.004	0.311 ± 0.002	0.359 ± 0.000
Methanol extract (µg/mL)	125	0.114 ± 0.001	0.028 ± 0.004	0.111 ± 0.004
	250	0.239 ± 0.000	0.173 ± 0.004	0.216 ± 0.002
	500	0.263 ± 0.004	0.241 ± 0.004	0.279 ± 0.002
	1000	0.311 ± 0.002	0.290 ± 0.004	0.363 ± 0.002
Petroleum ether extract (µg/mL)	125	0.111 ± 0.002	0.099 ± 0.000	0.147 ± 0.000
	250	0.212 ± 0.002	0.134 ± 0.000	0.194 ± 0.000
	500	0.259 ± 0.016	0.199 ± 0.000	0.267 ± 0.000
	1000	0.289 ± 0.006	0.275 ± 0.000	0.324 ± 0.004

p53-dependent apoptosis together with abnormalities in various physiological processes (WHO 1986; Dillon and Dillon 2004; Kunwar and Priyadarsini 2011). This indicates that there should be a specific balance between ROS-detoxifying responses and ROS-generating systems which are indispensable for retaining homeostasis in organisms (Oliveira et al. 2017). The present study uses this loophole as an intervention to control vector mosquitoes by inducing phytochemical prompted free radical production. In this study, the aforementioned phyto-compounds prompted reactive oxygen species were considered as a double-edged sword since their elevated level induce oxidative stress eventually causing cell damage

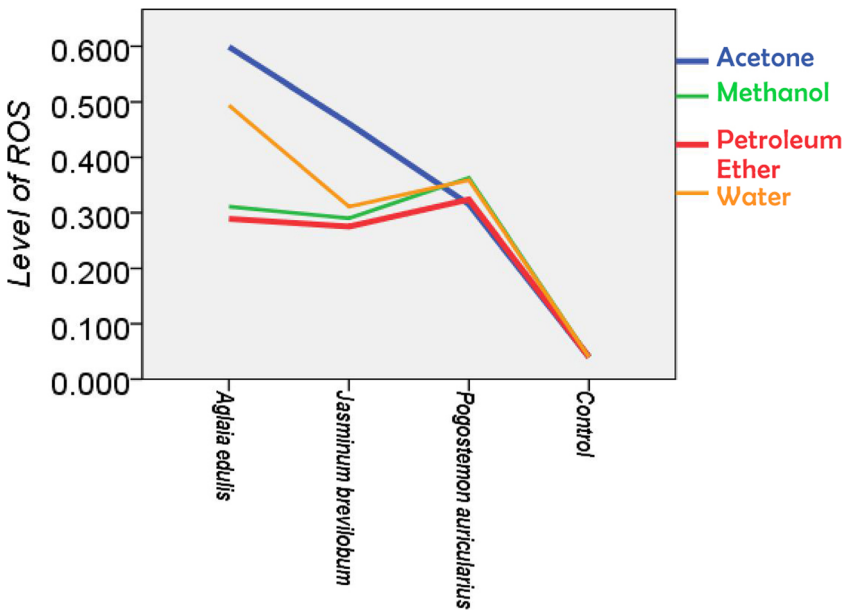


Fig. 5 Level of isolated bioactive compounds prompted reactive oxygen species generated in *Aedes aegypti* larvae

Table 6 Mode of action of isolated bioactive compounds prompted reactive oxygen species as oxidative stress in *Aedes aegypti* larvae

Compound	Dose (μg/mL)	Absorbance Mean ± SD
Aedes aegypti Larvae + Distilled water (μg/mL)	125	0.005 ± 0.000
	250	0.016 ± 0.000
	500	0.022 ± 0.002
	1000	0.040 ± 0.000
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	125	0.301 ± 0.000
	250	0.400 ± 0.000
	500	0.572 ± 0.002
	1000	0.600 ± 0.000
Jatamansone	125	0.203 ± 0.000
	250	0.371 ± 0.004
	500	0.433 ± 0.000
	1000	0.552 ± 0.006
3-hydroxy-2,2,4-trimethylpentyl isobutyrate	125	0.240 ± 0.000
	250	0.356 ± 0.002
	500	0.460 ± 0.006
	1000	0.559 ± 0.004

and death in *Ae. aegypti* larvae. Therefore, we suspected that the excessive production of free radicals by these three compounds was responsible for the death of *Ae. aegypti* fourth instar larvae.

Phytochemistry and toxicity effects of plants extracts and its fraction on mosquito larvae

The present investigation explored the use of three phytochemical compounds as safer insecticides for the sustainable approach of mosquito control. In which jatamansone exhibited pharmacological benefits (Sahu et al. 2016) and 3-hydroxy-2,2,4-trimethylpentyl isobutyrate was previously reported as strong antibacterial agents from *Tasmannia lanceolata* which impede the growth of *Clostridium perfringens*. This has confirmed their toxicity towards microorganisms (Wright et al. 2017). Incorporated with their findings, for the first time, we have reported its toxic effects against mosquito vectors of epidemiological significance. Similarly, 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate exhibit significant range of toxicity. Previous reports on 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate revealed that its higher concentration possesses a considerable range of toxicity (Astill et al. 1972). The various phytochemical constituents including defense-related enzymes and toxic chemicals in plants offer defense mechanisms for them to cope with the vast diversity of adverse biotic conditions (Jones and Dangl 2006). The production of toxic substance for defense mechanism is usually accompanied with the tannins, secondary metabolites, and pain-sensation prostaglandins that can be characterized in three clusters: phenolics, terpenoids, and alkaloids (Bennett and Wallsgrove 1994). In which, the phenolics are recognized as signaling constituents generated in response to the incursion of aggressors (Mandal et al. 2010). The various groups of phytochemical constituents including steroids, phenolics, aromatic compounds, alkaloids, terpenoids of botanical origin has been noted previously for their toxicity towards mosquitoes (Shaalan et al. 2005; Wang et al. 2019). This indicates that the phytochemical constituents responsible for the well-developed immune system in plants can be used as an excellent source of toxic substance for natural insecticides. We define the application of three compounds with potent toxicity against fourth instar larvae of *Ae. aegypti*.

Our findings underpin that these three isolated compounds are potentially effective and toxic and can be considered for natural vector control approaches. Despite numerous studies on the larvicidal efficacy of plant extracts against mosquito vectors, it was found to be that most of all the studies only focussed on the crude extracts of plants, and none piloted a study of *Jasminum brevilobum*, *Aglaia edulis,* and *Pogostemon auricularius* induced free radical formation with special inference on microorganism concerned vector control strategies. Therefore, there are no other investigations are reported concerning the bioactive compound isolation and its mode of action on reactive oxygen species generation for mosquito vector control strategies development using the aforementioned plants.

It is very familiar in the mosquito control research to reports on the arthropod-borne viruses and its impacts in the global human population together with various infectious diseases including dengue, yellow fever, Zika, and chikungunya (Joseph et al. 2004; Anoopkumar et al. 2017b). Zika infection in the fetal stage can cause high risk by microcephaly and birth defects. These abnormalities might also link to Guillain-BarreÂ syndrome in most of the patients (Mittler et al. 2004; Tilak et al. 2004). In this regard, to gain extensive perceptions on the vector control strategies and the development of novel vector control approaches for preventing the severe mosquito-borne diseases, the potent phytochemical constituents of the selected medicinal plants can be used. An investigation on the mode of action of phytochemicals constituents from *J. brevilobum*, *A. edulis* and *P. auricularius* against *Ae. aegypti* larvae with special emphasis on oxidative stress and microorganism link can provide sufficient evidence to develop effective vector control strategies alternatives with new notions, as well as for verifying the potent medicinal plants for their future commercial benefits in mosquito-borne diseases eradication programmes. The output from the present investigation can be used for the development of mosquito larvicidal products, and are of more interest since they are the botanical origin and naturally non-persistent. These potential phytochemical constituents can, therefore, be applied to different habitats of *Ae. aegypti* mosquitoes larvae in an identical way as conventional larvicides in the future. This will destruct their life forms and prevents the rapid spread of mosquito-borne diseases such as Dengue, yellow fever, zika, and Chikungunya.

Conclusion

An inevitable concern of bioactive elements induced toxic effects in the target organism is the formation and accumulation of unstable and reactive intermediates of oxygen. Despite the prospective significance of bioactive elements induced oxidative stress, it remains relatively unexamined in microbes based vector control perspectives. In this study, we made several observations on how the accumulation of free radicals interrelates with the microbial consortia targeted vector control. Our findings add to the existing knowledge of mosquito vector-related microbiomes, principally the eukaryotes, to aid divulge microbial consortia with potential to prevent arboviral diseases and to highlight the need to comprehend how the free radicals and symbiotic microbial consortia influences the control of vector-borne disease transmission. We suggest a possibility that the annihilation of microbial, symbiotic inhabitants of mosquito vectors using the phytochemicals may potentially reduce the mosquito vector population thereby diminishing the increased epidemics of mosquito-borne diseases especially the Dengue, yellow fever, zika, and Chikungunya. Greater understanding of bioactive elements induced free radical formation and oxidative stress together with the elements underlying the mode of action are the significant implications for future vector control strategies.

Acknowledgments The authors would like to thank Principal, St. Joseph's College, Irinjalakuda for the laboratory facilities provided. The author Embalil Mathachan Aneesh thanks University Grants Commission, Government of India (UGC Research Award - F30-6/20-16(SA-II)) and Department of Biotechnology (BT/IN/Indo-US/Foldscope/39/2015) for funding.

Funding information The study was financed by UGC Research Award (F30–6/20–16(SA-II) and DBT Foldscope project (BT/IN/Indo-US/Foldscope/39/2015).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abirami P, Rajendran A (2012) GC-MS analysis of methanol extracts of *Vernonia cinerea*. Euro J Exp Biol 2:9–12

Aivazi AA, Vijayan VA (2009) Larvicidal activity of oak *Quercus infectoria* Oliv.(Fagaceae) gall extracts against Anopheles stephensi Liston. Parasitol Res 104(6):1289

Anoopkumar AN, Puthur S, Rebello S, Aneesh EM (2017a) Screening of a Few traditionally used Medicinal Plants for their Larvicidal Efficacy against *Aedes aegypti* Linn (Diptera: Culicidae), a Dengue Fever Vector. SOJ Microbiol Infect Dis 5:1–5. https://doi.org/10.15226/sojmid/5/4/00181

Anoopkumar AN, Puthur S, Varghese P, Rebello S, Aneesh EM (2017b) Life cycle, bio-ecology and DNA barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). J Commun Dis 49. https://doi.org/10.24321/0019.5138.201719

Anoopkumar AN, Puthur S, Rebello S, Aneesh EM (2019) Molecular characterization of *Aedes*, *Culex*, *Anopheles*, and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis. Biologia 1–14. https://doi.org/10.2478/s11756-019-00231-0

Arora R, Sharma P, Kapila K (1958) Antiarrhythmic and anticonvulsant activity of jatamansone. Indian J Med Res 46:782–791

Astill B, Terhaar C, Fassett D (1972) The toxicology and fate of 2, 2, 4-trimethyl-1, 3-pentanediol diisobutyrate. Toxicol Appl Pharmacol 22:387–399

Azambuja P, Garcia ES, Ratcliffe NA (2005) Gut microbiota and parasite transmission by insect vectors. Trends Parasitol 21:568–572. https://doi.org/10.1016/j.pt.2005.09.011

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New Phytol 127:617–633

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013) The global distribution and burden of dengue. Nature 496:504–507. https://doi.org/10.1038/nature12060

Bussmann R et al (2010) Minimum inhibitory concentrations of medicinal plants used in northern Peru as antibacterial remedies. J Ethnopharmacol 132:101–108. https://doi.org/10.1016/j.jep.2010.07.048

Cantrell CL, Dayan FE, Duke SO (2012) Natural products as sources for new pesticides. J Nat Prod 75:1231–1242. https://doi.org/10.1021/np300024u

Chen KKBM, Oxford UK (1965) Pharmacology of oriental plants vol 51. Pergamon Press Ltd

Coon KL, Valzania L, McKinney DA, Vogel KJ, Brown MR, Strand MR (2017) Bacteria-mediated hypoxia functions as a signal for mosquito development. Proc Natl Acad Sci U S A 114:E5362–E5369. https://doi.org/10.1073/pnas.1702983114

Devasagayam T, Tilak J, Boloor K, Sane KS, Ghaskadbi SS, Lele R (2004) Free radicals and antioxidants in human health: current status and future prospects Japi 52:4

Dillon R, Dillon V (2004) The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol 49:71–92. https://doi.org/10.1146/annurev.ento.49.061802.123416

Gallicchio V, Casale G, Watts T (1987) Inhibition of human bone marrow-derived stem cell colony formation (CFU-E, BFU-E, and CFU-GM) following in vitro exposure to organophosphates. Exp Hematol 15:1099–1102

Gulland A (2016) Zika virus is a global public health emergency, declares WHO. British Medical Journal Publishing Group https://doi.org/10.1136/bmj.i657

Jayakrishnan L, Sudhikumar AV, Aneesh EM (2018) Role of gut inhabitants on vectorial capacity of mosquitoes. J Vector Borne Dis 55:69. https://doi.org/10.4103/0972-9062.242567

Jones JD, Dangl JL (2006) The plant immune system nature 444:323. https://doi.org/10.1038/nature05286

Joseph C, Ndoile M, Malima R, Nkunya M (2004) Larvicidal and mosquitocidal extracts, a coumarin, isoflavonoids and pterocarpans from Neorautanenia mitis. Trans R Soc Trop Med Hyg 98:451–455. https://doi.org/10.1016/j.trstmh.2003.10.008

Kodak (1971) Company Reports 21MAY 21MAY

Kunwar A, Priyadarsini K (2011) Free radicals, oxidative stress and importance of antioxidants in human health. J Med Allied Sci 1:53–60. https://doi.org/10.1007/s11746-998-0032-9

Lenaz G, Bovina C, D'aurelio M, Fato R, Formiggini G, Genova ML, Giuliano G, Pich MM, Paolucci UG, Castelli GP, Ventura B (2002) Role of mitochondria in oxidative stress and aging. Ann N Y Acad Sci 959(1):199–213. https://doi.org/10.1111/j.1749-6632.2002.tb02094.x

Mandal SM, Chakraborty D, Dey S (2010) Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant Signal Behav 5:359–368. https://doi.org/10.4161/psb.5.4.10871

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498. https://doi.org/10.1016/j.tplants.2004.08.009

Oliveira Melo AS, Malinger G, Ximenes R, Szejnfeld PO, Alves Sampaio S, Bispo de Filippis AM (2016) Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: tip of the iceberg? Ultrasound Obstet Gynecol 47(1):6–7. https://doi.org/10.1002/uog.15831

Oliveira JHM, Talyuli OA, Goncalves RL, Paiva-Silva GO, Sorgine MHF, Alvarenga PH, Oliveira PL (2017) Catalase protects *Aedes aegypti* from oxidative stress and increases midgut infection prevalence of Dengue but not Zika. PLoS Negl Trop Dis 11:e0005525. https://doi.org/10.1371/journal.pntd.0005525

Pitarokili D, Michaelakis A, Koliopoulos G, Giatropoulos A, Tzakou O (2011) Chemical composition, larvicidal evaluation, and adult repellency of endemic Greek Thymus essential oils against the mosquito vector of West Nile virus. Parasitol Res 109:425–430. https://doi.org/10.1007/s00436-011-2271-1

Poole SK, Dean TA, Oudsema JW, Poole CF (1990) Sample preparation for chromatographic separations: an overview. Anal Chim Acta 236:3–42. https://doi.org/10.1016/S0003-2670(00)83297-3

Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem 269:337–341. https://doi.org/10.1006/abio.1999.4019

Puthur S, Anoopkumar AN, Rebello S, Aneesh EM (2018) Hypericum japonicum: a double-headed sword to combat vector control and cancer Applied biochemistry and biotechnology:1–11. https://doi.org/10.1007/s12010-018-2713-7

Puthur S, Anoopkumar AN, Rebello S, Aneesh EM (2019) Synergistic control of storage pest rice weevil using Hypericum japonicum and deltamethrin combinations: a key to combat pesticide resistance Environmental Sustainability:1–7. https://doi.org/10.1007/s42398-019-00086-w

Rao KVB, Munjal M, Patnayak A, Karthik L, Kumar G (2013) Phytochemical composition, antioxidant, antimicrobial and cytotoxic potential of methanolic extracts of *Adhatoda vasica* (Acanthaceae). Asian J Res Chem 6:1004

Rebello S, Anoopkumar AN, Puthur S, Sindhu R, Binod P, Pandey A, Aneesh EM (2018) Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour Technol Rep 3:1–6. https://doi.org/10.1016/j.biteb.2018.05.007

Rebello S, Balakrishnan D, Anoopkumar AN, Sindhu R, Binod P, Pandey A, Aneesh EM (2019a) Industrial enzymes as feed supplements—advantages to nutrition and global environment. In: Green Bio-processes. Springer, pp 293–304. https://doi.org/10.1007/978-981-13-3263-0_15

Rebello, S., Anoopkumar, A., Aneesh, E. M., Sindhu, R., Binod, P., & Pandey, A. (2019b). Sustainability and life cycle assessments of lignocellulosic and algal pretreatments. Bioresour Technol, 122678. https://doi.org/10.1016/j.biortech.2019.122678

Rosenberg E, Zilber-Rosenberg I (2011) Symbiosis and development: the hologenome concept Birth Defects Research Part C: Embryo Today: Reviews 93:56–66. https://doi.org/10.1002/bdrc.20196

Sahu R, Dhongade H, Pandey A, Sahu P, Sahu V, Patel D (2016) Medicinal Properties of Nardostachys jatamansi (A Review). Orient J Chem 32:859–866. https://doi.org/10.13005/ojc/320211

Sarwar M, Ahmad N, Toufiq M (2009) Host plant resistance relationshiphs in chickpea (*Cicer arietinum* l.) against gram pod borer (*Helicoverpa armigera*) hubner. Pak J Bot 41:3047–3052

Shaalan EA-S, Canyon D, Younes MWF, Abdel-Wahab H, Mansour A-H (2005) A review of botanical phytochemicals with mosquitocidal potential. Environ Int 31:1149–1166. https://doi.org/10.1016/j.envint.2005.03.003

Sies H (1997) Oxidative stress: oxidants and antioxidants experimental physiology: translation and. Integration 82:291–295

Team RC (2013) R: a language and environment for statistical computing

Thongwat D, Lamlertthon S, Pimolsri U, Bunchu N (2017) Larvicidal activity of endocarp and seed crude extracts of *Dracaena loureiri* Gagnep against *Aedes aegypti* (L.) mosquito. Asian Pac J Trop Biomed 7:222–226. https://doi.org/10.1016/j.apjtb.2016.12.005

Tilak JC, Adhikari S, Devasagayam TP (2004) Antioxidant properties of Plumbago zeylanica, an Indian medicinal plant and its active ingredient, plumbagin. Redox Rep 9:219–227

Valzania L, Martinson VG, Harrison RE, Boyd BM, Coon KL, Brown MR, Strand MR (2018) Both living bacteriaand eukaryotes in the mosquito gut promote growth of larvae. PLoS Negl Trop Dis 12:e0006638. https://doi.org/10.1371/journal.pntd.0006638

Vijayan V (2010) Laboratory selection of carbofuran tolerant line of Culex quinquefasciatus say, the filarial vector at Mysore. J Commun Disord 42(3):201–207

Wang Z, Perumalsamy H, Wang X, Ahn Y-J (2019) Toxicity and possible mechanisms of action of honokiol from *Magnolia denudata* seeds against four mosquito species. Sci Rep 9:411. https://doi.org/10.1038/s41598-018-36558-y

WHO (1986) Resistance of vectors and reservoirs of disease to pesticides: tenth report of the WHO Expert Committee on Vector Biology and Control [meeting held in Geneva from 5 to 11 November 1985]

WHO (2005) World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides Geneva: World Health Organization:p 1–41. https://apps.who.int/iris/handle/10665/69101

WHO (2018) World health statistics 2018: monitoring health for the SDGs, sustainable development goals. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO. Available at https://www.who.int/gho/publications/world_health_statistics/2018/en/

Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3:163. https://doi.org/10.1038/nprot.2007.521

Wright MH, Lee CJ, Arnold MSJ, Shalom J, White A, Greene AC, Cock IE (2017) GC-MS analysis of Tasmannia lanceolata Extracts which Inhibit the Growth of the Pathogenic Bacterium *Clostridium perfringens*. Pharmacogn J 9. https://doi.org/10.5530/pj.2017.5.100

Yadav KK, Bora A, Datta S, Chandel K, Gogoi HK, Prasad G, Veer V (2015) Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India Parasites & vectors 8:641. https://doi.org/10.1186/s13071-015-1252-0, 8

Yang Y-C, Lee S-G, Lee H-K, Kim M-K, Lee S-H, Lee H-S (2002) A piperidine amide extracted from Piper longum L. fruit shows activity against *Aedes aegypti* mosquito larvae. J Agric Food Chem 50:3765–3767. https://doi.org/10.1021/jf011708f

A novel intervention on the inhibiting effects of Catunaregam spinosa *induced free radical formation and DNA damage in* Aedes aegypti *(Diptera: Culicidae): a verdict for new perspectives on microorganism targeted vector control approach*

A. N. Anoopkumar, Sharrel Rebello, Embalil Mathachan Aneesh

International Journal of Tropical Insect Science

e-ISSN 1742-7592

Int J Trop Insect Sci
DOI 10.1007/s42690-020-00157-0

Springer

International Journal of Tropical Insect Science
https://doi.org/10.1007/s42690-020-00157-0

ORIGINAL RESEARCH ARTICLE

A novel intervention on the inhibiting effects of *Catunaregam spinosa* induced free radical formation and DNA damage in *Aedes aegypti* (Diptera: Culicidae): a verdict for new perspectives on microorganism targeted vector control approach

A. N. Anoopkumar[1,2] · Sharrel Rebello[2] · Ambalaparambil Vasu Sudhikumar[1] · Sreedev Puthur[2] · Embalil Mathachan Aneesh[2]

Received: 21 November 2019 / Accepted: 30 April 2020

Abstract

Plant extracts prompting free radical formation causing DNA damage among especially symbiotic microorganisms of *Aedes aegypti* has not been extensively examined in previous studies. Here, investigated whether: (1) it was possible to reduce the vectorial capacity by eliminating the microbial consortia of *Aedes aegypti* larvae, (2) the excess formation of free radical could induce damages of genomic DNA and alter the morphological appearance of *Aedes aegypti* larvae, and (3) which are the probable mechanisms driving the death of mosquitoes that has been treated with phytochemicals. The toxicity of *Catunaregam spinosa* seed extracts on the microbial community of *Aedes aegypti* larvae was determined as previously described. The formation of free radicals was confirmed using the ammonium molybdate method. While the GCMS method was used to assess the phytochemical analysis and the DNA lysing potential. The petroleum ether, ethanol, acetone and water extracts of *Catunaregam spinosa* has exhibited potent toxicity towards *Aedes aegypti* larvea's gut bacterial microbiota, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumonia*, and *Proteus mirabilis* with strong MIC values ranging from 0.07 mg/ml to 5.9 mg/ml. Plant extract induced free radical formations and oxidative damage to DNA. The presence of the bioactive element squalene justifies the insecticidal proprieties of *Catunaregam spinosa* extract. This study reflects the probable mechanisms underlying the lethal effect of *Catunaregam spinosa* extract on *Aedes aegypti*, and it potential as a novel biological vector control approach.

Keywords *Catunaregam spinosa* · *Aedes aegypti* · Larvicidal · Gut bacterial microbiota · Oxidative damage

✉ Embalil Mathachan Aneesh
aneeshembalil@stjosephs.edu.in

A. N. Anoopkumar
anoopkumar.247@gmail.com

Sharrel Rebello
sharrel.rebello@gmail.com

Ambalaparambil Vasu Sudhikumar
avsudhi@rediffmail.com

Sreedev Puthur
sreedevputhur@gmail.com

[1] Department of Zoology, Christ College Irinjalakuda, University of Calicut, Thenhipalam, Kerala, India

[2] Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Joseph's College (Autonomous), Irinjalakuda, Thrissur, Kerala, India

Introduction

Extension of mosquito-borne diseases into tropical and sub-tropical regions of the world exposed the medical importance of mosquitoes since they designated as a public threat over 100 countries (Kadir et al. 2013). The emergence of arboviral diseases such as yellow fever, dengue fever, Japanese encephalitis, dengue haemorrhagic fever, West Nile fever, chikungunya, and zika within the urban and rural regions was primarily triggered by the mosquito species *Aedes aegypti* (Souza 2016; Anoopkumar et al. 2017a; Anoopkumar et al. 2019). Regarding dengue fever infections, more reliable epidemiological data are accessible, and the rate of infections has to remain increased in recent years (Anoopkumar et al. 2017b; WHO 2017). Thus, control of the vector *Aedes aegypti* at the

Published online: 23 May 2020

larval stage would be a promising strategy to prevent the onset of such diseases than curing (AhbiRami et al. 2014).

The synthetic insecticides are profoundly potent in their part as an effective option in the prevention of various mosquito-borne diseases. However, the frequent use of man-made insecticides has disrupted the biological control systems and ecosystems; and has resulted in the emergence of insecticide resistance in mosquito vectors. The emergence of insecticide resistance in mosquitoes coupled with the economic and environmental burden calls for an exigent alternative to conventional synthetic approaches (Nwabor et al. 2017). Recent research trends consider the bioactive elements retrieved from plants as alternative products to synthetic larvicides for the reason that the plant extract comprises a plethora of phytochemical constituents that are specific in mosquito larvae (Govindarajulu et al. 2015; Tiwary et al. 2007). The use of bioactive elements from locally available medicinal plants as natural larvicides for mosquito vector control would generate inexpensive methods and reduce dependence on imported synthetic products (Bowers et al. 1995; Puthur et al. 2018; Puthur et al. 2019). The phytochemical constituents that are responsible for the pharmacological property of *Catunaregam spinosa* has been verified in previous literature by Patil and Khan 2017. The various phytochemical constituents of the medicinal plants could also induce apoptosis in tumor cells by means of generating excessive free radicals (Forcados et al. 2017).

Several studies from all over the world are trying to analyse the mechanisms leading the development of insecticide resistance (Aivazi and Vijayan 2010; Aneesh and Vijayan 2010; Liu 2015). Established documents on the aforesaid aspect have exposed the links between microbial consortia (especially bacteria) and insecticide resistance (Kikuchi et al. 2012). Previous studies have also confirmed that the mosquitoes host specific groups of living microbes including bacterial population for their life cycle completion (Vogel et al. 2017). For instance, about approximately100 kinds of bacterial species have been harbored within the *Aedes aegypti* fourth instar larvae (Vogel et al. 2017) and they can also induce the vector susceptibility to disease-causing microorganisms.

The excessive productions of free radicals can alter DNA, disturbing the normal biological activities and triggering oxidative stress (Neumann et al. 2003). The elevated oxidative DNA damage together with the oxidation of DNA has been noted as the prominent modifications induced by oxidative stress (Cooke et al. 2006). The increased reactive oxygen species (ROS) in animals has been associated with shorter lifespan (Sinha et al. 2014) including the shortening of telomeres responsible for cellular aging, gradually contributing to lethal condition (Kawanishi and Oikawa 2004). Considering the increasing global vector-borne disease burden, a systematic understanding of the elements causing larval death and insecticide resistance is essential to allay its growing risk to mosquito control strategies (Dada et al. 2018).

Therefore, we cooperatively inferred and interpreted these aspects and assign that several disease-causing mosquito vector species necessitate living microbial consortia in their body for developmental processes. This investigation targets those microorganisms exist inside the mosquito larvae. The impact of plant extract induced oxidative stress and the influence of microbial consortia over mosquito larvae with special reference to epidemiological significance has not been well established in previous studies. Therefore, to eradicate the rapid proliferation of mosquito transmitting disease we need to reveal the probable mode of mechanisms of mosquito larval death against botanical insecticides. The main focus of this study is to disclose the probable mechanisms that could drive the death of plant extract exposed mosquito larvae, and to open new perspectives and notion for mosquito vector control by targeting the microbial consortia.

Materials and methods

Collection and preparation of plant material

Healthy seeds of *Catunaregam spinosa* (Fig. 1) were collected from Thirunelly, Wayanad a part of Western Ghats, Kerala, India. The GPS coordinates (11°53′30.25″ N, 76°0′52.25″E) of the selected plant was also recorded. The powdered material was extracted successively by using different solvents (petroleum ether, ethanol, acetone, and water) in Soxhlet apparatus.

Mosquito culture and larval bioassay

Aedes aegypti larvae were collected from Communicable Diseases Research Laboratory department of Zoology

Fig. 1 *Catunaregam spinosa*

(CDRL), St. Joseph's College (Autonomous), Irinjalakuda, Thrissur, Kerala, India (15-May-2018). The experimental insects were reared under the following conditions; 55–60% relative humidity, 12 L:12 D photoperiod 110 cycle followed by 27 ± 2 °C temperature (Anoopkumar et al. 2020). Larvicidal efficacy was evaluated according to standard guidelines provided by World Health Organization (WHO 2005). Triplicates were set up for each concentration with corresponding controls.

Mechanism of larvicidal action

Toxicity towards microorganisms

The screening of antibacterial potential of the seed extracts of *C. spinosa* was done against human pathogenic bacteria including the bacterial population that reside inside the mosquito larval gut viz., *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *E. coli* (MTCC 443), *Klebsiella pneumonia* (MTCC 661) and *Proteus mirabilis* (MTCC 442). The petroleum ether, ethanol, acetone and water extracts of *C. spinosa* seeds at different concentrations (10, 25, 50, and 100 micrograms) were loaded on 10-mm discs placed on microbe plated muller hinton agar plates along with suitable controls and incubated for 15 to 18 h at 37 °C. The diameter of inhibition zones (in millimetre) was noted and compared against positive standard (chloramphenicol). All executed experiments were maintained triplicates and values were expressed as the mean ± standard deviation (SD). Broth microdilution method was employed for the determination of minimum inhibitory concentrations. The Minimum inhibitory concentration (MIC) of petroleum ether, ethanol, acetone, and water extracts of *C. spinosa* seeds was determined using the method prescribed by (Wiegand et al. 2008) and (Bussmann et al. 2010). Triplicates were maintained for each assay and potent activity was expressed as MIC.

Phytochemicals induced free radical generation and DNA lysing potential

The lethal effects of petroleum ether, ethanol, acetone, and water extracts of *C. spinosa* seed induced free radical formation was manipulated by verifying the morphological changes in the treated and untreated fourth instar *Ae. aegypti* mosquito larvae. Morphological changes in the head, abdomen and anal gills region of *Ae. aegypti* fourth instar larvae were examined by Olympus CH -20i Microscope connected to the digital camera. The oxidative stress in the treated and untreated larval tissues was verified by estimating their free radicals by ammonium molybdate method (Prieto et al. 1999). Twenty fourth instar larvae of *Ae. aegypti* were treated with varying concentrations of seed extracts (125 mg/L, 250 mg/L, 500 mg/L, and 1000 mg/L) against untreated controls of larvae grown in similar laboratory conditions. The treated and untreated larvae after 24 h of exposure were sacrificed, homogenized in distilled water and centrifuged to obtain free radicals released from the body. The level of free radicals generated in the untreated and treated mosquito larvae was calculated by treating 1 ml of larval suspension with 3 ml reaction mixture (10 ml H_2SO_4 + 1.47 g ammonium molybdate +1.005 g sodium phosphate monobasic +290 ml of distilled water) and incubated in water bath at 95 °C for 1 h. One millilitre of distilled water dissolved in 3 ml of reaction mixture served as the blank solution. The extent of free radicals formed in the untreated and treated samples were recorded at 695 nm in Ultraviolet-visible spectroscopy.

The DNA lysing potential of the extract was assessed with the seed extract and analyzing the extent of damage by agarose gel electrophoresis. The untreated DNA served as a negative control; whereas 3% H_2O_2 treated DNA served as a positive control for DNA damage (Guha et al. 2011).

Analysis of bioactive compounds

The active chromatographic fraction of the plant extract was further analyzed for its phytocompounds by GC-MS (Al Yahya et al. 2018). The seed extracts of *C. spinosa* were analyzed by GC-MS (gas chromatography-mass spectrometry) (GC-7890 A, Agilent Technologies). We employed the approaches used by previously reported findings (Cheng et al. 2009; Mdoe et al. 2014), which used DB 5-MS capillary standard column. The length, diameter and film thickness of the column was 30 m, 0.25 mm and, 0.25 μm respectively.

Helium was chosen as a carrier gas (flow rate- 1.0 ml/min). The ideal mass spectrum was gained at 70 eV. The individual constituents were recognized and identified using RRPLIB and MAINLIB database library. The isolated compounds were compared with the retention time (RT) values of reference compounds from MAINLIB and REPLIB library for identification.

Statistical analysis The log-probit regression analysis was employed for calculating LC_{50}, LC_{90} and 95% Confidence limit (upper confidence and lower confidence). The other statistics including the calculation of Chi-square values, analysis of absorbance of free radicals and antibacterial activity has been performed using SPSS Statistical software package version 24.0.0.

Results

Toxicity towards microorganisms

The petroleum ether, ethanol, acetone and water seed extracts of *C. spinosa* were firstly tested using Disc diffusion method

against *S. aureus* (MTCC 3160), *B. subtilis* (MTCC 441), *E. coli* (MTCC 443), *K. pneumonia* (MTCC 661) and *P. mirabilis* (MTCC 442). The initial screening included four different seed extracts of *C. spinosa* with potent antibacterial activity which was preferred for the present study to find their MIC values. Tables 1 and 2 shows the potent antibacterial activity of *C. spinosa* seed extracts against different types of pathogenic bacteria that reside inside the mosquito larvae. The sensitivity of Gram negative and Gram-positive bacteria towards the *C. spinosa* seed extract varied amid the tested strains and all the five strains were conspicuously sensitive to the different extracts (Fig. 2). The extracts in different concentration were also subjected to the MIC value determination. The petroleum ether, ethanol, acetone, and water seed extracts of *C. spinosa* inhibited *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumonia*, and *P. mirabilis*. The low MIC values against these bacteria species indicate potent antibacterial property. The water extracts of *C. spinosa* showed potent activity towards *S. aureus* (19.25 ± 0.430, MIC = 0. 07 mg/ml), *B. subtilis* (19.00 ± 1.732, MIC = 0.08 mg/ml), *E. coli* (15.83 ± 0.886, MIC = 0.88 mg/ml), *K. pneumonia* (17.67 ± 0.577, MIC = 3.0 mg/ml), and *P. mirabilis* (16.33 ± 1.155, MIC = 0.73 mg/ml). The acetone extracts of *C. spinosa* exhibited effective activity against Gram positive and Gram-negative bacteria, with MIC values ranging from 0.5 mg/ml to 3.2 mg/ml. The entire sample belonging to the ethanol extracts of *C. spinosa* was characterized by better antibacterial potential towards all the bacteria population with MIC values ranging from 0.4 mg/ml to 2.1 mg/ml. The petroleum-ether seed extracts also exhibited a considerable range of MIC values from 0.8 to 5.9 mg/ml. The water seed extract shows lowest MIC values ranging from 0.07 mg/ml to 3 mg/ml towards the bacteria population and would be renowned as remarkable candidates for future studies.

Larvicidal efficacy

The larvicidal efficacy was accomplished by using seed extracts of *C. spinosa*. All the extracts revealed potent toxic effect on *Ae. aegypti* fourth instar larvae after 24 h of continuous exposure. The highest larvicidal efficacy was observed in the petroleum ether (Table 3 and Fig. 3) seed extracts of *C. spinosa* (LC50–184.257 mg/L and LC_{90}–944.595 mg/L). The water seed extract of *C. spinosa* also exhibits significant larvicidal efficacy with an LC_{50} of 210.212 mg/L on 24-h exposure against *Ae. aegypti* fourth instar larvae (Fig. 3). Alike trends have been renowned for the acetone (LC50–248.680 mg/L and LC_{90}–2415.714 mg/L) and ethanolic (LC50–465.224 mg/L and LC_{90}–5993.348 mg/L) extracts of *C. spinosa* against fourth instar larvae of *A. aegypti*.

Mechanism of larvicidal action

The microscopic examination of *C. spinosa* seed extract treated larvae of *Ae. aegypti* showed structural damages on the head (Fig. 4(a-1), abdomen (Fig. 4(a-2) and thorax (Fig. 4(a-3); the untreated larvae did not exhibit any external variance in their body (Fig. 4(b-1-3). During the present research, the morphological changes of the *C. spinosa* seed extract treated larvae have also been observed. Moreover, the larvae administrated with *C. spinosa* showed striking activities in terms of behavioral aspects such as sluggishness, restless movement followed by paralysis and death.

Administration of *C. spinosa* (seed) water extracts to fourth instar larvae of *Ae. aegypti* made prominent alterations in the naturally produced reactive oxygen species and it causes oxidative stress, leads to the death of the organism. The absorbance levels of free radicals at 695 nm were significantly increased in the water extract of *C. spinosa* (seed) (Fig. 5(a) than the extract homogenized with larvae unexposed to the plant extract treatment (Table 4). *C. spinosa* (seed) petroleum ether extracts were comparatively showed the low level of absorbance (Fig. 5(b) than the water extract. Very few free radicals were generated in homogenized extract containing larvae and distilled water (Fig. 5(c). The ethanol (Fig. 5(d), and acetone extract (Fig. 5(e) of *C. spinosa* exhibited a considerable degree of free radical generation. This indicated that the free radicals generated by *C. spinosa* (seed) water extract

Table 1 Determination of toxic effects of *Catunaregam spinosa* seed extracts against pathogenic bacteria at 50 µl. (Zone of inhibition measured in mm)

Human pathogenic Bacteria Sp.	*Type*	*Catunaregam spinosa* seed (Zone of inhibition (mm)			
		Petroleum ether	Ethanol	Acetone	water
Staphylococcus aureus	Gram-positive	9.02 ± 0.462	13.56 ± 0.224	12.01 ± 1.24	19.25 ± 0.430
Bacillus subtilis	Gram-positive	10.20 ± 0.900	14.45 ± 0.338	15.57 ± 1.020	19.00 ± 1.732
Escherichia coli	Gram-negative	10.34 ± 0.820	9.87 ± 0.240	13.45 ± 1.34	15.83 ± 0.886
Klebsiella pneumonia	Gram-negative	9.66 ± 0.678	10.91 ± 0.543	12.01 ± 0.210	17.67 ± 0.577
Proteus mirabilis	Gram-negative	16.67 ± 1.528	14.24 ± 0.166	10.44 ± 0.590	16.33 ± 1.155

Table 2 Minimum inhibitory concentrations of *Catunaregam spinosa* seed extracts of increasing polarity against tested bacteria

	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumonia*	*Proteus mirabilis*
Catunaregam spinosa seed Petroleum ether extract	1.04	1.32	1.9	5.9	0.81
Catunaregam spinosa seed Ethanol extract	0. 40	0.80	1.83	2.1	0.96
Catunaregam spinosa seed Acetone extract	0.50	0.60	0.90	3.2	1.3
Catunaregam spinosa seed Water extract	0. 07	0.08	0.88	3	0.73

MIC minimum inhibitory concentration mg/ml

cause oxidative stress and recognized as one of the reasons for the death of *Ae. aegypti* fourth instar larvae. These free radicals were also inducing oxidative damage to DNA when treated with plasmid DNA as an in vitro experiment to detect DNA damage (Fig. 6).

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of petroleum ether seed extract of *C. spinosa* showed 31 peaks which specify the presence of 31 phytochemical constituents (Fig. 7(a), Table 5). Based on the evaluation of the mass spectra of the constituents with the MAINLIB, REPLIB library, 31 compounds were characterized and recognized (Table 5). The major chemical compounds were identified based on the highest peaks are Heptacosane (4.499%) (Fig. 7(b), Hexanedioic acid (1.03%) (Fig. 7(c), 2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-(Squalene)(20.782%)(Fig. 7(d), Octacosane (8.489%)(Fig. 7(e), Tetratetracontane (54.058%), and Decane (6.134%).

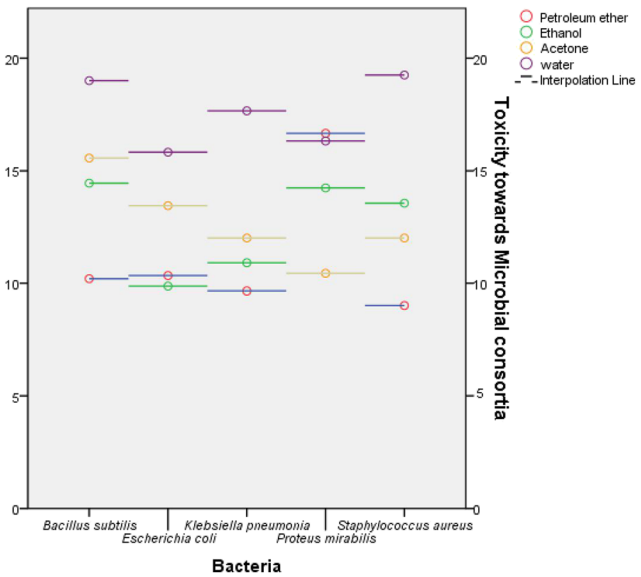


Fig. 2 Antibacterial activity of petroleum ether and water extracts of *Catunaregam spinosa* seed

Discussion

The principal focus of this study was to explicate the underlying mechanisms of the plant extract induced mosquito larval death since the mode of action, chemical framework, toxicity towards mosquito population have previously not been studied. There has been extensive apprehension regarding the forecast of mosquito-borne diseases eradication in the recent years (Ioos et al. 2014). In addition, previous studies have shown that the mosquito vector responsible for the transmission of zika virus infection have interrelated with several microorganisms including bacteria surrounding their environment, and which might lead to the establishment of an effective symbiotic association throughout their lifetime (Villegas et al. 2018). Until now, microorganisms specifically bacteria are documented as well studied part of the microbial research based on mosquito model aspects (Saldaña et al. 2017; Rebello et al. 2018; Rebello et al. 2019a; Rebello et al. 2019b). Results from the several vector models have consistently verified the symbiotic relationship between arthropod hosts and bacterial consortia (Dong et al. 2017).

In this study, the bacterial action of *C. spinosa* seed extracts was determined against Gram-negative and Gram-positive bacteria viz., *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumonia*, and *P. mirabilis*. Previous studies have shown that the cell wall of gram-negative bacteria is persisted as an effective obstacle against candidate extract since the cell wall comprises efflux pumps responsible for expelling the elements that pass over the membrane (Brown et al. 2014). In our study, the water extract of *C. spinosa* seeds has shown a high degree of toxicity towards the bacterial species (Tables 1 and 2). These results are in a pact with previously published reports that revealed the high antimicrobial activity of plant extracts against microbes of epidemiological significance (Hsueh et al. 2015; Vu et al. 2017). Mostafa et al. 2018 reported that *Cuminum cyminum*, *Punica granatum*, *Syzygium aromaticum*, *Thymus vulgaris*, *and Zingiber officinale* extracts exhibit antibacterial activity against Gram positive and Gram-negative bacteria including *E. coli* and *S. aureus* with strong MIC values (0.1 to 2.31 mg/ml). The presence of *S. aureus*, *K. pneumonia*, and *Bacillus* sp., inhabiting the *Ae.*

Table 3 Larvicidal efficacy of *Catunaregam spinosa seed extract against* the fourth instar larvae of *Aedes aegypti*

Plant	Sample	LC_{50} (ppm LCL-UCL	LC_{90} (ppm) LCL-UCL	Chi Square value (χ^2)	*P* value
Catunaregam spinosa	Petroleum ether	184.257 (105.869–261.856)	944.595 (589.288–2651.425)	2.19	.000
	Ethanol	465.224 (273.753–1115.004)	5993.348 (1891.211–588,581.062)	0.311	.001
	Acetone	248.680 (124.335–398.460)	2415.714 (1077.393–31,257.200)	0.334	.001
	Water	210.212 (66.898–364.010)	3205.896 (1172.989–248,686.198)	0.214	.002

*Values were significant at the $P < 0.05$ level

LC_{50} – lethal concentration that kills 50% of the exposed larvae, LC_{90} – lethal concentration that kills 90% of the exposed larvae

UCL upper confidence limit, *LCL* lower confidence limit, χ^2 – chi square

aegypti mosquito larvae has a prominent impact in host-parasite interaction and they would influences the vectorial capacity of vector-borne diseases (Yadav et al. 2015). Our study suggests that the microorganisms targeted mosquito vector control strategies will never eradicate the whole mosquito population, and this approach will provide a priority for the sustained conservation of mosquito population. This approach will also reduce the chance of insecticide resistance development (Kikuchi et al. 2012). The toxic property of the *C. spinosa* seed extract towards the microbial population (*S. aureus*, *B. subtilis*, *E. coli*, *K.pneumonia*, and *P.mirabilis*) would considered as a prominent reason for the death of mosquito larvae since previous studies have mentioned that the bacterial density (Valzania et al. 2018) have strongly interlinked with the rate of the larval growth of *Ae. aegypti* mosquitoes. This may be an innovative approach to prevent the rapid proliferation of mosquitoes as for the reason that the seed extract can exterminate the microorganisms that share the symbiotic relationships with *Ae. aegypti* mosquito population.

The use of plant-derived products recognized with antimicrobial activities might exhibit much more significance in therapeutic purposes since the extract constitute different kinds of phytochemical constituents that have different biological functions (Howard et al. 2007; Iloki-Assanga et al. 2015). For instance, the phytochemicals such as saponins, alkaloids, and tannins have medicinal and pesticidal property. The phytochemical constituents such as flavonoids, alkaloids, tannins, and saponins from plants are known by their insecticidal toxicity to other organisms (Valgas et al. 2007). This study has also reported the presence of the bioactive compound squalene (Table 5) and this justifies the medicinal, insecticidal, therapeutic benefits of our plant extract. Previously published reports (Adeosun et al. 2017) have mentioned that this compound exhibit the aforementioned activities. Our findings might also produce a link between previous reports that revealed the leaf extract of *C. spinosa* shown the antioxidant property for DPPH and FRAP assays (Surabhi and Leelavathi 2010). Previous studies produced a review of plant extracts as

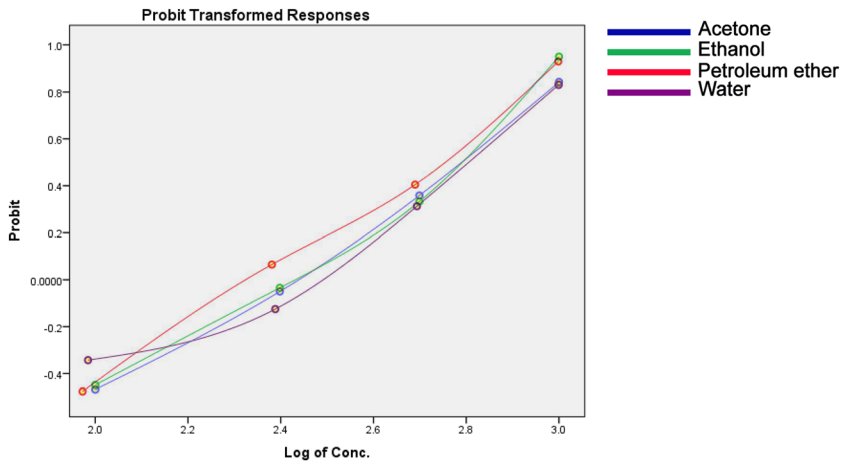


Fig. 3 Regression graph showing mortality of different solvent extracts of *Catunaregam spinosa* seed against *Aedes aegypti* fourth instar larvae

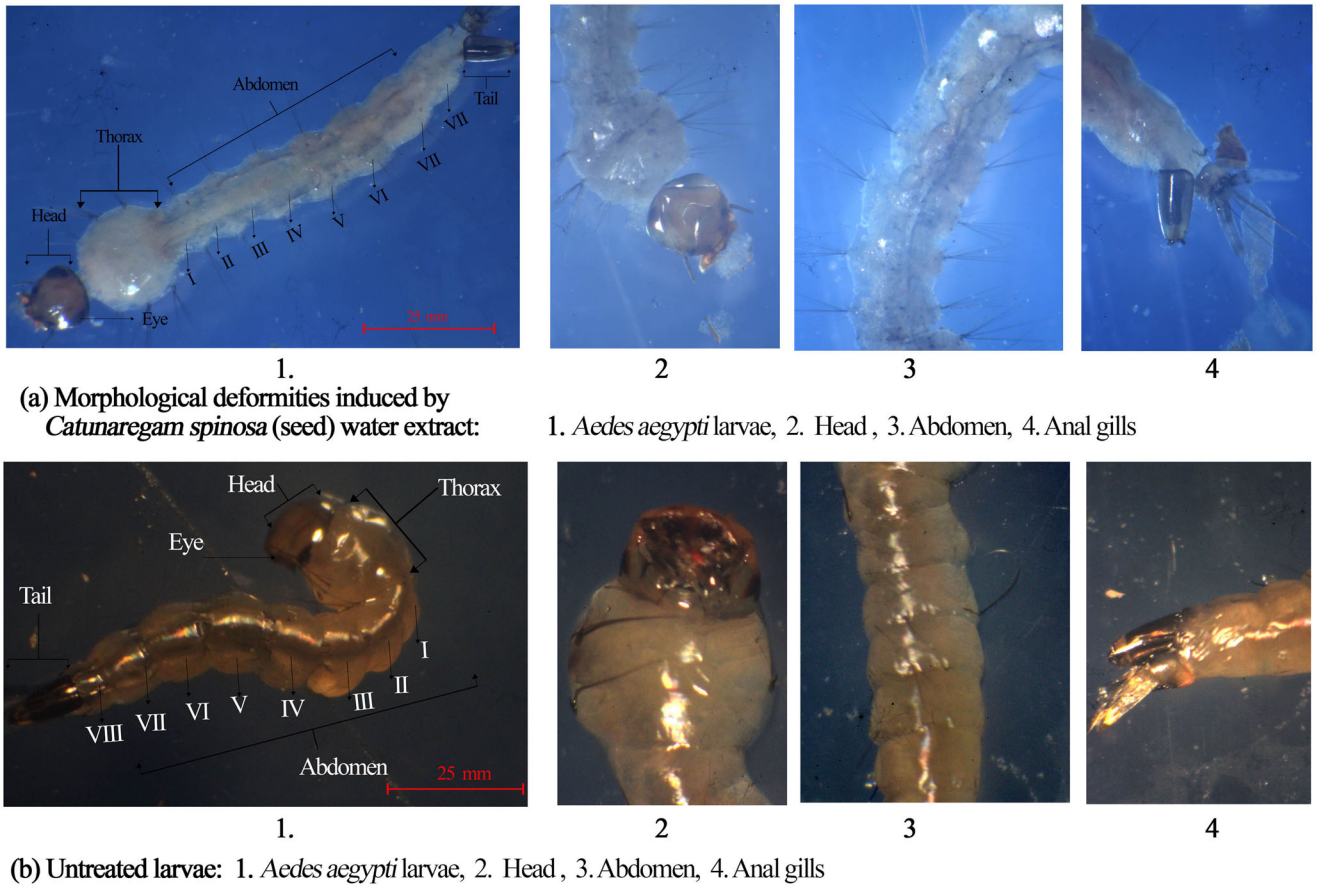


Fig. 4 *Catunaregam spinosa* seed extract induces histological modifications on *Ae. aegypti* larvae fourth instar larvae. Light micrographs of head, thorax, abdomen, and anal gills regions of *Aedes aegypti* fourth instar larvae with treatment (**a**) and without (**b**) ×40 magnification

mosquito larvicides and reported that octacosane (also predominant in our extract- Fig. 7(e), germacrene D and azadirachtin, found to be poisonous to multiple species (Ghosh et al. 2012). The phytochemicals of plant origin such as octacosane, falcarcinol, granial, azadirachtin, pipernonaline, plumabagin etc. are active larvicide (Kishore et al. 2011). The presence of these compounds in our extract justifies its insecticidal property. The tetratetracontane confirmed has antioxidant and cytoprotective activities (Ertas et al. 2014) and this justifies the medicinal properties of our extract too. Phenolic compounds such as tannins, flavonoids, and furanocoumarins retrieved from plants might generate free radicals in the insect species (Chaitanya et al. 2016).

This study also reports the larvicidal efficacy of *C. spinosa* seed extracts for the first time. In our study, the potential larvicidal efficacy of petroleum ether seed extract (184.257 mg/L) is recognized as prominent and effective against *Ae. aegypti* fourth instar larvae. In addition, the ability of the water seed extract of *C. spinosa* with an LC_{50} of 210.212 mg/L on 24-h exposure to cause mortality of *Ae. aegypti* is an added advantage to the layman to prepare mosquito larvicidal extracts rather than using organic chemicals. These results are corroborated to the formerly reported findings who described the larvicidal efficacy of *Murraya koenigii* (Kumar et al. 2012) plant extracts against yellow fever mosquito vector *Aedes aegypti*. We are also able to verify the probable mode of action of the seed extract on mosquito larval death. The modes of action of the seed extract in this study can principally attribute to the formation of free radicals followed by DNA damage. The extracts of botanical origin are reputed to have eco-friendly and prominent high toxicity compared to chemical insecticides for mosquito vectors of epidemiological significance (Sarwar et al. 2009).

The excessive formation of free radicals results in disparity between the antioxidants and pro-oxidants. The ROS metabolism strongly influences the vector competence, immune response, and fecundity in mosquitoes (Ha et al. 2005; Kumar et al. 2003; Oliveira et al. 2011) and the increased levels of ROS might interrupt the redox signaling pathways (Jones 2006). The enhanced generation of these reactive oxygen species (ROS) along with simultaneous damage to proteins, nucleic acids, and cell organelles might leads to the death of the organism (Kodrík et al. 2015). In our study, it was observed that the free radicals generated by *C. spinosa* (seed)

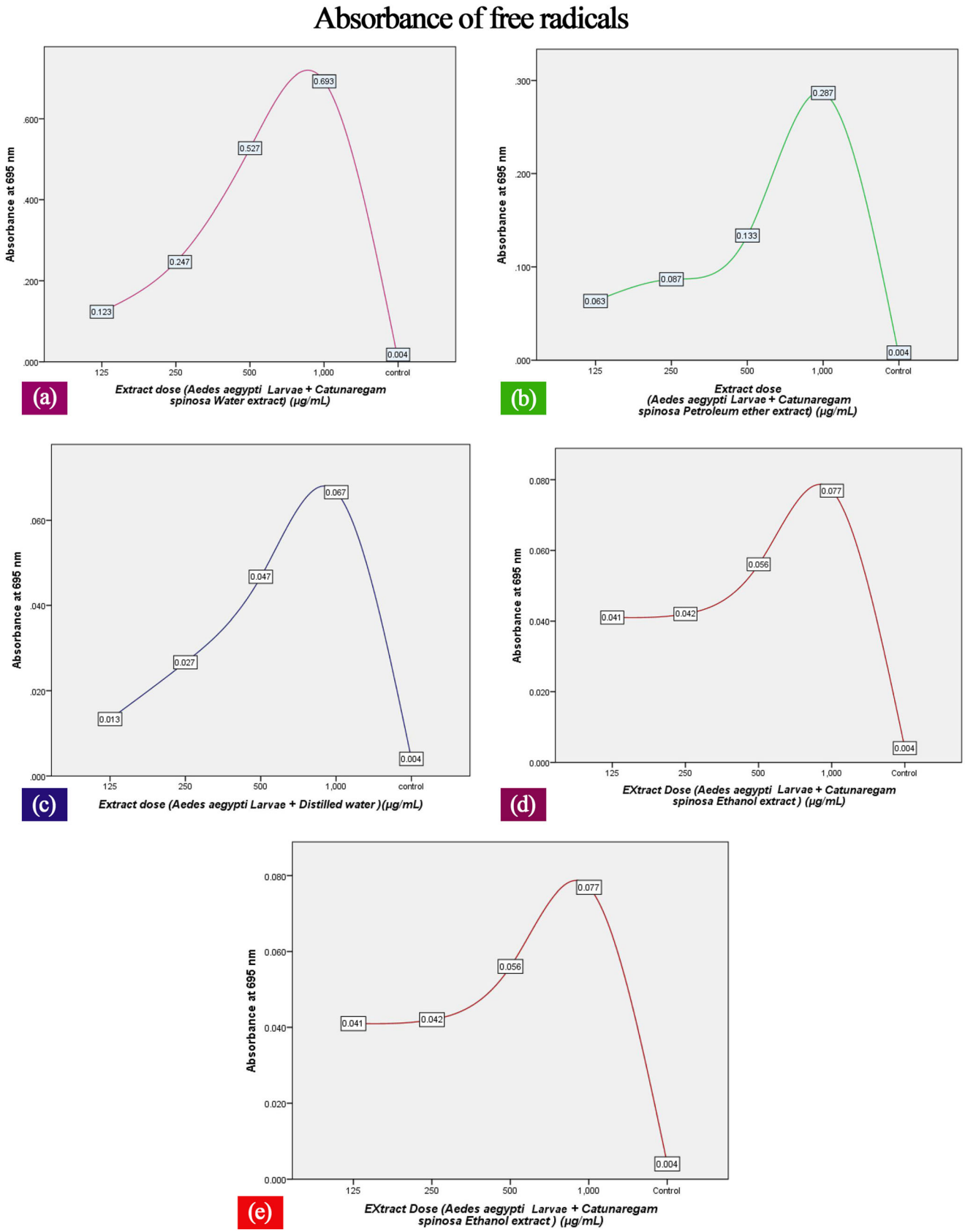


Fig. 5 Mechanism of larvicidal action. Determination of free radicals of *Catunaregam spinosa* seed extract exposed *Aedes aegypti* larvae

Table 4 Determination of free radicals from plant extract exposed *Aedes aegypti larvae*. Experiments were executed in triplicates

Sample	Extract Dose(μg/mL)	Absorbance Mean ± SD
Aedes aegypti Larvae + Distilled water (μg/mL)	125	0.013 ± 0.006
	250	0.027 ± 0.006
	500	0.047 ± 0.006
	1000	0.067 ± 0.006
Aedes aegypti Larvae + *Catunaregam spinosa* Acetone extract (μg/mL)	125	0.063 ± 0.005
	250	0.087 ± 0.005
	500	0.133 ± 0.010
	1000	0.287 ± 0.005
Aedes aegypti Larvae + *Catunaregam spinosa* Water extract (μg/mL)	125	0.123 ± 0.006
	250	0.247 ± 0.005
	500	0.527 ± 0.005
	1000	0.693 ± 0.006
Aedes aegypti Larvae + *Catunaregam spinosa* Ethanol extract (μg/mL)	125	0.017 ± 0.05
	250	0.024 ± 0.002
	500	0.037 ± 0.002
	1000	0.051 ± 0.005
Aedes aegypti Larvae + *Catunaregam spinosa* Petroleum ether extract (μg/mL)	125	0.041 ± 0.002
	250	0.042 ± 0.002
	500	0.056 ± 0.016
	1000	0.077 ± 0.006

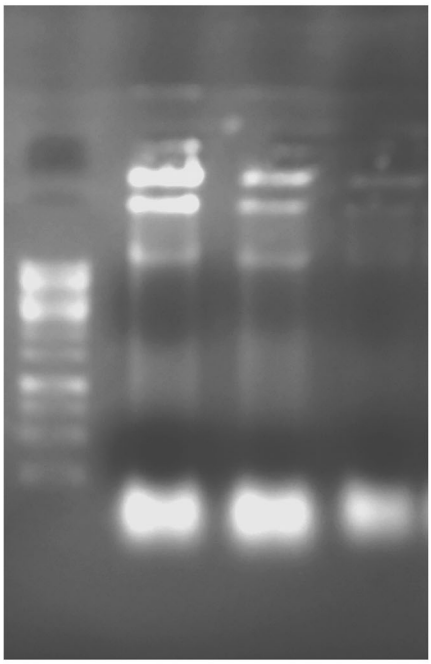
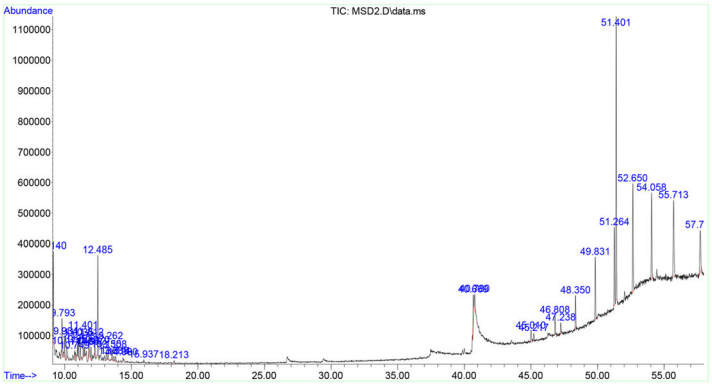


Fig. 6 Mechanism of larvicidal action- *Catunaregam spinosa* seed extract induces DNA damage

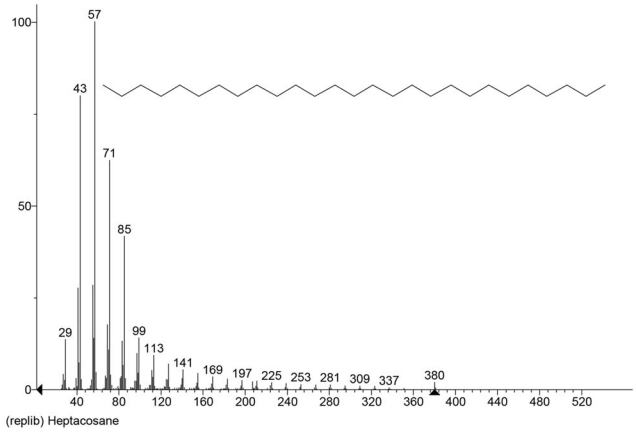
petroleum ether, ethanol, acetone, and water, extract cause oxidative stress, and recognized as one of the reasons for the death of *Ae. aegypti* fourth instar larvae. Our results validated that the free radicals generated by *C. spinosa* (seed) water extract also induce oxidative damage to DNA when treated with plasmid DNA as an in vitro experiment to detect DNA damage. Any impairment of the DNA might cause severe alterations in the encoded proteins, resulting in the protein production inhibition. Deoxyribose oxidation, nucleotide removal, strand breakage and alterations in the nitrogenous bases are recognized as the different forms of DNA oxidative damage induced by free radicals (Sharma et al. 2012). Hence, DNA oxidative damage induced by the free radicals from *C. spinosa* (seed) water extract resulting in cellular damage might be recognized as another reason for the death of *Ae. aegypti* fourth instar larvae.

In addition, the microscopic analysis of this study clearly specifies that the excessive formation of free radicals caused morphological alterations in head, abdomen, and thorax, and anal gills regions in the fourth instar larvae of *Ae. aegypti*. Similar to our results, several previously published reports revealed alterations induced by candidate drugs of synthetic origins (Farnesi et al. 2012; Wallis and Griffin 1973). Our findings indicate that the various phytochemical constituents in the plant extract triggered the morphological alterations and inhibition of the normal functioning of the mosquito larvae. These observations are in line with the previously published report made by Bekele et al. 2014 who found that the breeding sites of mosquitoes easily be discovered for spraying plant-derived products and use of this inexpensive botanical extract will destruct their life cycle and securely diminish the rapid multiplication of mosquito population (Bekele et al. 2014). Therefore, the plant-derived products can either be used as insecticides or repellent for killing mosquito larvae or for the protection from mosquito bite respectively depending on the type of activity they exhibited. The biological control of vector mosquito population is more effective and eco-friendly. The recommendation offered by our study is that the water extract of *C. spinosa* seed can be directly prepared in the home with minimum cost and struggle and might be used as a potential natural larvicide against *Ae. aegypti* in future. We are also able to give the possible mode of action of the plant extract as well as the phytochemical constituents against the mosquito larval death. This study demonstrates a novel linkage between the mosquito larval response and the various mechanisms involved in the larval death to plant extract exposure with special reference to the impeding effects of various phytochemical constituents induced free radical formation against the yellow fever vector *Ae. aegypti* (Diptera: Culicidae).

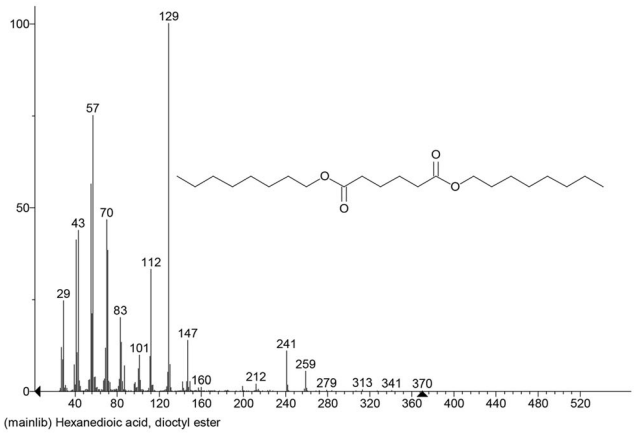


(a) GC-MS Chromatogram of Chemical compounds identified in petroleum ether extract of *Catunaregam spinosa*.

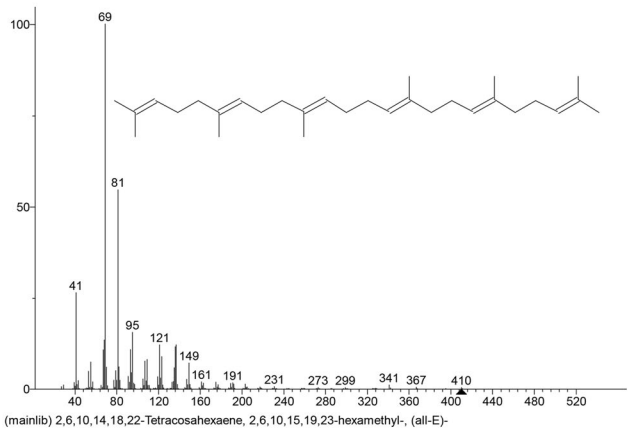
GCMS Spectrum of compounds having larvicidal property



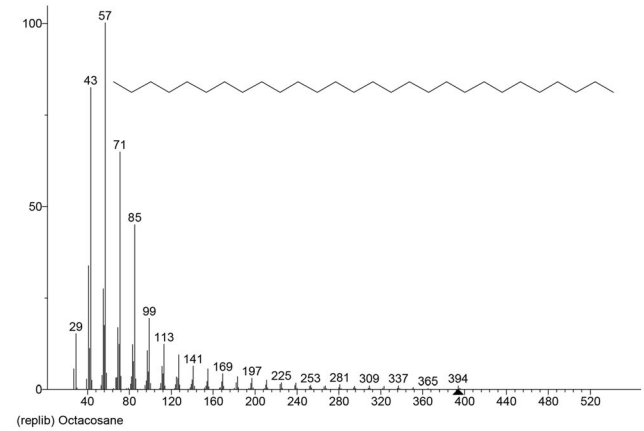
(b) GC-MS Spectrum of Heptacosane



(c) GC-MS Spectrum of Hexanedioic acid, dioctyl



(d) 2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-, (Squalene)



(e) GC-MS Spectrum of Octacosane

Fig. 7 GC-MS analysis of Chemical compounds identified in petroleum ether extract of *Catunaregam spinosa* seed

Conclusion

In summary, the present study made a first and foremost report on the impeding effects of plant extracts induced free radical formation and DNA damage toward *Ae. aegypti* (Diptera: Culicidae) mosquito larvae. It was noted that the water extract of *C. spinosa* seed had the utmost toxic effects on the microbial consortia which maintain a symbiotic relationship with arthropod diseases vectors especially the dengue fever vector *Ae. aegypti* in all executed experiments. The toxic effects on the microbial consortia (*S. aureus*, *B. subtilis*, *E. coli, K. pneumonia, P. mirabilis*) have also been renowned as a probable

Table 5 Chemical composition of Petroleum ether seed extract from *Catunaregam spinosa*

Sl. no	RT	Area %	Compound name
1	9.140	5.507	Oxirane, 2,2-dimethyl-3-propyl-
2	9.793	2.306	2-Hexene, 1-(1-ethoxyethoxy)-
3	9.984	1.152	Octane, 3,6-dimethyl-
4	10.171	1.363	Heptane, 3-ethyl-2-methyl-
5	10.749	0.284	2-Nonen-1-ol,
6	10.953	0.672	Trichloroacetic acid, 6-ethyl-3-octyl ester
7	11.035	1.366	Nonane, 4-methyl-
8	11.173	1.104	Nonane, 2-methyl-
9	11.401	1.755	Octane, 4-ethyl-
10	11.488	0.491	Cyclohexane, 1-ethyl-2,3-dimethyl-
11	11.812	1.435	Cyclohexane, 1-methyl-2-propyl-
12	11.972	1.142	m-Menthane
13	12.270	0.606	Sulfurous acid, cyclohexylmethyl octadecyl ester
14	12.485	6.134	Decane
15	13.130	0.822	3-Trifluoroacetoxydodecane
16	13.262	1.244	Octane, 1,1′-oxybis-
17	13.558	0.616	Cyclohexane, butyl-
18	13.688	0.365	Cyclopentane, pentyl-
19	13.814	0.401	(Z)-4-Decen-1-ol, methyl ether
20	14.380	0.200	Naphthalene, decahydro-, trans-
21	15.937	0.198	Undecane
22	18.213	0.250	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-
23	40.669	2.337	9,12-Octadecadienoic acid (Z,Z)-
24	45.010	1.030	Hexanedioic acid, dioctyl ester
25	46.808	1.387	Heptadecane, 9-hexyl-
26	47.238	0.853	1,2-Benzenedicarboxylic acid, diisooctylplasticiser
27	48.350	2.247	Heptadecane, 9-hexyl-
28	49.831	4.499	Heptacosane
29	51.401	20.782	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl- (Squaline)
30	52.650	8.489	Octacosane
31	54.058	7.536	Tetratetracontane

reason for the death of *Ae. aegypti* larvae. Here, we found that the release of reactive oxygen species in excess amount together with the DNA damage induced by the plant extract has triggered the formation oxidative stress results in mosquito larval death; and these two mechanisms were probably distinguished as another reason for mosquito larval death. The microorganism targeted vector control approaches also offer sustained mitigation of mosquito vectors with special priority on insect conservation. The combinatorial role as a mosquitocidal agent and its toxicity towards microorganisms made this plant as an effective candidate as a natural insecticide for mosquito vector control. Interestingly, this seed extract can also be used in various industrial researches including cosmetic, pharmacological, and therapeutic research as for the reason that the extract contains various phyto chemical compounds including the important bioactive element squalene.

Acknowledgments The authors would like to thank Principal, St. Joseph's College, Irinjalakuda for the laboratory facilities provided. The authors Embalil Mathachan Aneesh thank University Grants Commission, Government of India (UGC Research Award - F30-6/20-16(SA-II).

Funding information The study was funded by UGC (UGC Research Award - F30–6/20–16(SA-II).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Adeosun C, Bamidele I, Ayorinde A (2017) Essential oil from the stem bark of *Casuarina equisetifolia* exhibits mosquitocidal potential. J Phytopathol Pst 3:53–62

AhbiRami R, Zuharah WF, Thiagaletchumi M, Subramaniam S, Sundarasekar J (2014) Larvicidal efficacy of different plant parts of railway creeper, *Ipomoea cairica* extract against dengue vector mosquitoes, *Aedes albopictus* (Diptera: Culicidae) and *Aedes aegypti* (Diptera: Culicidae). J Insect Sci 14. https://doi.org/10.1093/jisesa/ieu042

Aivazi A-A, Vijayan V (2010) Efficacy of *Ruta graveolens* extract and its synergistic effect with cypermethrin against *Anopheles stephensi* Liston larvae. Toxicol Environ Chem 92:893–890. https://doi.org/10.1080/02772240903092306

Al Yahya NA, Alrumman SA, Moustafa MF (2018) Phytochemicals and antimicrobial activities of *Rumex nervosus* natural populations grown in Sarawat Mountains Kingdom of Saudi Arab. J Sci Eng 43:3465–3476. https://doi.org/10.1007/s13369-018-3136-z

Aneesh E, Vijayan V (2010) Laboratory selection of carbofuran tolerant line of *Culex quinquefasciatus* say, the filarial vector at Mysore. J Commun Disord 42(3):201–207

Anoopkumar AN, Puthur S, Rebello S, Aneesh EM (2017a) Screening of a few traditionally used medicinal plants for their larvicidal efficacy against *Aedes aegypti* Linn (diptera: culicidae), a dengue fever vector. SOJ Microbiol Infect Dis 5(4):1–5. https://doi.org/10.15226/sojmid/5/4/00181

Anoopkumar AN, Puthur S, Varghese P, Rebello S, Aneesh EM (2017b) Life cycle, bio-ecology and DNA barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). J Commun Disord 49. https://doi.org/10.24321/0019.5138.201719

Anoopkumar AN, Puthur S, Rebello S, Aneesh EM (2019) Molecular characterization of *Aedes, Culex, Anopheles,* and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis. Biologia:1–14. https://doi.org/10.2478/s11756-019-00231-0

Anoopkumar AN, Aneesh EM, Sudhikumar AV (2020) Exploring the mode of action of isolated bioactive compounds by induced reactive oxygen species generation in *Aedes aegypti*: a microbes based double-edged weapon to fight against Arboviral diseases. Int J Trop Insect Sci 1–13. https://doi.org/10.1007/s42690-020-00104-z

Bekele D, Petros B, Tekie H, Asfaw Z (2014) Larvicidal and adulticidal effects of extracts from some indigenous plants against the malaria vector, *Anopheles arabiensis* (Diptera: Culicidae) in Ethiopia. J Biofertil Biopestici 5:1. https://doi.org/10.4172/2155-6202.1000144

Bowers WS, Sener B, Evans PH, Bingol F, Erdogan I (1995) Activity of Turkish medicinal plants against mosquitoes *Aedes aegypti* and *Anopheles gambiae*. Int J Trop Insect Sci 16:339–341. https://doi.org/10.1017/S1742758400019299

Brown DG, Lister T, May-Dracka TL (2014) New natural products as new leads for antibacterial drug discovery. Bioorg Med Chem Lett 24:413–418. https://doi.org/10.1016/j.bmcl.2013.12.059

Bussmann R et al (2010) Minimum inhibitory concentrations of medicinal plants used in northern Peru as antibacterial remedies. J Ethnopharmacol 132:101–108. https://doi.org/10.1016/j.jep.2010.07.048

Chaitanya R, Shashank K, Sridevi P (2016) Oxidative stress in invertebrate systems. In: Free Radicals and Diseases InTech https://doi.org/10.5772/64573

Cheng S-S, Chua M-T, Chang E-H, Huang C-G, Chen W-J, Chang S-T (2009) Variations in insecticidal activity and chemical compositions of leaf essential oils from *Cryptomeria japonica* at different ages. Bioresour Technol 100:465–470. https://doi.org/10.1016/j.biortech.2007.11.060

Cooke MS, Olinski R, Evans MD (2006) Does measurement of oxidative damage to DNA have clinical significance? Clin Chim Acta 365:30–49. https://doi.org/10.1016/j.cca.2005.09.009

Dada N, Sheth M, Liebman K, Pinto J, Lenhart A (2018) Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors. Sci Rep 8:2084. https://doi.org/10.1038/s41598-018-20367-4

Dong S, Behura SK, Franz AW (2017) The midgut transcriptome of *Aedes aegypti* fed with saline or protein meals containing chikungunya virus reveals genes potentially involved in viral midgut escape. BMC Genomics 18:382. https://doi.org/10.1186/s12864-017-3775-6

Ertas A, Yilmaz MA, Firat M (2014) Chemical profile by LC–MS/MS, GC/MS and antioxidant activities of the essential oils and crude extracts of two species. Nat Prod Res 29(6):529–534

Farnesi LC, Brito JM, Linss JG, Pelajo-Machado M, Valle D, Rezende GL (2012) Physiological and morphological aspects of *Aedes aegypti* developing larvae: effects of the chitin synthesis inhibitor novaluron. PLoS One 7:e30363. https://doi.org/10.1371/journal.pone.0030363

Forcados GE, James DB, Sallau AB, Muhammad A, Mabeta P (2017) Oxidative stress and carcinogenesis: potential of phytochemicals in breast cancer therapy. Nutr Cancer 69:365–374. https://doi.org/10.1080/01635581.2017.1267777

Ghosh A, Chowdhury N, Chandra G (2012) Plant extracts as potential mosquito larvicides. Indian J Med Res 135:581

Govindarajulu B, Srimathi A, Bhuvana R, Karthikeyan J (2015) Mosquito larvicidal efficacy of the leaf extracts of *Annona reticulata* against *Aedes aegypti*. Int J Curr Microbiol App Sci 4:132–140. https://doi.org/10.5376/jmr.2015.05.0019

Guha G, Rajkumar V, Mathew L, Kumar RA (2011) The antioxidant and DNA protection potential of Indian tribal medicinal plants. Turk J Biol 35:233–242. https://doi.org/10.3906/biy-0906-64

Ha E-M, Oh C-T, Bae YS, Lee W-J (2005) A direct role for dual oxidase in Drosophila gut immunity. Science 310:847–850. https://doi.org/10.1126/science.111731

Howard AF, Zhou G, Omlin FX (2007) Malaria mosquito control using edible fish in western Kenya: preliminary findings of a controlled study. BMC Public Health 7:199. https://doi.org/10.1186/1471-2458-7-199

Hsueh Y-H, Lin K-S, Ke W-J, Hsieh C-T, Chiang C-L, Tzou D-Y, Liu S-T (2015) The antimicrobial properties of silver nanoparticles in *Bacillus subtilis* are mediated by released Ag+ ions. PLoS One 10:e0144306. https://doi.org/10.1371/journal.pone.0144306

Iloki-Assanga SB, Lewis-Luján LM, Lara-Espinoza CL, Gil-Salido AA, Fernandez-Angulo D, Rubio-Pino JL, Haines DD (2015) Solvent effects on phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and *Phoradendron californicum*. BMC Res Notes 8:396. https://doi.org/10.1186/s13104-015-1388-1

Ioos S, Mallet H-P, Goffart IL, Gauthier V, Cardoso T, Herida M (2014) Current Zika virus epidemiology and recent epidemics. Med Mal Infect 44:302–307. https://doi.org/10.1016/j.medmal.2014.04.008

Jones DP (2006) Redefining oxidative stress. Antioxid Redox Signal 8:1865–1879. https://doi.org/10.1089/ars.2006.8.1865

Kadir SLA, Yaakob H, Zulkifli RM (2013) Potential anti-dengue medicinal plants: a review. J Nat Med 67:677–689. https://doi.org/10.1007/s11418-013-0767-y

Kawanishi S, Oikawa S (2004) Mechanism of telomere shortening by oxidative stress. Ann N Y Acad Sci 1019:278–284. https://doi.org/10.1196/annals.1297.047

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T (2012) Symbiont-mediated insecticide resistance. Proc Natl Acad Sci U S A 109:8622. https://doi.org/10.1073/pnas.1200231109

Kishore N, Mishra BB, Tiwari VK, Tripathi V (2011) A review on natural products with mosquitosidal potentials. Opportunity, challenge and scope of natural products in medicinal chemistry. Kerala: Rese Signpost 11:335–365

Kodrík D, Bednářová A, Zemanová M, Krishnan N (2015) Hormonal regulation of response to oxidative stress in insects—an update. Int J Mol Sci 16:25788–25816. https://doi.org/10.3390/ijms161025788

Kumar S et al (2003) The role of reactive oxygen species on Plasmodium melanotic encapsulation in *Anopheles gambiae*. Proc Natl Acad Sci 100:14139–14144. https://doi.org/10.1371/journal.pone.0057014

Kumar PM, Murugan K, Kovendan K, Subramaniam J, Amaresan D (2012) Mosquito larvicidal and pupicidal efficacy of *Solanum xanthocarpum* (family: Solanaceae) leaf extract and bacterial insecticide, *Bacillus thuringiensis*, against *Culex quinquefasciatus* say (Diptera: Culicidae). Parasitol Res 110:2541–2550. https://doi.org/10.1007/s00436-011-2797-2

Liu N (2015) Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Annu Rev Entomol 60:537–559. https://doi.org/10.1146/annurev-ento-010814-020828

Mdoe FP, Cheng S-S, Lyaruu L, Nkwengulila G, Chang S-T, Kweka EJ (2014) Larvicidal efficacy of *Cryptomeria japonica* leaf essential oils against *Anopheles gambiae*. Parasit Vectors 7:426. https://doi.org/10.1186/1756-3305-7-426

Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM (2018) Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J Biol Sci 25:361–366. https://doi.org/10.1016/j.sjbs.2017.02.004

Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, van Etten RA (2003) Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature 424:561. https://doi.org/10.1038/nature01819

Nwabor OF, Nnamonu EI, Emenike MP, Odiachi O (2017) Synthetic insecticides, phytochemicals and mosquito resistance. Acad J Biotechnol 5(8):118–125. https://doi.org/10.15413/ajb.2017.239

Oliveira JHM, Gonçalves RL, Oliveira GA, Oliveira PL, Oliveira MF, Barillas-Mury C (2011) Energy metabolism affects susceptibility of *Anopheles gambiae* mosquitoes to Plasmodium infection. Insect Biochem Mol 41:349–355. https://doi.org/10.1016/j.ibmb.2011.02.001

Patil MB, Khan PA (2017) Primary phytochemical studies of *Catunaregam spinosa* (Thunb) Tirven for secondary metabolites. Int J Pharm Bio Sci 8(2):320–323. https://doi.org/10.22376/ijpbs.2017.8.2.p320-323

Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem 269:337–341. https://doi.org/10.1006/abio.1999.4019

Puthur S, Anoopkumar A N, Rebello S, Aneesh EM (2018) *Hypericum japonicum*: a Double-Headed Sword to Combat Vector Control and Cancer. Appl Biochem Biotech 1–11. https://doi.org/10.1007/s12010-018-2713-7

Puthur S, Anoopkumar AN, Rebello S, Aneesh EM (2019) Synergistic control of storage pest rice weevil using *Hypericum japonicum* and deltamethrin combinations: a key to combat pesticide resistance. Environ Sustain 2(4):411–417. https://doi.org/10.1007/s42398-019-00086-w

Rebello S, Anoopkumar AN, Puthur S, Sindhu R, Binod P, Pandey A, Aneesh EM (2018) Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour Technol Rep 3:1–6. https://doi.org/10.1016/j.biteb.2018.05.007

Rebello S, Balakrishnan D, Anoopkumar AN, Sindhu R, Binod P, Pandey A, Aneesh EM (2019a) Industrial enzymes as feed supplements—advantages to nutrition and global environment. In: Green Bio-processes. Springer, pp 293–304. https://doi.org/10.1007/978-981-13-3263-0_15

Rebello, S., Anoopkumar, AN., Aneesh, E. M., Sindhu, R., Binod, P., & Pandey, A. (2019b). Sustainability and life cycle assessments of lignocellulosic and algal pretreatments. Bioresour Technol 122678. https://doi.org/10.1016/j.biortech.2019.122678

Saldaña MA, Hegde S, Hughes GL (2017) Microbial control of arthropod-borne disease. Mem Inst Oswaldo Cruz 112(2):81–93. https://doi.org/10.1590/0074-02760160373

Sarwar M, Ahmad N, Toufiq M (2009) Host plant resistance relationshiphs in chickpea (*Cicer arietinum* l.) against gram pod borer *Helicoverpa armigera* hubner. Pak J Bot 41:3047–3052

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot. https://doi.org/10.1155/2012/217037.

Sinha JK, Ghosh S, Swain U, Giridharan NV, Raghunath M (2014) Increased macromolecular damage due to oxidative stress in the neocortex and hippocampus of WNIN/Ob, a novel rat model of premature aging. Neuroscience 269:256–264. https://doi.org/10.1016/j.neuroscience.2014.03.040

Souza VM (2016) Identifying *Aedes aegypti* mosquitoes by sensors and one-class classifiers. In: Iberoamerican Congress on Pattern Recognition. Springer, pp 10–18. https://doi.org/10.1007/978-3-319-52277-7_2.

Surabhi S, Leelavathi S (2010) Anti-oxidant property of ethanolic extract of *Catunaregam spinosa* Thunb. Int J Drug Dev 2:399–403

Tiwary M, Naik S, Tewary DK, Mittal P, Yadav S (2007) Chemical composition and larvicidal activities of the essential oil of *Zanthoxylum armatum* DC (Rutaceae) against three mosquito vectors. J Vector Borne Dis 44:198

Valgas C, SMD S, Smânia EF, Smânia A Jr (2007) Screening methods to determine antibacterial activity of natural products. Braz J Microbiol 38:369–380. https://doi.org/10.1590/S1517-83822007000200034

Valzania L, Martinson VG, Harrison RE, Boyd BM, Coon KL, Brown MR, Strand MR (2018) Both living bacteria and eukaryotes in the mosquito gut promote growth of larvae. Plos Neglect Trop D 12: e0006638. https://doi.org/10.1371/journal.pntd.0006638

Villegas LE et al (2018) Zika virus infection modulates the bacterial diversity associated with *Aedes aegypti* as revealed by metagenomic analysis. PLoS One 13:e0190352. https://doi.org/10.1371/journal.pone.0190352

Vogel KJ, Valzania L, Coon KL, Brown MR, Strand MR (2017) Transcriptome sequencing reveals large-scale changes in axenic *Aedes aegypti* larvae. Plos Negl Trop D 11:e0005273. https://doi.org/10.1371/journal.pntd.0005273

Vu TT, Kim H, Tran VK, Vu HD, Hoang TX, Han JW, Choi YH, Jang KS, Choi GJ, Kim JC (2017) Antibacterial activity of tannins isolated from *Sapium baccatum* extract and use for control of tomato bacterial wilt. PLoS One 12:e0181499. https://doi.org/10.1371/journal.pone.0181499

Wallis M, Griffin R (1973) A routine method for embedding animal tissues in Spurr resin for electron microscopy. J Clin Pathol 26:77. https://doi.org/10.1136/jcp.26.1.77

WHO (2005) World Health Organization. Guidelines for laboratory and field testing of mosquito larvicides Geneva: World Health Organization:p 1–41. https://apps.who.int/iris/handle/10665/69101

Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3(163):175. https://doi.org/10.1038/nprot.2007.521

World Health Organization (2017) Global vector control response 2017–2030 Geneva, Switzerland: WHO. Licence: CC BY-NC-SA 3.0

IGO. Available at: https://www.who.int/vector-control/publications/global-control-response/en/. (Last Accessed on 01-04-2020)

Yadav KK, Bora A, Datta S, Chandel K, Gogoi HK, Prasad G, Veer V (2015) Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India. Parasit Vectors 8:641. https://doi.org/10.1186/s13071-015-1252-0

Journal of Hazardous Materials 402 (2021) 123474

Contents lists available at ScienceDirect

Journal of Hazardous Materials

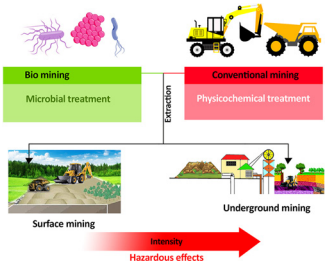
journal homepage: www.elsevier.com/locate/jhazmat

Hazardous minerals mining: Challenges and solutions

Sharrel Rebello[a], A.N. Anoopkumar[a,b], Embalil Mathachan Aneesh[a], Raveendran Sindhu[c], Parameswaran Binod[c], Sang Hyoun Kim[d], Ashok Pandey[e,f,*]

[a] Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, India
[b] Department of Zoology, Christ College, Irinjalakuda, University of Calicut, India
[c] Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum, 695 019, India
[d] School of Civil and Environmental Engineering, Yonsei University, Seoul, South Korea
[e] Center for Innovation and Translational Research, CSIR- Indian Institute of Toxicology Research, Lucknow, 226 001, India
[f] Frontier Research Lab, Yonsei University, Seoul, South Korea

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Rinklebe Jörg

Keywords:
Minerals
Mining
Hazards
Bioleaching

ABSTRACT

Minerals are valuable resources gifted to man from the Mother Earth and quite often they need to be dug out from deep down with much effort to utilize them in many of our anthropogenic activities. The fascinating nature, colours as well as the physicochemical properties of minerals has extended their prospective value in the synthesis of various household and industrial products. However, knowledge of the mostly explored minerals, associated products, and their hazardous nature becomes relevant to its prevalence in our daily life. The harmful effects of some minerals are mostly evident from its site of occurrence, process of mining, post mining wastes left over and even in finished products. The current review focuses to evaluate the hazardous nature of minerals, cautions associated with its mining, drastic effects on human health, and ecosystem as an eye-opener to us. Finally, the effective remedies that could be implemented in the exploration of minerals are also discussed to the best of our knowledge. Bioleaching methods of rare earth elements and copper have been discussed briefly to explain the pros and cons of biological methods over conventional chemical leaching methods.

1. Introduction

With extensive growth in economic aspects, the prominent use of hazardous chemicals has persistently augmented in the current scenario subsequently caused chemical accidents such as leakages, fires, and explosions which can result in disability, injury, illness, and death of individuals in various circumstances (Han and Park, 2018). These hazardous chemicals induce a life risk and can result in a plethora of health complications in individuals who are living near to chemical industries.

* Corresponding author at: Center for Innovation and Translational Research, CSIR- Indian Institute of Toxicology Research, Lucknow, 226 001, India.
E-mail address: ashok.pandey1@iitr.res.in (A. Pandey).

https://doi.org/10.1016/j.jhazmat.2020.123474
Received 30 April 2020; Received in revised form 5 July 2020; Accepted 10 July 2020
Available online 24 July 2020

Nevertheless, it is noted that even various naturally occurring elements themselves are found to be hazardous nature to the environment and ecosystem irrespective of their presence in meager quantities, and most of the minerals that we harness from the earth come under this category. Mainly 23 elements that are of special interest for us because of occupational and residential exposure and they are; platinum, arsenic, chromium, antimony, copper, iron, nickel, manganese, cadmium, gold, vanadium, thallium, bismuth, mercury, cerium, gallium, tellurium, uranium, zinc, cobalt, silver, lead and tin (Mosby et al., 1996). The higher dose of these aforementioned elements may instigate hazardous effects in the human population whereas long-term exposure may progressively develop severe health effects including muscular, physical and certain neurological degenerative processes that mimic muscular dystrophy, multiple sclerosis, Alzheimer's disease and Parkinson's disease (Järup, 2003).

The studies of Markov also identified various minerals and their components with special inference to selenium, silica minerals, asbestos (Markov, 2012). Among them asbestos is recognized as an excellent insulator; and as for the reason that it has been prominently used for construction proposes; however, it has been banned and regulated in various regions over the world, especially in European countries and the United States owing to associated toxicity.

In addition to the above-mentioned members there exist few rare hazardous elements that simultaneously induce severe health threats as well as play a prominent role in human days to-day lives. They include cinnabar, thorium, galena, hutchinsonite, orpiment, torbernite, uraninite, fluorite (fluorspar), quartz, and K-feldspar. Table 1 enlists some of these hazardous minerals that are explored and used by mankind. Many of them even have medicinal value and are used in primitive medicine. For instance, powdered form of the stibnite used in 'Surma', an eye cosmetic also served as an effective candidate for skin ailments in 1 st century A.D. (Antimony, 2015). Cinnabar renowned as a naturally occurring mercuric sulfide (HgS) was prominently used for therapeutic purposes in China for 2000 years (Huang et al., 2012). However, it is noted that in excess quantities they are toxic to mankind and thus identification of such minerals becomes trivial.

The concept of hazardous minerals often does not indicate that all minerals are hazardous but at least sometimes even the most valuable minerals might add more hazardous chemicals to the environment due to the processes involved in their extraction. Various anthropogenic activities to harness these minerals by conventional mining methods leave back an uncompromising amount of residual toxic xenobiotics that leads to the generation of a hazardous environment. The current review comprehensively lays out the prevalence of various hazardous minerals worldwide, their mining strategies, and hazards associated with them. Finally, the concept of bioleaching that could aid to overcome various problems associated with conventional mining is addressed stating examples of rare earth element as well as pyrite based mining.

2. Uses and harmful effects of minerals to mankind

2.1. Asbestos

There exists an open debate concerning the diverse hazardous effects and carcinogenic potential of several hazardous elements. In this regard, several countries over the world have banned the extensive use of few hazardous elements like asbestos. The use of asbestos has been forbidden in many European countries. The Canadian government has followed the same pattern to prohibit the use of products containing asbestos; however, certain essential use of asbestos is still legal in the country. For example, the Canadian government has allowed the use of asbestos in nuclear energy industries and Canadian military services through 2029. But, the United States Government has provided priority for the reduced use of asbestos instead of a complete ban (Visonà et al., 2018).

Table 1
Hazardous minerals- uses and associated hazards.

Mineral name	Colour	Chemical name	Source of metal	Use	Bad effect
Chalcanthite	blue	$CuSO_4 5H_2O$	copper	gemstone	Poisonous in large concentrations
Stibnite	Silver	Sb_2S_3	Sb	cosmetics to darken eyebrows & lashes, make eating utensils, fireworks, matches	lung diseases, heart problems, diarrhoea, severe vomiting and stomach ulcers.
Asbestos	Greyish white	$Mg_3Si_2O_5(OH)_4$	Si	Useful in insulation, fire resistance, and sound absorption	Lung cancer, mesothelioma, asbestosis
Arsenopyrite	brilliant steel metallic color	FeAsS	Arsenic	Usually close to gold mines	Leads to arsenic water and acid drainage
Cinnabar	Bright red	HgS	mercury	fluorescent lighting, including compact fluorescent light bulbs (CFLs)	Deadly to humans
Galena -	Bright silvery white	PbS	Pb	Primary ore of lead	Inhalation causes lung cancer
Hutchinsonite	Silver and brown crystals	$(Tl,Pb)_2As_5S_9$	Pb	Thallium in rat poison, Pb	Deadly
Orpiment	Orangish yellow colour	As_2S_3	arsenic	Found in hydrothermal vents	Arsenic poisoning
Torbernite	green	$Cu(UO_2)_2(PO_4)_2 \cdot 8 - 12\ H_2O$	Cu	Found in granite that contain uranium	Radioactive and long exposure is not safe
Uraninite	silver	UO2	uranium	Ammunition and fuel nuclear power plants.	Radon is produced by natural radioactive decay of uranium (and/or thorium) which is a carcinogen.
Fluorite (fluorspar)	Purplish green	CaF_2	Fluorine	ornaments and lapidary works, in the flux for smelting, in the production of certain glasses, enamels, microscopic & telescopic lenses.	Causes skeletal fluorosis
Quartz	Wide range colours (greyish to brown)	silicon dioxide	Si		Silicosis, lung cancer, kidney disease and immunological problems
K-Feldspar	brown	$KAlSi_3O_8$, group of potassium aluminium silicate minerals including orthoclase, microcline and adularia	K, Al, Si	Manufacture glass and ceramic products, artificial teeth and scouring powders, gemstones.	Contains small quantities of radioactive uranium that forms radon gas, a major cause of lung cancer.

Many developing countries over the world depend on the asbestos business for their economic needs. The high demand for low-cost roofing and piping products is recognized as the driving force behind the success of this big business over the world. Due to these reasons, the following countries are becoming the foremost world leaders in the production of asbestos for the past few years: Kazakhstan, China, India, and Russia (Visonà et al., 2018). Previous reports indicated that the continuous exposure of asbestos has resulted in pulmonary fibrosis, pleural plaques, asbestosis, pleural effusion, mesothelioma, and lung cancer (Nynäs et al., 2017; Kamp, 2009; Liu et al., 2013). The most common health effects instigated by silica include silicosis, chronic renal disease, lung cancer, and autoimmune disorders (Sato et al., 2018).

2.2. Arsenic

Arsenic compounds like arsenopyrite have been principally used for centuries for various purposes such as glass-making, wood preservatives, semiconductor, and metallurgical industries, pharmaceuticals, and agricultural chemicals. The wide spectrum of applications of arsenic compounds in the agricultural industry for pesticides, insecticides, defoliants, herbicides, and cotton desiccants has been previously reported by several studies (Gomez-Caminero et al., 2001). In addition to this, the arsenic compounds are known for the manufacture of leather preservatives, sheep-dips, and pigments. Because of its photovoltaic, electromagnetic, light-emitting properties, it is used as an important element in high-speed semiconductor devices, optoelectronic devices, high-power microwave devices, fibre-optic sources, and detectors; as well as for the manufacture of fibre optics and computer chips. They are also used in alloys (radiators and automotive), pharmaceutical substances, ceramics, dyes and soaps, antifouling agents in paints as well as in electro-photography. Arsenic compounds are used in combination with lead and copper for the manufacture of toys (Gomez-Caminero et al., 2001).

Frequent exposure to arsenic compounds may instigate gastrointestinal symptoms like vomiting, abdominal pain, nausea, stomach ulcers, and anorexia. Furthermore, arsenic compounds are also allied to several respiratory disorders (bronchitis, pneumoconiosis, respiratory irritation, chronic cough, pleural adhesions, and rhinitis), reproductive effects (spontaneous abortions and menstrual irregularities) and cardiovascular problems linked to electrocardiographic (ECG) changes and increased blood pressure (Saerens et al., 2019). The major arsenic compounds such as arsenate and arsenic trioxide cause cancer of the skin, urinary bladder, and lung. In addition to this, there was a positive correlation has perceived between exposure to arsenic elements and cancer of the prostate, kidney and liver.

2.3. Antimony

The early use of Stibnite (antimonite) for medical purposes spanned about 600 years since the element was introduced in internal treatment (Cooper and Harrison, 2009). The hazardous effects of Stibnite allied elements like Antimony may happen due to frequent exposure during therapy or occupational exposure. The occupational exposure may result in pneumoconiosis, gastrointestinal symptoms, respiratory irritation, and spots on the skin. Mainly, antimony has been used for the treatment of Schistosomiasis and Leishmaniasis; and most of the patients that are frequently exposed with the antimonials have faced threats from pancreatitis and cardiotoxicity (Choi et al., 2018). Therefore, it is necessary to maintain unceasing quality control of antimonials based drugs to ensure safety.

2.4. Lead

The high density, low strength, electrochemical reaction with sulphuric acid, acid resistance, low melting point, ease of fabrication along with chemical stability in water, soil, and air of lead has augmented the economic importance of lead allied hazardous elements. Commercial applications of lead and its ore has included from the manufacture of storage batteries to pipe and sheets for x-ray and nuclear shielding (Hara et al., 2005). Galena is the most important ore of lead.

Despite the benefits from the lead, it also exhibited many hazardous effects through gifts, toys as well as through jewellery. The lead-based elements exposure may severely instigate harmfulness to the health of kids since the main constituent for most of all the toys were lead-based elements (Greenway and Gerstenberger, 2010). The continuous exposure to galena results in anaemia, heart disease, stroke, hypertension, skin cancer, and chronic kidney disease (Neuberger et al., 1990).

2.5. Mercury and cinnabar

The adverse effects in the human population due to frequent exposure of cinnabar and mercury allied elements remain a major subject of debate among the scientific community since the aforementioned elements have a prominent role in the day to day life. The presence of mercuric content in cinnabar has indicated its toxicity to organisms (Pajączkowska, 1970). Despite the toxic effects of cinnabar, it has a history of being an extensive role in Chinese traditional medicines (Jain et al., 2019). It is considered as the major ore of mercury and the long term use of the same has resulted in the accumulation of mercury in the kidney thereby leads to renal dysfunction. A review by Jain et al., 2019 (Jain et al., 2019) reported the neurotoxic aspects of cinnabar with special inference on the significant human health consequences from mercury allied elements.

Several previous studies have reported that the level of mercuric elements in blood and urine has prominently linked with dental amalgam exposure in the human body (Kostyniak, 1998). The positive correlation between the urine mercury level and the dental amalgam filling has been previously reported by (Jung et al., 2012). However, it is prominently accepted that the mercuric elements have maintained their foremost role in therapeutic purposes. In addition to this, the role of cinnabar in CFL making has been previously reported by Johnson et al., 2008 (Johnson et al., 2008).

2.6. Thorium

The human intoxication of other rare hazardous minerals like thorium may lead to the accumulation of the same in lungs, bones, and liver. The fact is that the thorium may absorb through the skin or open wounds. The toxic effects of thorium in the various organs like kidney and lungs may depend on the routes of inhalation. Contaminated water and food could be recognized as another route of thorium exposure like other radio-nuclides. The radiological half-life of thorium is approximately 14 billion years; however, it's biological half-life in human organs has varied (liver- ~2 y, skeleton ~20 y, lungs - ~2) (Kumar et al., 2013). Evidence revealed that frequent deposition of thorium and other rare hazardous minerals in the various organs of the human body can increase the chance of development of cancer (Akhter et al., 2003). Despite the abovementioned dangerous facts, the importance of thorium has prominently increased in nuclear research since the thorium can be recognized as an alternative source of fuel against the natural uranium. It may be so, the scientific community has now focussed on the largest deposits of natural thorium that is found in the Kerala state of India (Kumar et al., 2013).

2.7. Rare earth elements

The 17 member group of rare metals include members of the lanthanides as well as other members such as scandium and yttrium; most often associated with thorium and occasionally uranium. Hazardous elements such as hutchinsonite, orpiment, torbernite, uraninite, fluorite, quartz, K-feldspar, and thorium have found to exhibit benefits

for human life in several ways. Fluorite is also considered as a part of the environment; hence it continuously exists in people's lives since it is used for the manufacture of ornaments, glasses, and telescopic and microscopic lenses for the past years. Both the hazardous elements such as uranium and thorium have received unique characteristic features driving their application in nuclear reactors. However, thorium is also recognized as a sustainable energy resource since it exhibits excellent fuel performance in nuclear reactor with lower nuclear waste (Dietrich, 1968).

But most of the individuals are not aware of the drastic and poisoning effects of rare hazardous elements. It is noted that exposure to extremely high concentrations of the fluorite may lead to severe toxic effects (Davies, 1994). Exposure to quartz dust particles through respiration may result in deposition of the same in the lungs, thereby leads to nodule formation, alveolar proteinosis, suppressed immune functions, and cellular proliferation. There exists sufficient data on the risk factors that are linked with quartz exposure and silicosis (W.H. Organization, 2000). The hazardous effects linked with the dermal or oral exposure to uraninite found to be not radiological while the exposure through inhalation may instigate a slight radiological effect (Keith et al., 2013). The hazardous nature of the uranium-based elements is always identical in nature regardless of its specific action.

3. Conventional mineral mining techniques

The mineral mining in India is recognized as a foremost activity which contributes to the Indian economy. The density of mineral distribution in India may vary in different regions. The major rare hazardous elements and their localities over India and other regions of the world were noted in Table 2 and Supplementary Table 1 respectively (Antimony, 2015). A close look at the data reveals that most of the locations like Rajasthan are a good source of minerals and are explored for numerous minerals. The occurrence of mines is scanty only in some states of India and different states adopt different mining techniques as per the mineral explored. The next section explains some of the conventional mining techniques.

3.1. Surface mining

The demand for mining of natural elements is increased in the current scenario. The surface of the earth is therefore considered as an important source for minerals and mining is an effective way to collect those minerals. Global Positioning Systems [GPS] has turned into a vital component of vast surface mines for tracking the trucks and other equipment, precision positioning of shovels, and guiding equipment. There exist several surface mining strategies such as blasting, hauling, drilling, and loading (Ramani, 2012). Surface mining is recognized as a predominant method over the world (Hartman and Mutmansky, 2002). It always provides high productivity at a low cost. The surface mining technologies have been broadly divided into two; 1) Mechanical excavation methods and 2) Aqueous methods (Harraz, 2010).

3.1.1. Mechanical excavation methods

The mechanical excavation involving breaking of rock masses for the production of ore. The Mechanical excavation methods include Terrace mining, Open-pit mining, Auger Mining, Quarrying, Contour strip (hilly terrain) mining, Strip (flat terrain) mining, Contour strip (hilly terrain) mining, and Glory Holing. The open-pit mining is performed through rock and soil peeled off from the topside of deposit to dump. The following factors may affect the safety of open-pit mining: blasting operation safety, slope stability, and road conveyance safety (Kaihuan and Fuchuan, 2012). Terrace Mining is considered as a variation of the Open-Pit mining method which is usually employed in mineral deposits of thicker overburdens. The whole mine is subjected to multiple stages of operation (Souza et al., 2018). The auger mining is usually related to the contour strip (hilly terrain) mining.

3.1.2. Aqueous extraction methods

The Aqueous extraction methods use either water or any other convenient liquid such as weak cyanide solution, ammonium carbonate, or dilute sulphuric acid to extract the minerals. Aqueous methods divided into Placer (Panning, Sluicing, Hydraulic Mining, and Dredging) and solution methods (In-situ leaching (ISL) and Heap Leaching). Among them, solution mining and placer mining are renowned as the best methods; however, it is limited to specific types of mineral deposits (Harraz, 2010).

3.2. Underground mining

To eliminate the various disadvantages concerned with the traditional mining approaches, it is essential to follow effective mining technology. Rock drilling is an important process involved in underground mining and it offers high productivity and high efficiency at a low cost. However, the mining methods may vary in different geological conditions and a hydraulic rock-drilling jumbo is required for drilling. In addition to this, in certain industries, a virtual reality display along with remote control has been implanted. The parameters for rock drilling can be adjusted to the different geological and rock conditions. The latest intelligent rock-drilling jumbo is included with certain specific components that are essential for preventing the blockage, frequency matching, and rock-characteristic acquisition (Prasad et al., 2016). Since the successful testing of scrapers for underground mining in the 1960s by Wagner, they have been principally used for mining because of their manoeuvrability, high efficiency, low cost together with flexibility. With the advanced developments in the information technology and electronics, novel advanced technologies for scrapers have been developed. Now the scraper has changed from conventional manual mode to fully automatic with remote control and it is renowned as the fourth-generation scraper (Dindarloo, 2016). Besides this, an underground mining truck is required as a key transport vehicle to complete the tasks concerned with underground trackless mining that offers high efficiency, mobility, economy, high efficiency, and flexibility. The major function of the mining truck is the transportation of ore and its use can significantly offer maximum labour productivity and production capacity, improve the transportation system and mining technology, and upsurge the production scale. To achieve maximum energy conservation and protection for the environment, the double-power transmission mining truck can be used with a diesel engine driving generator (Li and Zhan, 2018).

4. Hazards associated with conventional mineral mining

4.1. Hazards to the environment

Mining of Minerals has a great role in contributing to a country's economy, yet it is noted that these economic thrust areas are converted to graveyard laden with the most toxic elements after years of mining. The Tinto River of Spain was converted to an acidic pool with a pH of 1.5–2.5 along with prominent occurrence of pyrite pellets and heavy metals (Pb, Ba, As, Cu, Zn, Sn, Tl, Cd, Ag, Hg, Au) as a result of mining activities (Leblanc et al., 2000). A comparative analysis of the Numerow's pollution index of various areas of Shizishan mining area in Tongling, China indicated values are 4 times higher in heap mining area compared to vegetable area and the heavy metal pollution of these areas are found even to drastically affect the microbial flora of soil (Zhao et al., 2020).

The impact of mining on the environment is always wide affecting every sector of the environment. The construction of infrastructure and associated buildings leads to violation of the balanced ecosystem leading to soil erosion, deforestation, habitat loss, decline species diversity as noted in Fig. 1. Studies indicate that the survival of fish, their growth and hatching are critically affected in water streams used for acid waste disposal if pH lowers below a value of 5 (Nemerow, 2007).

Table 2
A close look on various mineral deposits harnessed in India.

Rare hazardous elements	Locations in India
Chalcanthite	1. Rakha Cu deposit, Jharkhand, India 2. Sambhar Salt Lake, Ajmer Division, Rajasthan, India
Antimony/stibnite	1. Rampura-Agucha Zn-(Pb) deposit, Bhilwara District, Rajasthan 2. Askot, Pithoragarh District, Uttarakhand, India
Asbestos	1. Malkapuram mine, Pulivendla asbestos belt, Kadapa (Cuddapah), YSR District (Cuddapah District), Andhra Pradesh, India 2. Jharkhand Singhbhum (East), Singhbhum (West) 3. Karnataka–Chikkamagaluru,Hassan,Mandya, Mysuru, Shivamogga 4. Odisha – Kendujhar 5. Rajasthan –Ajmer, Bhilwara, Dungarpur, Pali, amphibole minerals, Rajsamand, Udaipur 6. Uttarakhand - Chamoli
Arsenopyrite	1. Mallappakonda deposit, South Schist Belt, Kolar Gold Fields, Chittoor District, Andhra Pradesh, India 2. Tosham Sn-Cu Prospect, Bhiwani District, Haryana, India 3. Bokaro coalfield, Ramgarh District, Jharkhand, India 4. Ingaldhal copper deposits ("Ingladhal" copper deposit; Ingaldal), Chitradurga (Chittaldurg; Chitaldurg), Chitradurga District, Karnataka, India 5. Hutti-Maski belt, Karnataka, India 6. Champion lode, Central Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 7. McTaggart West lode, Central Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 8. Nundydroog Mine (Coromandal Mine; Oriental Lode), Central Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 9. West Prospect lode, Central Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 10. Hutti Mine, Raichur District, Karnataka, India 11. Uti Mine, Raichur District, Karnataka, India 12. Ajjanahalli mine, Chitradurga belt, Tumkur District, Karnataka, India 13. Rampura-Agucha Zn-(Pb) deposit, Bhilwara District, Rajasthan, India 14. Saladipura Cu-Zn deposit, Jaipur district, Rajasthan, India 15. Khetri Mines (Ketri Mine), Khetri, Sikar District, Jaipur Division, Rajasthan, India 16. Kolihan, Jhunjhunu District, Jaipur Division, Rajasthan (Rajputana), India 17. Deri-Ambaji Zn-Pb-Cu deposit, Sirohi District, Jodhpur Division, Rajasthan, India 18. Ambaji deposit, Deri-Ambaji Zn-Pb-Cu deposit, Sirohi District, Jodhpur Division, Rajasthan, India 19. Rajpura-Dariba deposit, Udaipur District, Udaipur Division, Rajasthan, India 20. Dariba Mine, Rajpura-Dariba deposit, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 21. Sindesar Khurd prospect, Rajpura-Dariba deposit, Udaipur District, Udaipur Division, Rajasthan, India 22. Mochia Mine, Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 23. Yenambail, Palwancha (Palvancha; Paloncha), Khammam District, Telangana, India 24. Askot, Pithoragarh District, Kumaun Division, Uttarakhand, India 25. Nawahatu, Purulia District, West Bengal, India
Cinnabar	Chandmari Deposit, Jhunjhunu District, Rajasthan, India
Galena	1. Agnigundala Mines, Guntur District, Andhra Pradesh, India 2. Nundydroog Mine (Coromandal Mine; Oriental Lode), Central Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 3. Chigargunta Mine (Chigarikunta Mine), South Schist Belt, Kolar Gold Fields, Kolar District, Karnataka, India 4. Sargipalli Mines, Sundargarh District (Sundergarh District), Odisha (Orissa), India 5. Kayad mine, Ajmer District, Ajmer Division, Rajasthan, India 6. Kayar-Ghugra mine, Ajmer District, Ajmer Division, Rajasthan, India 7. Balaria Mine, Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 8. Baroi Mine, Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 9. Dariba Mine, Rajpura-Dariba deposit, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 10. Zawarmala Mine (Zawar Mala Mine), Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan, India 11. Mochia Mine, Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 12. Baroi Mine, Zawar Mines, Udaipur, Udaipur District, Udaipur Division, Rajasthan (Rajputana), India 13. Nawahatu #1 Baryte Quarry, Nawahatu, Purulia District, West Bengal, India 14. Askot, Pithoragarh District, Kumaun Division, Uttarakhand, India
Hutchinsonite	1. Gowari Wadhona Mine, Chhindwara District (Chindawara District; Chindwara District), Madhya Pradesh, India 2. Tirodi Mine, Tirodi, Balaghat District, Jabalpur division, Madhya Pradesh, India 3. Kajlidongri Mine, Jhabua District, Madhya Pradesh, India 4. Sitasaongi mine, Bhandara District, Maharashtra, India 5. Koduru deposit, Vizianagaram District, Andhra Pradesh, India 6. Risara, Madhya Pradesh, India
Uraninite	1. Kudada, Jadugora (Jadugoda; Jaduguda), Singhbhum District, Jharkhand, India 2. Turamdih U-Cu(-Fe) deposit, Jadugora (Jadugoda; Jaduguda), Singhbhum District, Jharkhand, India 3. Tatanagar, Singhbhum District, Jharkhand, India 4. Chikkamagaluru District, Karnataka, India 5. Malanjkhand Mine, Padritola, Baihar Tehsil, Balaghat District, Madhya Pradesh, India 6. Samarkiya area, Bhilwara District, Ajmer Division, Rajasthan, India 7. Khetri Mines (Ketri Mine), Khetri, Sikar District, Jaipur Division, Rajasthan, India 8. Madan-Kudan deposit, Jhunjhunu District, Jaipur Division, Rajasthan, India 9. Umra, Udaipur District, Rajasthan (Rajputana), India

(*continued on next page*)

Table 2 (*continued*)

Rare hazardous elements	Locations in India
	10. Peddur, Karimnagar District, Telangana, India 11. Mussoorie phosphorite deposit, Dehradun District, Uttarakhand, India 12. Nawahatu, Purulia District, West Bengal, India 13. Koppunuru deposit, Guntur District, Andhra Pradesh, India 14. Bagjata Mine, Jadugora (Jadugoda; Jaduguda), Singhbhum District, Jharkhand, India
Fluorite	
	1 Dancherla Complex, Anantapur District, Andhra Pradesh, India 2 Danduvaripalli plug, Anantapur District, Andhra Pradesh, India 3 Reddypalle plug, Anantapur District, Andhra Pradesh, India 4 Pulikonda intrusion, Kadapa district, Andhra Pradesh, India 5 Govindpal pegmatite-1, Bastar district, Chhattisgarh, India 6 Ghatkachhar, Mahasamund District, Chhattisgarh, India 7 handi Dongri, Bagbahara, Dongargarh, Rajnandgaon district, Chattisgarh (Chhattisgarh), India
Quartz	
	1. Sitasaongi mine, Bhandara District, Maharashtra, India 2. Agargaon Mine, Nagpur District, Nagpur District, Maharashtra, India 3. Gowari Wadhona Mine, Chhindwara District (Chindawara District; Chindwara District), Madhya Pradesh, India 4. Girola area, Bhandara District, Maharashtra, India 5. Kakamunurle Mine, Karur District, Tamil Nadu, India 6. Chavara Alluvial Deposit, Karunagappalli, Kollam District, Kerala, India 7. Kollam Alluvial Deposit, Kollam (Quilon), Kollam District, Kerala, India 8. Peralimala Intrusion, Kannur district, Kerala, India 9. Ambalavayal intrusion, Wayanad district, Kerala, India
K-Feldspar	
	1. Kunavaram area, East Godavari, Andhra Pradesh, India 2. Giddalur, Prakasam District, Andhra Pradesh, India 3. Errakonda Pluton, Prakasam District, Andhra Pradesh, India 4. Pasupugallu pluton, Prakasam District, Andhra Pradesh, India 5. Koduru deposit, Vizianagaram District, Andhra Pradesh, India 6. Amba Dongar complex, Chhota Udaipur District, Gujarat, India 7. Hira Buddini Mine (Hira-Buddini Mine), Raichur District, Hutti-Maski belt, Karnataka, India 8. Hutti Mine, Raichur District, Karnataka, India 9. Perunthol-Kakkaponnu, Pathanapuram, Kollam District, Kerala, India 10. Ambalavayal intrusion, Wayanad district, Kerala, India 11. Tirodi Mine, Tirodi, Balaghat District, Jabalpur division, Madhya Pradesh, India 12. Sargipalli Mines, Sundargarh District (Sundergarh District), Odisha (Orissa), India
Thorium	
	1. Odisha, India 2. Andhra Pradesh, India 3. Tamil Nadu, India 4. Kerala, India 5. West Bengal, India 6. Jharkhand, India

Since the mining activity is a continuous process, the continuous generation of acid or alkalis occurs and the often incessant burden is added to water bodies of mining areas. The drain out of these minerals to aquatic environments has a drastic impact on the marine environment. For instance, Neodymium (Nd) a rare earth element used in permanent magnets and turbine motors severely affects the bivalves such as *Mytilus* at cellular level critically influencing its growth and reproduction (Freitas et al., 2020).

The blood, liver, muscle, and otolith of predatory fish species Greenland cod and shorthorn sculpin in areas of Arctic mines revealed the presence of high concentrations of heavy metals; thereby indicating the transfer of pollutants to the food chain in mining areas (Hansson and Desforges, 2020). The occurrence of 10,000-year old mine contaminants in Alpine Lakes even now in high concentrations, highlights how many years will be needed to nullify the affects of once active mines (Elbaz-Poulichet et al., 2020). The occurrence of elements like arsenic and cadmium in the brain of inhabitant muskrats and red squirrels of Canada (Amuno et al., 2020), the water of mining areas of Mali (Bokar et al., 2020) are yet other instances of metal toxicity due to mining activity.

Soil fertility greatly relies on the microbial flora and the infiltration of heavy metals in mining areas deteriorates the prevalence of a wide variety of microbes including *Mycorrhizae*; thereby depleting the soil quality and fertility in such areas. Studies indicate that the growth of organic plants in such abandoned mining areas as a remedial measure is found to revive the microbial activity as plants serve as heavy metal quenchers (Stefanowicz et al., 2020).

Apart from destroying various ecological niches of plants, animals, and microbial flora (Sonter et al., 2018), the occurrence of accidents in mining areas could dump a heap of toxic wastes to adjoining areas from waste storage dams. For instance, the collapse of the mining dam in Minas Gerais (Brazil) was an environmental disaster resulting in xenobiotic pollution in surrounding rivers, wildlife, and human loss, hampering the delicate balance of the ecosystem as well as inhabitant life forms (Sonter et al., 2018).

4.2. LCA of hazards during mining steps

The life cycle analysis of mining processes indicates that conventional mining involves a wide range of steps, each contributing to a wide range of aftermaths. The majority of mining activity is carried out *in situ* in open pits, apart from other strategies such as placer mining and underground mining (Navarro and Zhao, 2014). Whatever, be the strategy of digging the earth for mining it should be ensured that

Fig. 1. An overview of mining pollution and associated hazards.

restoration of the land after to its previous topological state is quite necessary. In an instance noted along the coast of Kerala (India) near Chavara where deep underground pits are laid for the Titanium exploration; unless until it is filled back after the exploration that would cause drastic instability to that landform forcing the sea to encroach the adjoining lands including residential areas. Such sinkholes could lead to various environmental hazards. Similar decline of Nile Delta, as well as Po Delta (Italy) by anthropogenic activities and its chances of sea encroachment, has been predicted in another study (Gebremichael et al., 2018; Teatini et al., 2011). Thus as the economic benefits of mining promote us to carry out such industrial processes one should ensure such land should be restored a balanced topological form with its environment to avoid natural disasters. Strict regulations from the judiciary as well as the Government should be adopted to ensure such violations against natural borders should be prevented worldwide.

The process of beneficiation of minerals can be carried out by various techniques like grinding, sifting, gravitational separation, magnetic separation, and froth floatation. The life cycle analysis of Scandium mining by smelting reactions indicates that almost 88 % of the environmental impact was caused by the beneficiation step (Wang et al., 2020a). The wastes from the beneficiation step and subsequent results in the generation of a waste pool of unearthly matter yet finds no other way than trailing on this earth (U. EPA). They are often laden with highly concentrated levels of heavy metals and radioactive substances which was once remain scattered on the earth (Vahidi and Zhao, 2017). The chemical treatment of minerals also leaves back a trail of compounds like hydrogen fluoride, sulphur dioxide, sulphur trioxides, silicon tetra fluorides, cyanides, rare earth minerals etc. (Protano and Riccobono, 2002; Sun et al., 2016). The tailing impoundments, heap leach and waste rocks often infiltrate even into groundwater and surface waters in this area giving even well water a typical red colour making it unfit to drink (Vahidi and Zhao, 2017). The accumulation of acid wastes in water bodies of mining areas also indicates that why the approaches to quench and detoxify such wastes should be encouraged.

A recent study on the fire gases released after coal mining revealed the presence of components of HCN, isocyanic acid, acetaldehyde, alcohols, and notably SF_6 a greenhouse gas having a wide impact on global warming (Kruszewski et al., 2020). While quite often the so-called 'cloud making factories' (nicknamed by resident children) of mining areas are valued for its economic benefits by adults, the unnoticed presence of toxic gases in exhausts is depriving the chances of clean air to next generations.

Directive methods using biological agents to treat such waste could serve as ideal remediation processes (Johnson, 2014; Mahajan et al., 2017). Apart from this, the incorporation of techniques of microfiltration as well as nanofiltration would be helpful to remove almost 99 % of metals from sludge discards, thereby reducing the environmental burden (Meschke et al., 2020). The use of adsorption based treatment of acid wastes, adopting bio-nanomining techniques (Wong-Pinto et al., 2020), development of composites (Khudyakova et al., 2020; Gryczak et al., 2020), production of coal based technosols (Weiler et al., 2020) etc, from mining wastes are some the adjoining efforts directed to reduce the aftermaths of mineral mining. The use of natural acid quenching agents such as marl and sandstone in the treatment of acid mining exudates could also aid to decelerate the effect of extensive waste disposal from such areas (García-Valero et al., 2020).

4.3. Occupational hazards

Regardless of the high incentives obtained by most of the mining workers, the hazards they get exposed during mining cannot be ignored. As noted in Fig. 2 the various stress factors the miners face are numerous which could lead to various diseases by the time they retire from work or even before (Fig. 3). Apart from this, the workers are also vulnerable to various health issues described in the previous sections.

5. Microbes utility in mining

Irrespective of the need for excessive acid use, harsh smelting conditions, and costly machinery needed for mining, all efforts in mineral

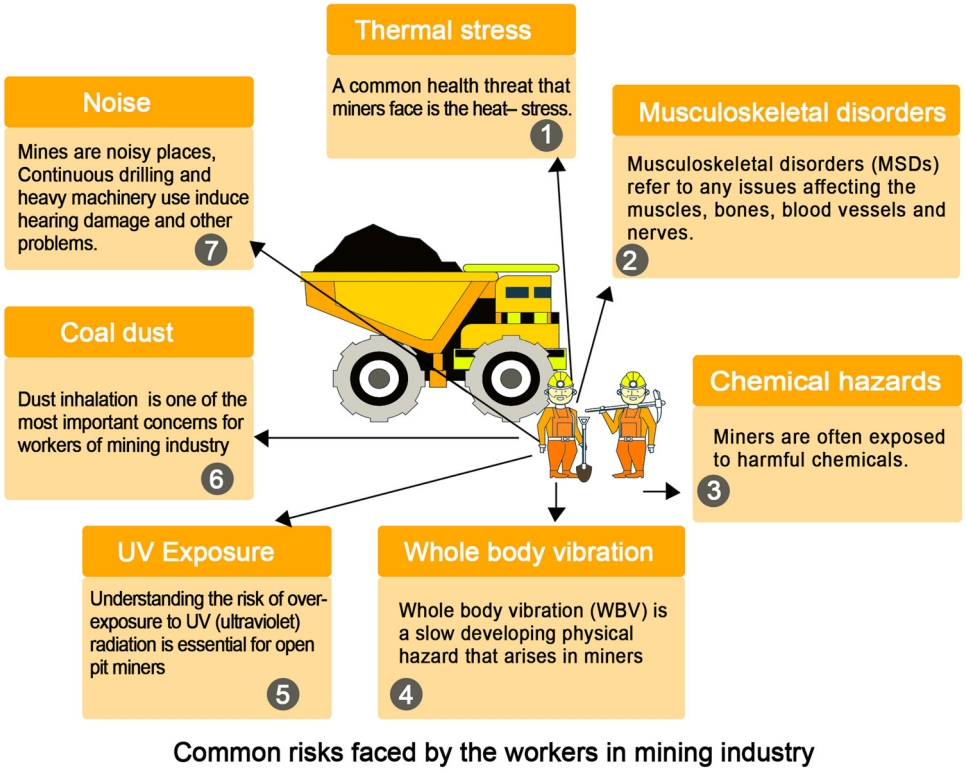


Fig. 2. Occupational Hazards at mining.

extraction are prioritized due to their economic value, hilarious utility in various sectors and contribution to a country's economy. In such a scenario, it's quite impossible to ban or discourage mining operations, but strategies to diminish its negative effects should be promoted. Process improvements including environmentally friendly strategies, new mine designs, effluent treatment methods etc, include some of such clean mining efforts (Hilson, 2003). The process of microbes at mining facilities come to play in two directions viz. to extract metals which are difficult to extract by conventional processes as well as to remediate the toxic wastes left over after mining (Godoy-Faúndez et al., 2015). The former referred to as microbial bioleaching involves the active or passive action of microbes and microbial products in the recovery of minerals from ores whereas the latter involves the microbe mediated treatment of exudates from mining leachates. Quite often drawing a line between these two processes is not practical as even while bioleaching remediation of exudates also happens.

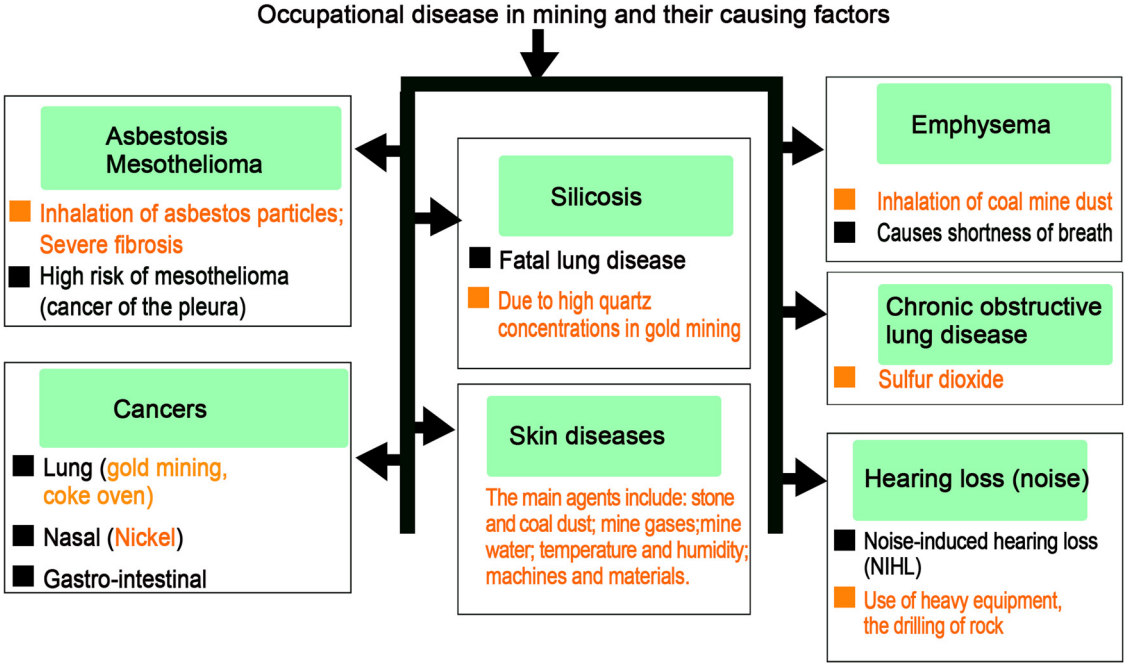


Fig. 3. Occupational diseases faced by miners.

5.1. Microbial bioleaching

Bioleaching turns out to be an eco-friendly alternative to conventional mining as well as an option even to treat toxic exudates from mines. The use of biomining in exploring the underground mines for metals are also encouraged owing to the ecological damage and high cost of conventional mining techniques (Johnson, 2015). The concepts of bioleaching involving microbes to leach minerals instead of using acids have thus attained much interest. However, the slow rates of extraction and long-time needed for extraction often diminishes its prospects for safe mining efforts. Thus efforts and progressive measures to promote bioleaching, extending its utility in treating other mining waste as well as its concomitant use with chemical methods need to be explored effectively in the near future at least (Asghari et al., 2013). Moreover, bioleaching finds its role in extraction of metals from smaller deposits, removal of arsenic or magnesium based contaminants and lacks the need for extreme treatment conditions compared to conventional methods.

The microbes oriented techniques to extract the elements from ores is getting much more significance in the mining research. The various microbes are known to participate in the solubilisation and deposition of chemicals in the earth's crust since ancient times (Clifford et al., 2018). The prominent use microorganisms in mining have some unique advantages over conventional strategies. Almost without exemption, the extraction of mineral elements using microbes is more eco-attractive. They do not instigate the production of sulphur dioxide or any other hazardous gaseous emissions during smelting or roasting.

Mainly there are two kinds of microbial mining strategies that have been reported in previous studies viz. stirred tank-type and irrigation-type processes. The former involves the growth of promising microbes in stirred tank type fermenters with ores of the metals and microbial interactions with them. The Irrigation processes comprise filtration of leaching solutions through concentrated ores that have been loaded in heaps, dumps, or columns (Silver, 1995). *Sulfobacillus, Sulfolobus, Ferroplasma, Metallosphaera*, are Ascidians recognized as the potentially significant microbial consortia in Bio-mining processes (Clifford et al., 2018; Cleaver et al., 2007). Table 3.reports some microbes used in bioleaching. Microbial leaching can be through either direct contactor noncontact modes with the ores and sometimes by both (Rawlings et al., 1999). Pyrite leaching involves the combinatorial action of iron oxidising and sulphur oxidising bacteria. The ferric ions act on the sulfide ores liberating thiosulfates and ferrous ions, the latter being converted to ferrous ions by iron oxidising bacteria. The sulphur oxidizers in turn oxidize thiosulphates liberating sulphuric acid thereby dissolving minerals (Li et al., 2020). The action of organic acids produced by the microbes as well as the involvement of various extracellular polysaccharides involved in biofilm formation is also suggested in some instances of rare earth element bioleaching (Wesam Abdel Ghany et al., 2013).

5.1.1. Rare earth element bioleaching

Rare Earth element based niches are the regions that are often the areas of high radioactivity due to the high content of thorium in majority areas. The wide utility of the rare earth elements in electronic devices and generated e-waste including mobile phones necessitates exploring better methods to track and utilize such mineral wastes rather than dumping them. Moreover, regardless of its limited occurrence quite often the spread occurrence of REE minerals is the major factor that limits its concentration and harnessing. Exploring the spent fly ashes of coal mines as noted in Wyoming's Powder River Basin (Huang et al., 2020), urban mines, e- wastes following the principles of biomining is found to be promising methods to mitigate the mineral waste toxicity.

The rare earth element (REE) mining is equally spread worldwide as other minerals and the requirement of energy-intensive techniques, strong chemicals, and resultant toxic end products are also evident in the case of these elements (Hewedy et al., 2013). The recovery of REE often involves the breakdown of ores such as phosphorus based monazite using organic acids and biotechnological agents are found more effective in this process compared to their chemical counterparts (Wesam Abdel Ghany et al., 2013). Moreover some bioleaching studies indicate that even precautions taken to overcome thorium infiltration is not needed absence of thorium was noted after bioleaching rare earth elements in their study (Fathollahzadeh et al., 2018a). The use of various organic acids such as citric acid, oxalic acid, citramalic, α- ketogluconic acid is found to influence the rate of REE leaching and radioactive elements removal with different organic acids with different microbes (Wesam Abdel Ghany et al., 2013; Brisson et al., 2020). Apart from organic acids various other components such as siderophores and complexing ligands to contribute to REE recovery by Actinobacteria (Zhang et al., 2018). Biological mineral recovery is more predominant in procedures permitting direct microbial interaction with the residual mixtures over the use of spent media or even contactless treatment using microbes (Fathollahzadeh et al., 2018a). The enhanced rate of bioleaching was also found by the combinatorial action of organic acids and sulphuric acid produced by A. ferrooxidans and E. aerogenes respectively in a consortium mediated REE recovery experiment from monazite ores (Fathollahzadeh et al., 2018b).

The use of various microbes such as *Penicillium tricolor* indicated that the leaching capacity of rare earth minerals from red mud was highest when the pulp ratio was 2–5 % and the microbe gave satisfactory radioactivity removal results as per Chinese standards (Qu and Lian, 2013), but pulp rates are found to be different for different microbes. The use of *Gluconobacter oxydans* feeded Continuous stirred tank fermentors to treat mining waste yielded 55 % efficiency in REE recovery; demanding more intensive research in this direction (Thompson et al., 2018). The recovery of REE from waste electronic devices has also been possible by the acidic environment provided by Acidithiobacillus thiooxidans aiding a recovery % greater than 99 % for

Table 3
Microbial Bioleaching agents an overview.

Microbe involved	Ore/substrate used for recovery	Metal	Reference
Acidithiobacillus caldus.	copper-bearing sulfide ore	Copper	(Feng et al., 2019)
Sulfobacillus and *Leptospirillum*	pyrite	Iron, copper	
Leptospirillum ferriphilum & Acidithiobacillus ferrooxidans	Low grade copper ores from mines	Copper	(Wang et al., 2020b)
Paecilomyces	Monazite	Rare earth elements	(Brisson et al., 2020)
Aspergillus ficuum and Pseudomonas aeruginosa	Monazite	Rare earth elements	(Wesam Abdel Ghany et al., 2013)
Penicillium tricolor	Red mud	Rare earth elements	(Qu and Lian, 2013)
Gluconobacter oxydans	retorted phosphor powder (RPP) and spent fluid catalytic cracking (FCC) catalyst.	Rare earth elements	(Reed et al., 2016)
Actinobacteria (Streptomyces)	Bastnaesite ($REE(CO_3)F$)	Rare earth elements (Ce, La or Y.)	(Zhang et al., 2018)
E. aerogenes and A. ferrooxidans	monazite	Rare earth elements (Ce, La, Nd, Pr, and Y)	(Fathollahzadeh et al., 2018b)

cerium, europium and neodymium (Marra et al., 2018). The ability to recover yet more metals from bio-remediated leachate at a later stage using a different microbe also increases the prospects of metal recovery even in the case of high risk elements. However, the need for long incubation times and low loading rates compared to chemical processes is a major challenge (Kucuker and Kuchta, 2018).

5.2. Copper bioleaching

The wide-spread prevalence of chalcopyrites ($CuFeS_2$) compared to other copper sulphides such as bornite (Cu_5FeS_4) and chalcocite (Cu_2S), often promotes copper recovery from chalcopyrite (Turan, 2014). Copper bioleaching from such ores thus are achieved by indirectly oxidising the iron as well as sulphur moieties of such ores indirectly yielding copper from ores. Indirect bio-oxidation of Copper from ores are brought about usually by the action of various iron oxidising bacteria which firstly oxidizes ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) and the latter in turn returns to its Fe^{2+} state after interacting with Cu thereby yielding copper from the ore (Yang et al., 2009). The indirect process of bioleaching is the most widely opted method and could be either with direct contact of bacterial biofilms with ores or mere oxidative action of ferric ions on ores through a bacterial noncontact mechanism (Silva et al., 2015). The involvement of extracellular exopolysaccharides in development of biofilms and aiding in copper bioleaching is also well noted (Feng et al., 2019).

The recovery of Cu from the wastes of mines from Brazil using Acidithiobacillus ferrooxidans assisted by silicate dissolution has aided to buffer the leachate pH to a great extent (Henne et al., 2018). Mostly the incorporation of autochthonous bacteria from leaching sites such as Leptospirillum ferriphilum and Acidithiobacillus ferrooxidans have proven noteworthy in regulating the pH and redox potential of leachate effectively by implementing techniques of cross bioleaching and microbial population regulatory strategies (Wang et al., 2020b).

Copper ore deposits are diminishing worldwide and thus recovery of copper from residual ores as well as e-waste using biological agents has achieved magnitude worldwide. The utility of *Leptospirillum ferriphilum* and *Sulfobacillus thermosulfidooxidans* culture-free supernatants were efficient in removing 100 % copper from 5 g/L of copper based e-waste; while almost 90 % of 100 g/L was recovered by a period of 9 days (Feng et al., 2019). Such studies open doors to consider developing e-waste managing units in different areas to manage their proper remediation. However, bioleaching of low grade copper sulfides by column floatation experiments has also been reported to take a long period of 75 days also even to 45 % recovery rates (Wang et al., 2014). Bioleaching of low grade copper sulfides and chalcopyrite by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* was found to be best at 30 ˚C at pH of 2.0 with pulp density of 5–10 % (Wang et al., 2014). Pyrite leaching using *Sulfobacillus* and *Leptospirillum* consortium indicates that the bioleaching properties of the former were directly influenced by the presence of active biofilms of the latter microbe and the inactivation of *Leptospirilllum* drastically affected the leaching efficiency of *Sulfobacillus* (Li et al., 2020). Comparative analysis of chemical leaching over bioleaching of copper sulphides indicates that bioleaching was advantageous for copper yields while it gave comparable results with iron yields (Bobadilla-Fazzini et al., 2017). In countries like Chile while Hydrometallurical copper recovery accounts for 23 %, bioleaching from copper leachates accounts for 42 % (Acevedo and Gentina, 2013).

6. Conclusion

A close look at every mining location worldwide is that they are either toxic by the presence of hazardous minerals or their environment is turned more toxic for living as a result of the mining. Thus whatever is the reason, the essentiality of such areas is to convert them to life-supporting nontoxic niches for life and bioleaching finds a reasonable solution in this direction. The establishment of an optimized bioleaching environment becomes essential for every mining location considering efforts of research using autochthonous microbes and practical expertise to implement successful remediation strategies of leachate. Apart from the general notion to gain economical benefit from mining, efforts in the direction of considering the delicate balance of Mother Earth should also be weighed by relying on eco-friendly bioleaching techniques concomitantly.

CRediT authorship contribution statement

Sharrel Rebello: Writing - original draft. **A.N. Anoopkumar:** Writing - original draft. **Embalil Mathachan Aneesh:** Writing - original draft, Validation. **Raveendran Sindhu:** Writing - original draft, Validation. **Parameswaran Binod:** Writing - review & editing. **Sang Hyoun Kim:** Writing - review & editing. **Ashok Pandey:** Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Raveendran Sindhu acknowledges Department of Science and Technology for sanctioning a project under DST WOS-B scheme.

References

Acevedo, F., Gentina, J.C., 2013. Application of Bioleaching to Copper Mining in Chile.
Akhter, P., Iqbal, S., Orfi, S., Kawamura, H., Ahmad, N., 2003. Thorium concentration in Pakistani diet. Health Phys. 84, 784–787.
Amuno, S., Shekh, K., Kodzhahinchev, V., Niyogi, S., 2020. Neuropathological changes in wild muskrats (*Ondatra zibethicus*) and red squirrels (Tamiasciurus hudsonicus) breeding in Arsenic endemic areas of Yellowknife, Northwest Territories (Canada): Arsenic and cadmium accumulation in the brain and biomarkers of oxidative stress. Sci. Total Environ. 704, 135426.
Antimony, H.D.W.U., 2015. Antimony—A Flame Fighter.
Asghari, I., Mousavi, S., Amiri, F., Tavassoli, S., 2013. Bioleaching of spent refinery catalysts: a review. J. Ind. Eng. Chem. 19, 1069–1081.
Bobadilla-Fazzini, R.A., Pérez, A., Gautier, V., Jordan, H., Parada, P., 2017. Primary copper sulfides bioleaching vs. chloride leaching: advantages and drawbacks. Hydrometallurgy 168, 26–31.
Bokar, H., Traoré, A.Z., Mariko, A., Diallo, T., Traoré, A., Sy, A., Soumaré, O., Dolo, A., Bamba, F., Sacko, M., Touré, O., 2020. Geogenic influence and impact of mining activities on water soil and plants in surrounding areas of Morila Mine, Mali. J. Geochem. Explor. 209, 106429.
Brisson, V.L., Zhuang, W.-Q., Alvarez-Cohen, L., 2020. Metabolomic analysis reveals contributions of citric and citramalic acids to rare earth bioleaching by a paecilomyces fungus. Front. Microbiol. 10.
Choi, S., Yoon, C., Kim, S., Kim, W., Ha, K., Jeong, J., Kim, J., Shin, J., Park, D., 2018. Comprehensive evaluation of hazardous chemical exposure control system at a semiconductor manufacturing company in South Korea. Int. J. Environ. Res. Public Health 15, 1162.
Cleaver, A.A., Burton, N.P., Norris, P.R., 2007. A novel Acidimicrobium species in continuous cultures of moderately thermophilic, mineral-sulfide-oxidizing acidophiles. Appl. Environ. Microbiol. 73, 4294–4299.
Clifford, Obi, Ejukonemu, Nkemnaso, Ejovwokoghene, Francis, Innocent, C.U., 2018. Biomining: the past, the present and the future. Biosci. Bioeng. 4, 52–60.
Cooper, R.G., Harrison, A.P., 2009. The exposure to and health effects of antimony. Indian J. Occup. Environ. Med. 13, 3.
Davies, T., 1994. Water quality characteristics associated with fluorite mining in the Kerio Valley area of western Kenya. Int. J. Environ. Health Res. 4, 165–175.
Dietrich, G., 1968. Uranium and Thorium Reserves of the World. Prospecting, Mining, and Costs.
Dindarloo, S., 2016. Reliability forecasting of a load-haul-dump machine: a comparative study of ARIMA and neural networks. Qual. Reliab. Eng. Int. 32, 1545–1552.
Elbaz-Poulichet, F., Guédron, S., Anne-Lise, D., Freydier, R., Perrot, V., Rossi, M., Piot, C., Delpoux, S., Sabatier, P., 2020. A 10,000-year record of trace metal and metalloid (Cu, Hg, Sb, Pb) deposition in a western Alpine lake (Lake Robert, France): deciphering local and regional mining contamination. Quat. Sci. Rev. 228, 106076.
Fathollahzadeh, H., Becker, T., Eksteen, J.J., Kaksonen, A.H., Watkin, E.L.J., 2018a. Microbial contact enhances bioleaching of rare earth elements. Bioresource Technol. Rep. 3, 102–108.
Fathollahzadeh, H., Hackett, M.J., Khaleque, H.N., Eksteen, J.J., Kaksonen, A.H., Watkin,

E.L.J., 2018b. Better together: potential of co-culture microorganisms to enhance bioleaching of rare earth elements from monazite. Bioresource Technol. Rep. 3, 109–118.
Feng, S., Li, K., Huang, Z., Tong, Y., Yang, H., 2019. Specific mechanism of *Acidithiobacillus caldus* extracellular polymeric substances in the bioleaching of copper-bearing sulfide ore. PloS one 14.
Freitas, R., Costa, S., Cardoso, C.E.D., Morais, T., Moleiro, P., Matias, A.C., Pereira, A.F., Machado, J., Correia, B., Pinheiro, D., Rodrigues, A., Colónia, J., Soares, A.M.V.M., Pereira, E., 2020. Toxicological effects of the rare earth element neodymium in *Mytilus galloprovincialis*. Chemosphere 244, 125457.
García-Valero, A., Martínez-Martínez, S., Faz, A., Rivera, J., Acosta, J.A., 2020. Environmentally sustainable acid mine drainage remediation: use of natural alkaline material. J. Water Process Eng. 33, 101064.
Gebremichael, E., Sultan, M., Becker, R., El Bastawesy, M., Cherif, O., Emil, M., 2018. Assessing land deformation and sea encroachment in the Nile Delta: a radar interferometric and inundation modeling approach. J. Geophys. Res.: Solid Earth 123, 3208–3224.
Godoy-Faúndez, A., Aitken, D., Reyes-Bozo, L., Rivera, D., 2015. Environmental-microbial biotechnology inside mining operations from an engineering viewpoint based on LCA. Environmental Microbial Biotechnology. Springer, pp. 133–158.
Gomez-Caminero, A., Howe, P.D., Hughes, M., Kenyon, E., Lewis, D., Moore, M., Aitio, A., Becking, G.C., Ng, J., 2001. Arsenic and Arsenic Compounds. World Health Organization.
Greenway, J.A., Gerstenberger, S., 2010. An evaluation of lead contamination in plastic toys collected from day care centers in the Las Vegas Valley, Nevada, USA. Bull. Environ. Contam. Toxicol. 85, 363–366.
Gryczak, M., Wong, J.W.Y., Thiemann, C., Ferrari, B.J.D., Werner, I., Petzhold, C.L., 2020. Recycled low-density polyethylene composite to mitigate the environmental impacts generated from coal mining waste in Brazil. J. Environ. Manage. 260, 110149.
Han, D.-H., Park, M.S., 2018. Survey of awareness about hazardous chemicals of residents living near chemical plants in South Korea. Ind. Health 56, 285–291.
Hansson, S.V., Desforges, J.-P., van Beest, F.M., Bach, L., Halden, N.M., Sonne, C., Mosbech, A., Søndergaard, J., 2020. Bioaccumulation of mining derived metals in blood, liver, muscle and otoliths of two Arctic predatory fish species (*Gadus ogac* and *Myoxocephalus scorpius*). Environ. Res. 183, 109194.
Hara, M., Honda, T., Nguyen, H.X., Nakazawa, K., Yamamoto, R., Yasui, I., 2005. toxicological profile for lead toxicological profile for lead, 1990. J. Mater. Cycles Waste Manage. 7, 78–87.
Harraz, H., 2010. Mining Methods: Part I-Surface Mining.
Hartman, H.L., Mutmansky, J.M., 2002. Introductory Mining Engineering. John Wiley & Sons.
Henne, A., Craw, D., Vasconcelos, P., Southam, G., 2018. Bioleaching of waste material from the Salobo mine, Brazil: recovery of refractory copper from Cu hosted in silicate minerals. Chem. Geol. 498, 72–82.
Hewedy, M.A., Rushdy, A.A., Kamal, N.M., 2013. Bioleaching of rare earth elements and uranium from Sinai Soil, Egypt using actinomycetes. Egypt. J. Hosp. Med. 53, 909–917.
Hilson, G., 2003. Defining “cleaner production” and “pollution prevention” in the mining context. Miner. Eng. 16, 305–321.
Huang, C.-F., Hsu, C.-J., Liu, S.-H., Lin-Shiau, S.-Y., 2012. Exposure to low dose of cinnabar (a naturally occurring mercuric sulfide (HgS)) caused neurotoxicological effects in offspring mice. Biomed Res. Int. 2012.
Huang, Z., Fan, M., Tian, H., 2020. Rare earth elements of fly ash from Wyoming’s powder River Basin coal. J. Rare Earths 38, 219–226.
Jain, A., Sarsaiya, S., Wu, Q., Shi, J., Lu, Y., 2019. New insights and rethinking of cinnabar for chemical and its pharmacological dynamics. Bioengineered 10, 353–364.
Järup, L., 2003. Hazards of heavy metal contamination. Br. Med. Bull. 68, 167–182.
Johnson, D.B., 2014. Biomining—biotechnologies for extracting and recovering metals from ores and waste materials. Curr. Opin. Biotechnol. 30, 24–31.
Johnson, D.B., 2015. Biomining goes underground. Nat. Geosci. 8, 165–166.
Johnson, N.C., Manchester, S., Sarin, L., Gao, Y., Kulaots, I., Hurt, R.H., 2008. Mercury vapor release from broken compact fluorescent lamps and in situ capture by new nanomaterial sorbents. Environ. Sci. Technol. 42, 5772–5778.
Jung, Y.-S., Sakong, J., An, S.-Y., Lee, Y.-E., Song, K.-B., Choi, Y.-H., 2012. The relationship between dental amalgam fillings and urinary mercury concentration among elementary school children in a metropolitan area. J. Dental Hygiene Sci. 12, 253–258.
Kaihuan, Z., Fuchuan, J., 2012. Research on intrinsic safety method for open-pit mining. Procedia Eng. 43, 453–458.
Kamp, D.W., 2009. Asbestos-induced lung diseases: an update. Transl. Res. 153, 143–152.
Keith, S., Faroon, O., Roney, N., Scinicariello, F., Wilbur, S., Ingerman, L., Llados, F., Plewak, D., Wohlers, D., Diamond, G., 2013. Toxicological Profile for Uranium.
Khudyakova, L., Kotova, I.Y., Timofeeva, S., 2020. Composite binders from mining waste. In: IOP Conference Series: Earth and Environmental Science. IOP Publishing. pp. 012053.
Kostyniak, P.J., 1998. Mercury as a potential hazard for the dental practitioner. N. Y. State Dent. J. 64, 40.
Kruszewski, Ł., Fabiańska, M.J., Segit, T., Kusy, D., Motyliński, R., Ciesielczuk, J., Deput, E., 2020. Carbon-nitrogen compounds, alcohols, mercaptans, monoterpenes, acetates, aldehydes, ketones, SF6, PH3, and other fire gases in Coal-mining waste heaps of Upper Silesian Coal Basin (Poland)–A re-investigation by means of in situ FTIR external database approach. Sci. Total Environ. 698, 134274.
Kucuker, M., Kuchta, K., 2018. Biomining–biotechnological systems for the extraction and recovery of metals from secondary sources. Glob. Nest J. 20, 737–742.
Kumar, A., Ali, M., Pandey, B.N., 2013. Understanding the biological effects of thorium and developing efficient strategies for its decorporation and mitigation. Barc Newsletter 335, 55–60.
Leblanc, M., Morales, J., Borrego, J., Elbaz-Poulichet, F., 2000. 4,500-year-old mining pollution in southwestern Spain: long-term implications for modern mining pollution. Econ. Geol. 95, 655–662.
Li, Q., Zhu, J., Li, S., Zhang, R., Xiao, T., Sand, W., 2020. Interactions between cells of *Sulfobacillus thermosulfidooxidans* and *Leptospirillum ferriphilum* during pyrite bioleaching. Front. Microbiol. 11, 44.
Li, J.-g., Zhan, K., 2018. Intelligent mining technology for an underground metal mine based on unmanned equipment. Engineering 4, 381–391.
Liu, G., Cheresh, P., Kamp, D.W., 2013. Molecular basis of asbestos-induced lung disease. Annu. Rev. Pathol.: Mech. Dis. 8, 161–187.
Mahajan, S., Gupta, A., Sharma, R., 2017. Bioleaching and biomining. Principles and Applications of Environmental Biotechnology for a Sustainable Future. Springer, pp. 393–423.
Markov, S., 2012. Toxic Minerals. pp. 519–522.
Marra, A., Cesaro, A., Rene, E.R., Belgiorno, V., Lens, P.N.L., 2018. Bioleaching of metals from WEEE shredding dust. J. Environ. Manage. 210, 180–190.
Meschke, K., Hofmann, R., Haseneder, R., Repke, J.-U., 2020. Membrane treatment of leached mining waste – a potential process chain for the separation of the strategic elements germanium and rhenium. Chem. Eng. J. 380, 122476.
Mosby, C., Glanze, W., Anderson, K., 1996. Mosby Medical Encyclopedia, The Signet: Revised Edition, St. Louis Vol. 16. pp. 875–882.
Navarro, J., Zhao, F., 2014. Life-cycle assessment of the production of rare-earth elements for energy applications: a review. Front. Energy Res. 2.
Chapter 3 - neutralization. In: Nemerow, N.L. (Ed.), Industrial Waste Treatment. Butterworth-Heinemann, Burlington, pp. 35–44.
Neuberger, J.S., Mulhall, M., Pomatto, M.C., Sheverbush, J., Hassanein, R.S., 1990. Health problems in Galena, Kansas: a heavy metal mining Superfund site. Sci. Total Environ. 94, 261–272.
Nynäs, P., Pukkala, E., Vainio, H., Oksa, P., 2017. Cancer Incidence in Asbestos-Exposed Workers: An Update on Four Finnish Cohorts, Safety and Health at Work 8. pp. 169–174.
Pajączkowska, A., 1970. Hydrothermal crystallization of cinnabar, HgS. J. Cryst. Growth 7, 93–96.
Prasad, B.S., Murthy, V., Pandey, S., 2016. Investigations on rock drillability applied to underground mine development vis-à-vis drill selection. Recent Advances in Rock Engineering (RARE 2016). Atlantis Press.
Protano, G., Riccobono, F., 2002. High contents of rare earth elements (REEs) in stream waters of a Cu–Pb–Zn mining area. Environ. Pollut. 117, 499–514.
Qu, Y., Lian, B., 2013. Bioleaching of rare earth and radioactive elements from red mud using *Penicillium tricolor* RM-10. Bioresour. Technol. 136, 16–23.
Ramani, R.V., 2012. Surface mining technology: progress and prospects. Procedia Eng. 46, 9–21.
Rawlings, D.E., Tributsch, H., Hansford, G.S., 1999. Reasons why ‘Leptospirillum’-like species rather than Thiobacillus ferrooxidans are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology 145, 5–13.
Reed, D.W., Fujita, Y., Daubaras, D.L., Jiao, Y., Thompson, V.S., 2016. Bioleaching of rare earth elements from waste phosphors and cracking catalysts. Hydrometallurgy 166, 34–40.
Saerens, A., Ghosh, M., Verdonck, J., Godderis, L., 2019. Risk of cancer for workers exposed to antimony compounds: a systematic review. Int. J. Environ. Res. Public Health 16, 4474.
Sato, T., Shimosato, T., Klinman, D.M., 2018. Silicosis and lung cancer: current perspectives. Lung Cancer: Targets Ther. 9, 91.
Silva, R.A., Park, J., Lee, E., Park, J., Choi, S.Q., Kim, H., 2015. Influence of bacterial adhesion on copper extraction from printed circuit boards. Sep. Purif. Technol. 143, 169–176.
Silver, S., 1995. Mining with microbes. Bio/Technology 13, 773–778.
Sonter, L.J., Ali, S.H., Watson, J.E., 2018. Mining and biodiversity: key issues and research needs in conservation science. Proc. R. Soc. B 285, 1926.
Souza, J.Cd., Rodrigues, H.C., Rocha, S.S., Bastos, Fd.F., 2018. mining scheduling of gypsum exploitation by terrace mining method: an eco-sustainable alternative. Tecnologia em Metalurgia, Materiais e Mineração 15, 175–181.
Stefanowicz, A.M., Kapusta, P., Zubek, S., Stanek, M., Woch, M.W., 2020. Soil organic matter prevails over heavy metal pollution and vegetation as a factor shaping soil microbial communities at historical Zn–Pb mining sites. Chemosphere 240, 124922.
Sun, Z., Xiao, Y., Agterhuis, H., Sietsma, J., Yang, Y., 2016. Recycling of metals from urban mines–a strategic evaluation. J. Cleaner Prod. 112, 2977–2987.
Teatini, P., Tosi, L., Strozzi, T., 2011. Quantitative evidence that compaction of Holocene sediments drives the present land subsidence of the Po Delta, Italy. J. Geophys. Res.: Solid Earth 116.
Thompson, V.S., Gupta, M., Jin, H., Vahidi, E., Yim, M., Jindra, M.A., Nguyen, V., Fujita, Y., Sutherland, J.W., Jiao, Y., Reed, D.W., 2018. Techno-economic and life cycle analysis for bioleaching rare-earth elements from waste materials. ACS Sustain. Chem. Eng. 6, 1602–1609.
Turan, M., 2014. Direct selective leaching of chalcopyrite concentrate. Can. Metall. Q. 53, 444–449.
U. EPA, in.
Vahidi, E., Zhao, F., 2017. Environmental life cycle assessment on the separation of rare earth oxides through solvent extraction. J. Environ. Manage. 203, 255–263.
Visonà, S.D., Villani, S., Manzoni, F., Chen, Y., Ardissino, G., Russo, F., Moretti, M., Javan, G.T., Osculati, A., 2018. Impact of asbestos on public health: a retrospective study on a series of subjects with occupational and non-occupational exposure to asbestos during the activity of Fibronit plant (Broni, Italy). J. Public Health Res. 7.
W.H. Organization, 2000. Crystalline Silica, Quartz. World Health Organization.

Wang, J., Zhu, S., Zhang, Y.-s., Zhao, H.-b., Hu, M.-h., Yang, C.-r., Qin, W.-q., Qiu, G.-z., 2014. Bioleaching of low-grade copper sulfide ores by *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. Journal of Central South University 21, 728–734.

Wang, L., Wang, P., Chen, W.-Q., Wang, Q.-Q., Lu, H.-S., 2020a. Environmental impacts of scandium oxide production from rare earths tailings of Bayan Obo Mine. J. Cleaner Prod., 122464.

Wang, X., Ma, L., Wu, J., Xiao, Y., Tao, J., Liu, X., 2020b. Effective bioleaching of low-grade copper ores: insights from microbial cross experiments. Bioresour. Technol. 308, 123273.

Weiler, J., Firpo, B.A., Schneider, I.A.H., 2020. Technosol as an integrated management tool for turning urban and coal mining waste into a resource. Miner. Eng. 147, 106179.

Wesam Abdel Ghany, H., Osman Abdel Naby, D., Shimaa Salah Eldien, H., 2013. Bioleaching of some rare earth elements from Egyptian monazite using *Aspergillus ficuum* and *Pseudomonas aeruginosa*. Walailak J. Sci. Technol. (WJST) 11.

Wong-Pinto, L.-s., Menzies, A., Ordóñez, J.I., 2020. Bionanomining: biotechnological synthesis of metal nanoparticles from mining waste—opportunity for sustainable management of mining environmental liabilities. Appl. Microbiol. Biotechnol. 1–11.

Yang, T., Xu, Z., Wen, J., Yang, L., 2009. Factors influencing bioleaching copper from waste printed circuit boards by *Acidithiobacillus ferrooxidans*. Hydrometallurgy 97, 29–32.

Zhang, L., Dong, H., Liu, Y., Bian, L., Wang, X., Zhou, Z., Huang, Y., 2018. Bioleaching of rare earth elements from bastnaesite-bearing rock by actinobacteria. Chem. Geol. 483, 544–557.

Zhao, X., Sun, Y., Huang, J., Wang, H., Tang, D., 2020. Effects of soil heavy metal pollution on microbial activities and community diversity in different land use types in mining areas. Environ. Sci. Pollut. Res. 27 (2020), 20215–20226.

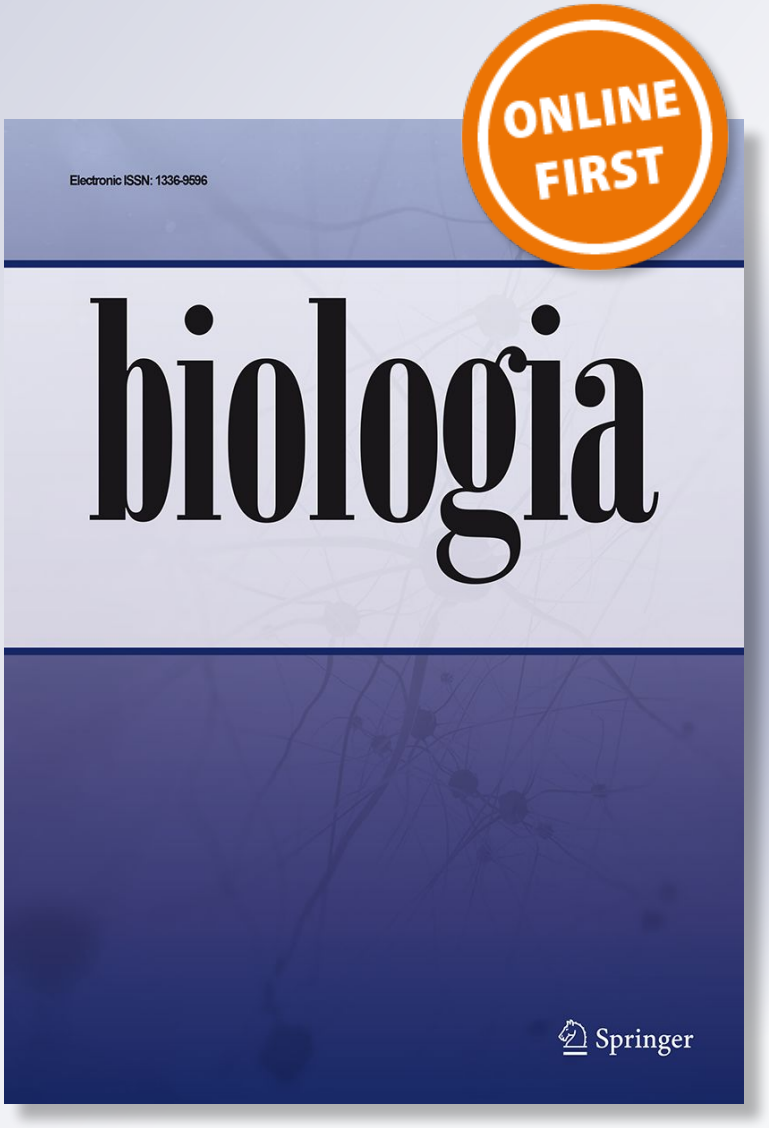
Molecular characterization of Aedes, Culex, Anopheles, *and* Armigeres *vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis*

Ammini Naduvanthar Anoopkumar, Sreedev Puthur, Sharrel Rebello & Embalil Mathachan Aneesh

Biologia
Botany, Zoology and Cellular and Molecular Biology

ISSN 0006-3088

Biologia
DOI 10.2478/s11756-019-00231-0



Springer

Biologia
https://doi.org/10.2478/s11756-019-00231-0

ORIGINAL ARTICLE

Molecular characterization of *Aedes*, *Culex*, *Anopheles*, and *Armigeres* vector mosquitoes inferred by mitochondrial cytochrome oxidase I gene sequence analysis

Ammini Naduvanthar Anoopkumar[1,2] · Sreedev Puthur[1] · Sharrel Rebello[1] · Embalil Mathachan Aneesh[1]

Received: 30 October 2018 / Accepted: 12 March 2019

Abstract

The current epidemics of vector-borne diseases in tropical countries, especially in India, have extended the need for a comprehensive understanding of the distribution of epidemiologically important vector mosquitoes. The advanced developments in genomic research including phylogenetic studies will empower the molecular studies that are vital to determine the genetic divergence and evolutionary history of mosquito vectors. The use of the cytochrome oxidase c subunit I (COI) gene-based approaches to outline the interrelationship of vector mosquitoes from different genera could also elucidate the obscurity that has risen from improper taxonomical classification. The mosquitoes were collected from Nelliyampathy, Kerala, India and identified using systematic keys and catalogues. The genomic DNA of the mosquitoes was extracted using NucleoSpin® Tissue Kit and PCR amplification of the mitochondrial COI gene was accomplished by using the following primers: Forward 5′- GGTCAACAAATCATAAAGATATTGG-3′ and reverse 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′. The genetic divergence was assessed by means of the NJ-K2P method. The neighbor-joining tree was constructed using the MEGA7 software. The diversity of mosquitoes was estimated using the Shannon index through SPSS and Venn diagram plotter. The COI gene-based mitochondrial DNA analysis revealed distinct clustering of individual mosquito species within every genus together with strong bootstrap support. In total, our investigation productively identified and confirmed the interrelationship between the COI gene sequences of ten epidemiologically important mosquito species. This study also discusses the substantial evidence of mosquito species complex formation. Our findings offer a base for future research which is essential for the better understanding of mosquito phylogeny and shed light upon, a novel vector control strategy.

Keywords DNA barcoding · COI · Mosquitoes · Phylogeny · Diversity

Introduction

Intensified movement of animals and humans builds rapid openings for the establishment of vector mosquito species. Hence, mosquitoes are known as the most important arthropods implicated in nearly 90% of major vector-borne diseases over the world (WHO 2011). Precise and accurate identification of the mosquitoes is imperative to develop the mosquito-control strategies (Otranto et al. 2009). The *Aedes*, *Anopheles*, and *Culex* genera are reflected as the principal vectors for the transmission of mosquito-borne diseases including malaria, West Nile fever, filariasis, dengue, and yellow fever. Extension of the geographic distribution of mosquitoes over the world increases the significance of epidemiologically important species identification (Tipayamongkholgul et al. 2009; Aivazi and Vijayan 2010; Do et al. 2014).

The morphological identification of mosquitoes became challenging in closely related species differing in host and ecological preferences (Apote 1947). The identification based on morphological characteristics of *Anopheles gambiae* (Giles, 1902) and *Culex pipiens* (Linnaeus, 1758) mosquitoes has been revealed as a crucial challenge since they exhibit several

✉ Embalil Mathachan Aneesh
aneeshembalil@stjosephs.edu.in

Ammini Naduvanthar Anoopkumar
anoopkumar.247@gmail.com

Sreedev Puthur
sreedevputhur@gmail.com

Sharrel Rebello
sharrel.rebello@gmail.com

[1] Communicable Disease Research Laboratory, Department of Zoology, St. Joseph's College, Irinjalakkuda, Kerala, India

[2] Department of Zoology, Christ College, Irinjalakkuda, Thrissur, Kerala, India

Published online: 23 April 2019

subtle differences in morphological characters (Börstler et al. 2014; Laurito et al. 2015). The conventional taxonomic method of mosquito identification relies on morphological characters of the organisms, is time-consuming, requires special knowledge, and might be problematic when damaged specimens are obtained for identification (Batovska et al. 2016; Wilke et al. 2016).

The cytochrome oxidase c subunit I (COI) gene-based methods for molecular phylogeny; genetic diversity (Low et al. 2014) and species identification have received more significance in the current mosquito research (Singh et al. 2004; Kang and Sim 2013). The 5′- segment is referred as the most commonly accepted barcode region for animal species identification using COI gene-based molecular methods (Ratnasingham and Hebert 2007). DNA barcoding of a wide range of mosquitoes offers insights into the mosquito genera composition of a particular geographic region (Reinert and Harbach 2005). In addition, the significant relationship between mosquito genotypes and their ability to transmit disease-causing pathogens has prompted the need of genetic diversity based studies on mosquitoes (Fansiri et al. 2013; Thongsripong et al. 2013). Molecular studies on species diversity and species composition of mosquito population in the local region have contributed to the public system to develop advanced strategies for the preclusion of mosquito-borne diseases (Mwangangi et al. 2013).

As per the statistical analysis of Directorate of Health Service, Kerala Government health department in 2017, several people (2287 people infected, 37 people died) near the study area were infected, threatened and died due to mosquito-borne diseases such as dengue, chikungunya, and malaria. This drastic situation indicated that the study area is prone to severe mosquito-borne diseases. The elevated number of foreigners and tourist visitors in this region might also contribute to increasing infection rate by mosquito-borne diseases. The importance of mosquitoes in the transmission of pathogens underscores the significance of accurate, comparable and precise approaches of population diversity determination and phylogenetic analysis among their species in Nelliyampathy. Many reports regarding the severe infections transmitted by dengue and malaria vectors necessitate advanced molecular studies to analyze the population diversity and extensive genetic divergence of mosquitoes occurring in the study region. Moreover, this region is an ecotone formed by two different ecosystems, particularly forest and semi-urban ecosystems which makes the study unique. There are only few research articles correlating molecular studies and species-specific similarity identification of mosquitoes in Nelliyampathy. Therefore a molecular study was required to determine the species-specific host-pathogen interaction with special reference to co-evolution. In this study, we focussed on the species diversity, distribution, medical importance, and phylogeny of selected mosquito species collected from Nelliyampathy, a part of Western Ghats, hotspot of biodiversity, Palakkad district of Kerala, India.

Materials and methods

Study area and mosquito sampling

The study area Nelliyampathy (Fig. 1) is a forest and semi-urban tourist place belonging to the Palakkad district of Kerala, India. Larvae, pupae, and adult mosquitoes were randomly collected during April to September 2017 by using white enameled dippers and mosquito traps. The collected mosquitoes were counted, kept in small plastic bottles and transferred individually to plastic trays for rearing in laboratory conditions. The adult mosquitoes that emerged from larvae and pupae were identified with the help of experienced taxonomists (using dichotomous keys) together with the COI gene-based molecular analysis (Clark-Gil and Darsie 1983; Ward 2005; Cywinska et al. 2006; Chan et al. 2014). A sample number was allotted to each adult mosquito specimen since the voucher specimen was deposited at Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Joseph's College, Irinjalakuda.

All mosquito specimens analysed in this study were collected as a part of UGC research award (UGC Research Award - F30–6/20–16(SA-II)). The CDRL (Communicable Disease Research Laboratory, Department of Zoology, St. Joseph's College, Irinjalakuda, Kerala, India) serves as the reference centre for the identification of field collected mosquito specimens under the above-mentioned programme. The collected mosquitoes were identified to species level using systematic keys and catalogues (Christophers 1933; Barraud 1934; Stone et al. 1959; Hayat and Subba Rao 1981).

DNA extraction, polymerase chain reaction and DNA sequencing

DNA from selected mosquito species was extracted from their legs. Sterilized forceps was used for removing the fore-, mid-, and hind legs. As per the manufacturer's instructions, the genomic DNA from the selected tissues was extracted using NucleoSpin® Tissue Kit (Macherey-Nagel). The extracted DNA was then stored at 20 °C for further analysis. The PCR amplification of the mitochondrial COI gene was performed by using the following primers: forward 5′- GGTCAACA AATCATAAAGATATTGG-3′ and reverse 5′- TAAACTTC AGGGTGACCAAAAAATCA-3′. The PCR reaction conditions were maintained as follows: initial denaturation at 98 °C for 30 s, followed by 10 cycles of denaturation at 98 °C for 5 s, annealing at 50 °C for 10 s, and extension at 72 °C for 10 s and 1 min. Another 35 cycles of amplification reaction were performed at 94 °C for 40 s, annealing at 51 °C for 1 min together

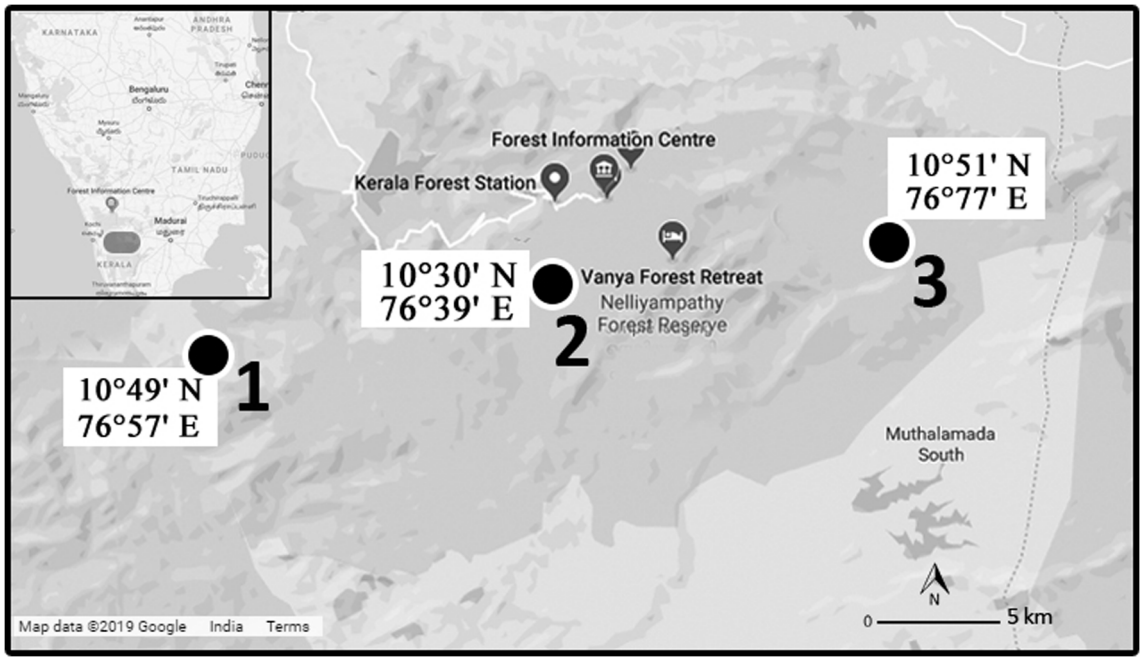


Fig. 1 Map of mosquito sampling locations in Nelliyampathy, Palakkad District of Kerala, India. 1 - Site 1, 2 - Site 2, 3 - Site3

with extension at 72 °C. The final extension was maintained at 72 °C for 10 min.

Agarose gel electrophoresis of PCR products

The amplicons were visualized in 1.2% agarose gels prepared by using TBE buffer (0.5X) encompassing 0.5 μg ml^{-1} ethidium bromide. Aliquots of the PCR products were loaded after mixing with 6X loading dye. The electrophoresis was done for about 1–2 h at 75 V in a power supply along with standard DNA markers. 2-log DNA ladder (NEB) was used as a molecular standard, and gels were visualized by using a UV transilluminator (Genei). ExoSAP-IT (GE Healthcare) treatment was employed for the removal of unwanted elements from PCR products, including dNTPs and primers.

The sequencing reactions of the DNA samples were carried out in a PCR thermal cycler with the help of BigDye Terminator v3.1 Cycle Sequencing Kit developed by Applied Biosystems, USA. The quality of the sequence was validated by using the Sequence Scanner Software v1. The obtained sequences from the selected mosquito species were forwarded to Geneious Pro v5.1 (Drummond et al. 2010) for editing and sequence alignment. The aligned COI sequences generated from the present investigation were deposited in NCBI (National Center for Biotechnology Information) GenBank (http://www.ncbi.nlm.nih.gov).

Sequence alignment and phylogenetic analysis

Clustal W version 1.6 was employed for DNA sequence alignment (Thompson et al. 1994). The phylogenetic relationships of the selected specimens were inferred using the neighbour-joining method (MEGA7 Version 7.0). Maximum Composite Likelihood method was employed for the evolutionary distance computation (Saitou and Nei 1987; Tamura et al. 2004). The Codon positions comprised were 1st + 2nd + 3rd + Noncoding. The positions encompassing gaps were eliminated and the evolutionary analyses were accomplished using MEGA7 (Kumar et al. 2016).

Statistical analysis

The statistical analysis was performed using the SPSS version 24.0.0. and Venn Diagram Plotter version 1.5.5228.29.250. The diversity index was calculated by using the following equation.

$$\text{Shannon Index } \left(H'\right) \text{ and Sorensen's Coefficient (CC)}$$

$$\text{Shannon Index } \left(H'\right) = \sum_{i=1}^{s} pi \ln pi$$

Where p is the proportion of individuals, ln is the natural log, and Σ is the Sum of the calculations.

Values of Shannon index ranged from 0.6 to 2.1. Larger Shannon index values represent greater diversity of mosquito species from the selected sites.

The Sorensen's Coefficient analysis was made to determine similarities between communities

$$\text{Sorensen's Coefficient (CC)} = \frac{2C}{S1 + S2}$$

The number of species of two communities is represented by C. S1 represents the total number of species in community 1; S2 represents the total number of species recorded in community 2.

Results

Of the 150 samples collected, 104 mosquito specimens belonging to 10 species of 4 genera were analysed at the molecular level. After the collection and molecular identification of mosquitoes (*Aedes*, *Culex*, *Anopheles,* and *Armigeres*), the data were arranged for the determination of species diversity in Nelliyampathy. Most of the mosquitoes that were collected as larvae were reared in laboratories to adults before identification. Advanced developments in molecular biology (Mitochondrial DNA Analysis) allows us to complement the conventional taxonomic methods of mosquito species identification. Our investigation included three species of *Aedes* ($n = 75$), three species of *Anopheles* ($n = 22$), three species of *Culex* ($n = 50$), followed by one species of *Armigeres* ($n = 3$). As shown in Figs. 2, 3, and 4, COI gene-based mitochondrial DNA analysis revealed the distinct clustering of individual mosquito species within every genus together with strong bootstrap support. The clustering patterns strongly approved the conventional morphological identification, permitting the differentiation of individual mosquito species based on mitochondrial DNA analysis (COI sequences). Moreover, the sequences from the present study clustered with those of other related species from various prevalent regions reported in the NCBI databases. It was noted that various morphologically similar *Culex* and *Aedes* species can be differentiated to species level based on mitochondrial DNA analysis. The COI sequences-based identification in the present study revealed the presence of 10 mosquito species such as *Aedes aegypti*, *Aedes albopictus*, *Aedes vittatus, Anopheles annularis, Anopheles stephensi, Anopheles culicifacies, Culex quinquefasciatus, Culex tritaeniorhynchus, Culex pipiens*, and *Armigeres subalbatus* (Table 1). *Aedes aegypti*, *Ae. albopictus,* and *Cx. quinquefasciatus* were the most widespread and were observed at all three study sites. *Aedes vittatus* and *An. annularis* were absent from site 2, whereas *Ar. subalbatus* was not recorded from site 3. Neighbour-joining (NJ) phylogenetic tree constructed using the COI sequences for *Aedes* species is shown in Fig. 2. Figures 3 and 4 show the NJ tree of *Anopheles* and *Culex* species together with *Armigeres,* respectively. The NJ tree for *Aedes* species formed 20 distinct clades with strongly supported bootstrap values, each representing an individual species (Fig. 2). The six morphologically identified *Anopheles* species were grouped into 21 clades in the NJ tree (Fig. 3). All of the aforementioned clades were strongly supported by bootstrap values. Figure 4 shows the phylogenetic analysis of *Culex* species using three *Culex* species COI sequences obtained in the present study together with sequences retrieved from NCBI database.

Genetic divergence was assessed based on the sequence of mosquito specimens using NJ-K2P method (Fig. 5). The average value for inter-specific genetic divergence was (0.0000–0.0020) 0.0018. The most genetically divergent clusters included *Cx. pipiens* (Cpn) with *An. annularis* (Ann) (0.1853) and *Cx. quinquefasciatus* (Cqf) with *An. annularis* (0.1801); while the most similar pairs were *Ae. albopictus* (Aps) (0.0017) and *Ae. aegypti* (Agt). *Anopheles stephensi* (Stp) showed relatively greater genetic (Table 2) divergence of 0.1494 (range 0.1214–0.1763) with respect to the other groups of field-collected mosquitoes from Nelliyampathy.

All the mosquito specimens analysed in this study can, therefore, be identified to species level based on their COI sequences, which offers a 100% compatibility between taxonomic and molecular identification and reveals that COI gene-based phylogenetic analysis is a convenient approach to complement conventional taxonomy.

Abundance and species richness

The present study revealed four mosquito genera, 10 species, and 150 individuals distributed among the three selected sites from Nelliyampathy. Of the 150 mosquito specimens collected, 50% belonged to the *Aedes* genus, followed by 14.66% of *Anopheles* spp., 33.33% of *Culex* spp., and 3% of *Armigeres* spp. The most abundant species were *Ae. aegypti* (26.66%), *Ae. albopictus* (20%), followed by *Cx. quinquefasciatus* (20%). Owing to their significance in the transmission of serious diseases including yellow fever, malaria, dengue fever, Japanese encephalitis, dengue hemorrhagic fever, West Nile fever, chikungunya, zika, and filariasis the aforementioned species are considered to be of specific interest. The mosquito species composition differed among the three study sites. This is illustrated and explained by diversity indices (Shannon Index), Sorensen's Coefficient (CC), and Venn-diagram. The Venn-diagram (Fig. 6) showed that 50% of the collected mosquito specimens were found in all three sites.

Aedes spp. diversity

Shannon index diversity values strongly differed between the three study sites (Fig. 7) ($\delta = 0.6–1.8$; $p < 0.001$). The highest Shannon index (Table 3) values were found at site 3 (Shannon Index ($H' = 1.8599$), followed by site 1 ($H' = 0.8602$), and site 2 ($H' = 0.6774$). The significant findings from the diversity index were reliable with the cluster analysis of the selected mosquito specimens.

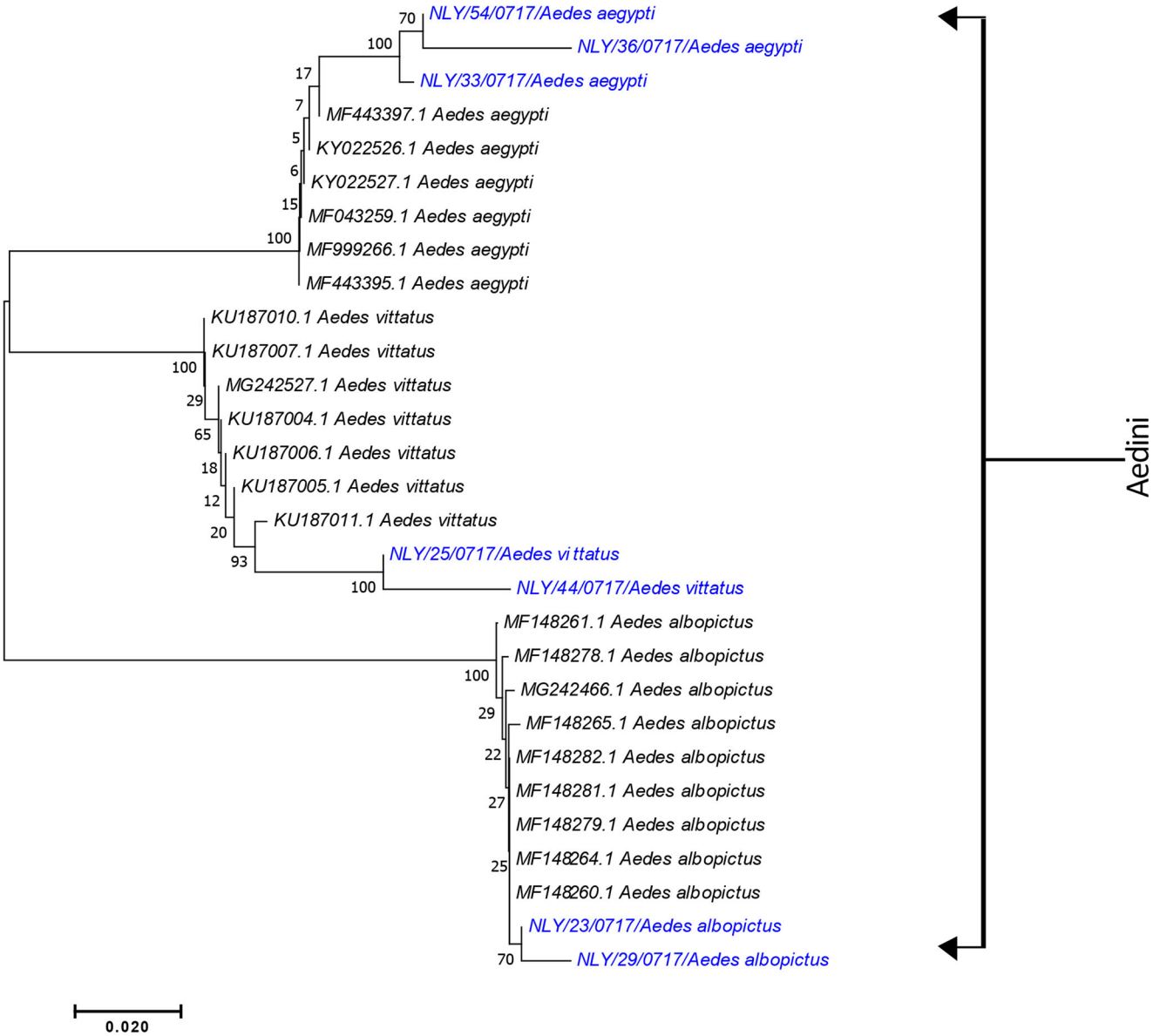


Fig. 2 Phylogenetic tree based on COI sequences of *Aedes* spp. A 450 bp-region of the COI mitochondrial gene was used to construct the Neighbour-joining tree in MEGA 7.00 software 7.0.9 beta version. The numbers displayed on branches were derived from the bootstrap method using 1000 replications. The gene sequences retrieved from NCBI are shown with accession numbers. The sequences starting with NLY were generated during the present study and are highlighted in blue colour

Culex spp. diversity

Prominent differences were noticed between diversity values in the three study sites. The Shannon index value at site 1 ($H' = 2.1846$) higher than at the remaining two sites (site 2: $H' = 0.8018$; site 3: $H' = 0.9291$) (Fig. 7).

Anopheles spp. diversity

Diversity analyses on *Anopheles* mosquito specimens indicated that site 1 had the highest Shannon index value ($H' = 0.9011$), followed by site 3 ($H' = 0.6931$) and site 2 ($H' = 0.6108$) (Fig. 7).

Sorensen's coefficient (CC)

Sorensen's Coefficient values for the comparison of mosquito species composition between three selected sites are presented in Fig. 8. Prominent similarity was found between mosquito communities at site 1 and site 2 ($CC = 0.87$). Similar results were obtained when comparing site 2 and site 3 ($CC = 0.66$). In addition, the CC value for site 3 and site 1 (0.82) also shows overlap between them. The aforesaid findings were strongly consistent with those of the statistical analysis together with the diversity analyses and the Venn diagram.

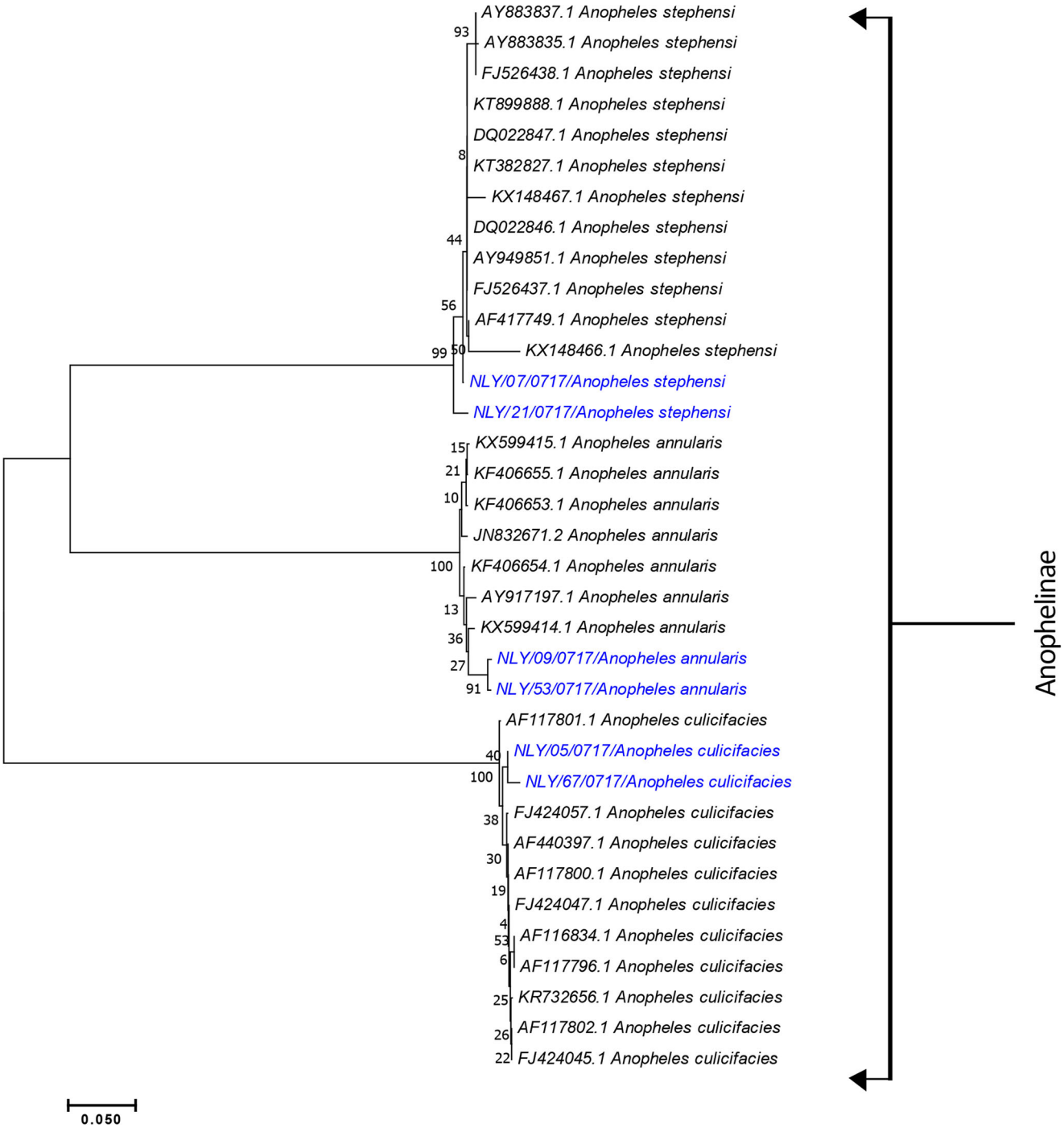


Fig. 3 Phylogenetic tree based on COI sequences of *Anopheles* spp.. A 432 bp-region of the COI gene was used to construct the Neighbor-joining tree in MEGA 7.00 software 7.0.9 beta version. The numbers on branches were derived from the bootstrap method using 1000 replications. The gene sequences retrieved from NCBI are shown with accession numbers. The sequences starting with NLY were generated during the present study and are highlighted in blue colour

Discussion

The present investigation offers molecular characterization and diversity of ten mosquito species belonging to four genera from three selected sites (site 1, site 2 and site 3) of Nelliyampathy. The increased level of mosquito-borne diseases epidemics is strongly influenced by tourist visitors. The study area Nelliyampathy is a well-known site for tourists, especially for foreigners. Anoopkumar et al. (2017) reported that about 8888 people were infected by dengue fever in Kerala from June 1st -30th 2017, of which one individual died due to severe dengue infection from Palakkad district

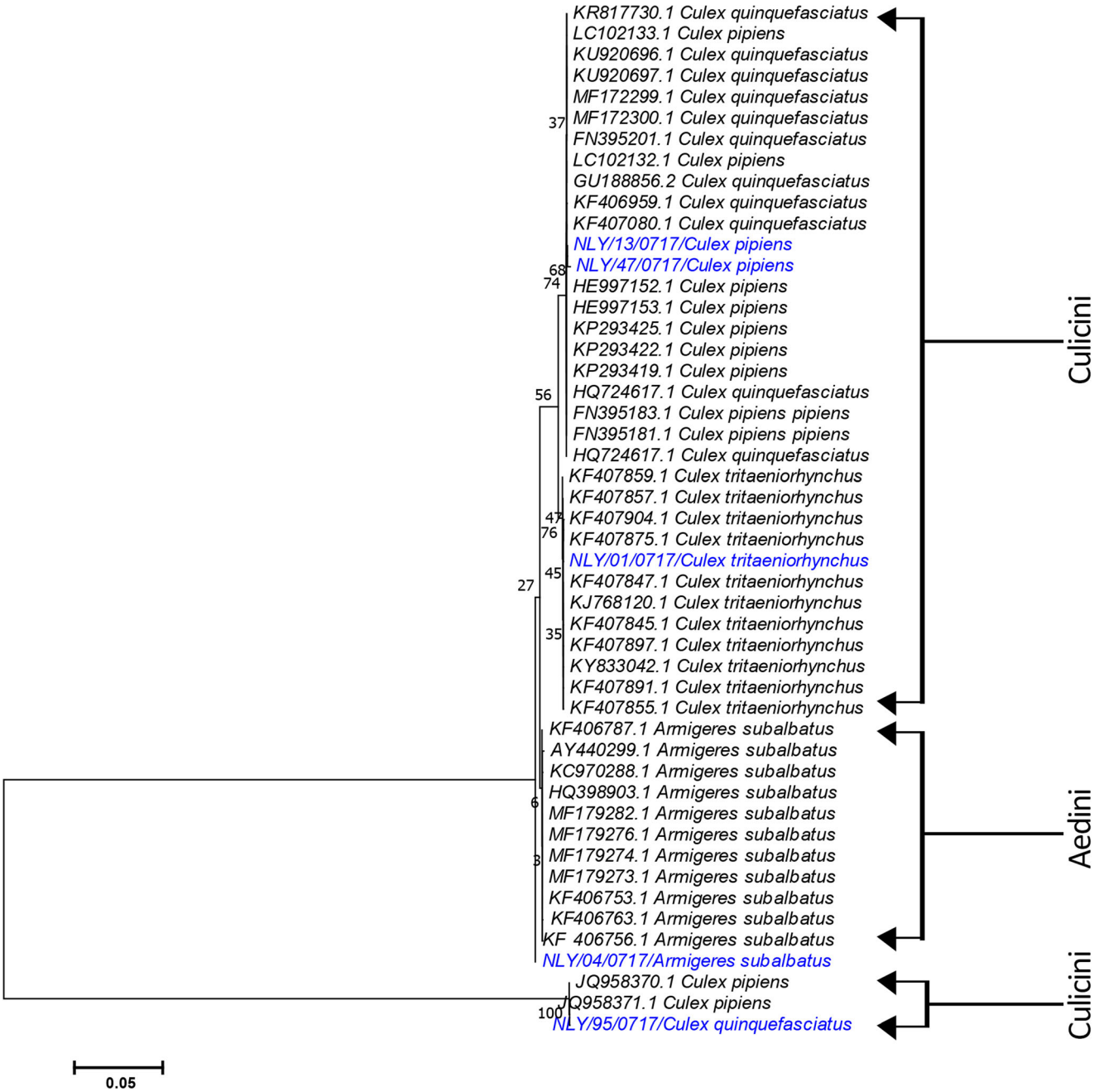


Fig. 4 Phylogenetic tree based on COI sequences of *Culex* spp. and *Armigeres* spp.. An alignment of COI gene sequences (616 bp) was used to generate the Neighbor-joining tree in MEGA 7.00 software 7.0.9 beta version. The numbers on branches were derived from the bootstrap method using 1000 replications. The sequences from NCBI are shown with accession numbers. The sequences starting with NLY were obtained during this study and are highlighted in blue colour

nearby the study site Nelliyampathy, Kerala. This has enhanced the importance of characterizing the molecular diversity and species divergence of mosquitoes from the study area.

The presence of foremost and potential vectors of various mosquito-borne diseases throughout the world regardless of ecological circumstances shows the prominent threat of severe diseases re-emerging over a region where they had been eradicated. Thus, the accurate and precise identification of vector mosquitoes is indispensable in vector control surveillance programmes (Higa et al. 2010; Chan et al. 2014; Puthur et al. 2018).

The mosquito species identification using conventional methods possess some complexity and it requires specimens in excellent condition, without any damages (Cywinska et al. 2006). During the present investigation, the mitochondrial COI gene was used to confirm the uniqueness of morphologically identified mosquitoes. Neighbor-Joining tree constructed using the COI sequences is the most widely accepted approach in

Table 1 Mosquito species collected from Nelliyampathy, Palakkad, Kerala, India in 2017

Mosquito species	Number of specimens collected			Total	Percentage of species	Percentage of each genera
	Jan-2017, to June-2017		July-2017 to December 2017			
	Site 1	Site 2	Site 3			
Aedes aegypti (Linnaeus, 1762)	15	10	15	40	26.66	50%
Aedes albopictus (Skuse, 1894)	7	7	16	30	20	
Aedes vittatus (Bigot, 1861)	2	0	3	5	3.33	
Anopheles annularis (Van der Wulp, 1884)	1	0	3	4	2.66	14.66%
Anopheles stephensi (Liston, 1901)	5	7	3	15	10	
Anopheles culicifacies (Giles, 1901)	3	0	0	3	2	
Culex quinquefasciatus (Barraud, 1934)	8	7	15	30	20	33.33%
Culex tritaeniorhynchus (Giles, 1901)	5	2	3	10	6	
Culex pipiens (Linnaeus, 1758)	2	0	8	10	6	
Armigeres subalbatus (Coquillett, 1898)	2	1	0	3	2	2%

several barcoding studies on mosquitoes since the method offers accurate and effective recognition of the targeted species (Kumar et al. 2007; Gunay et al. 2015; Rozo-Lopez and Mengual 2015). As formerly stated, all mosquitoes were identified to species level by comparing the sequences obtained in this study with the sequences available from the reference NCBI database, and most of the sequences had identity values above 96%. The COI gene-based phylogenetic analysis is

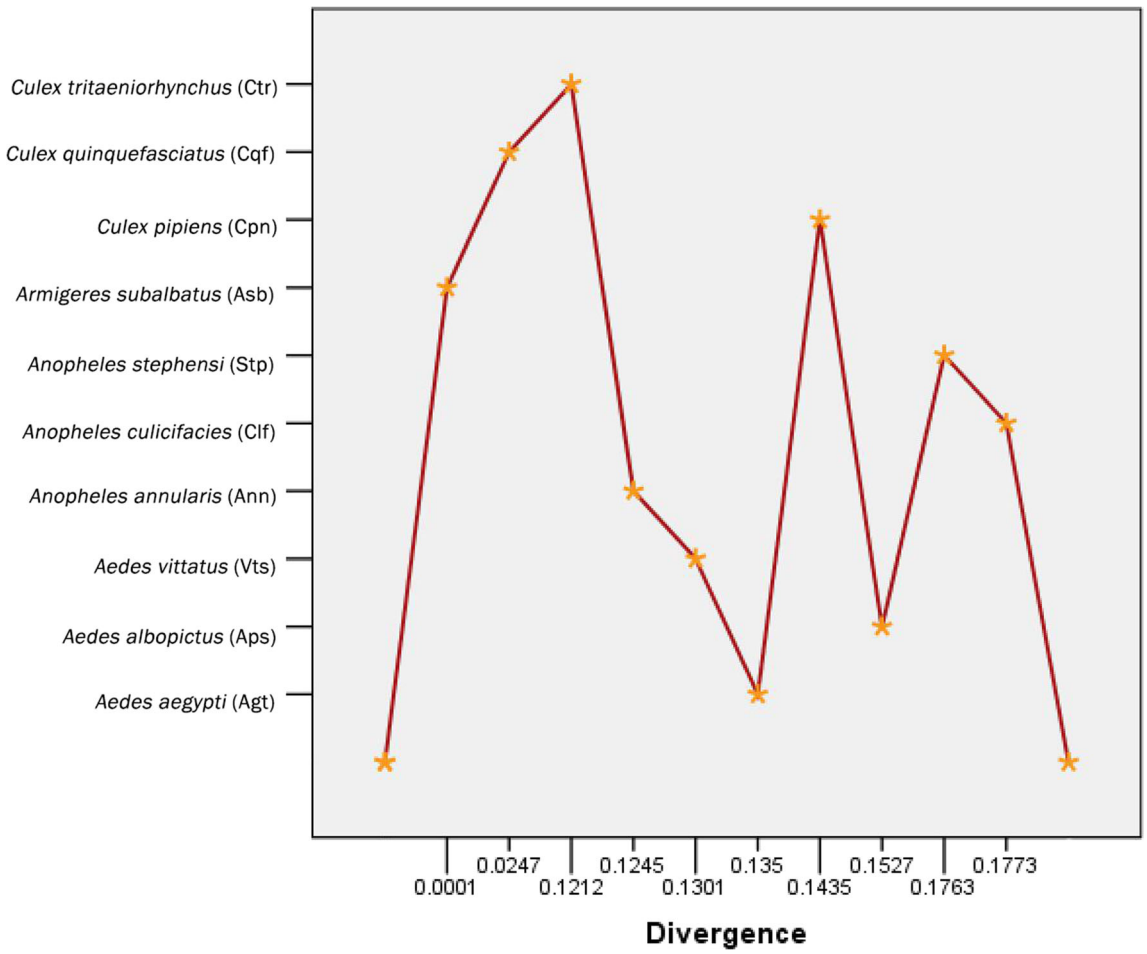


Fig. 5 Mean of K2P distances in ten mosquito species clusters. The species clusters were defined using the NJ-K2P pairwise distance method typically employed in barcoding using MEGA7 Software

Table 2 Mean of K2P distances in ten mosquito species clusters

Mosquito species	n	Asb	Cqf	Ctr	Cpn	Stp	Ann	Clf	Vts	Aps	Agt
Armigeres subalbatus (Asb)	*3*	**0.0001**									
Culex quinquefasciatus (Cqf)	*30*	0.0247	**0.0013**								
Culex tritaeniorhynchus (Ctr)	*10*	0.1212	0.0716	**0.0012**							
Culex pipiens (Cpn)	*10*	0.1435	0.0916	0.0501	**0.0020**						
Anopheles stephensi (Stp)	*15*	0.1763	0.1453	0.1545	0.1214	**0.0000**					
Anopheles annularis (Ann)	*4*	0.1245	0.1802	0.1715	0.1853	0.0175	**0.0100**				
Anopheles culicifacies (Clf)	*3*	0.1773	0.1311	0.0221	0.0114	0.0395	0.0599	**0.0010**			
Aedes vittatus (Vts)	*5*	0.1302	0.1250	0.1488	0.1530	0.1546	0.1204	0.1418	**0.0011**		
Aedes albopictus (Aps)	*30*	0.1528	0.1220	0.1481	0.1484	0.1229	0.1465	0.1106	0.0119	**0.0001**	
Aedes aegypti (Agt)	*40*	0.1350	0.1847	0.1169	0.1301	0.1272	0.1096	0.0967	0.0045	0.0017	**0.0010**

The species clusters were defined using the NJ-K2P pairwise distance method typically employed in barcoding. The K2P distance values highlighted in bold letters indicates inter-specific genetic divergence. The numbers of individual specimens were also included

renowned as the suitable method for mosquito species identification in this study. The tree-based analyses are renowned as a graphic criterion for the identification of species, which defines the genetic diversity in a visually satisfying elegance (Goldstein and DeSalle 2011). A significant benefit of using phylogenetic tree-based method is that it provides a direct logic

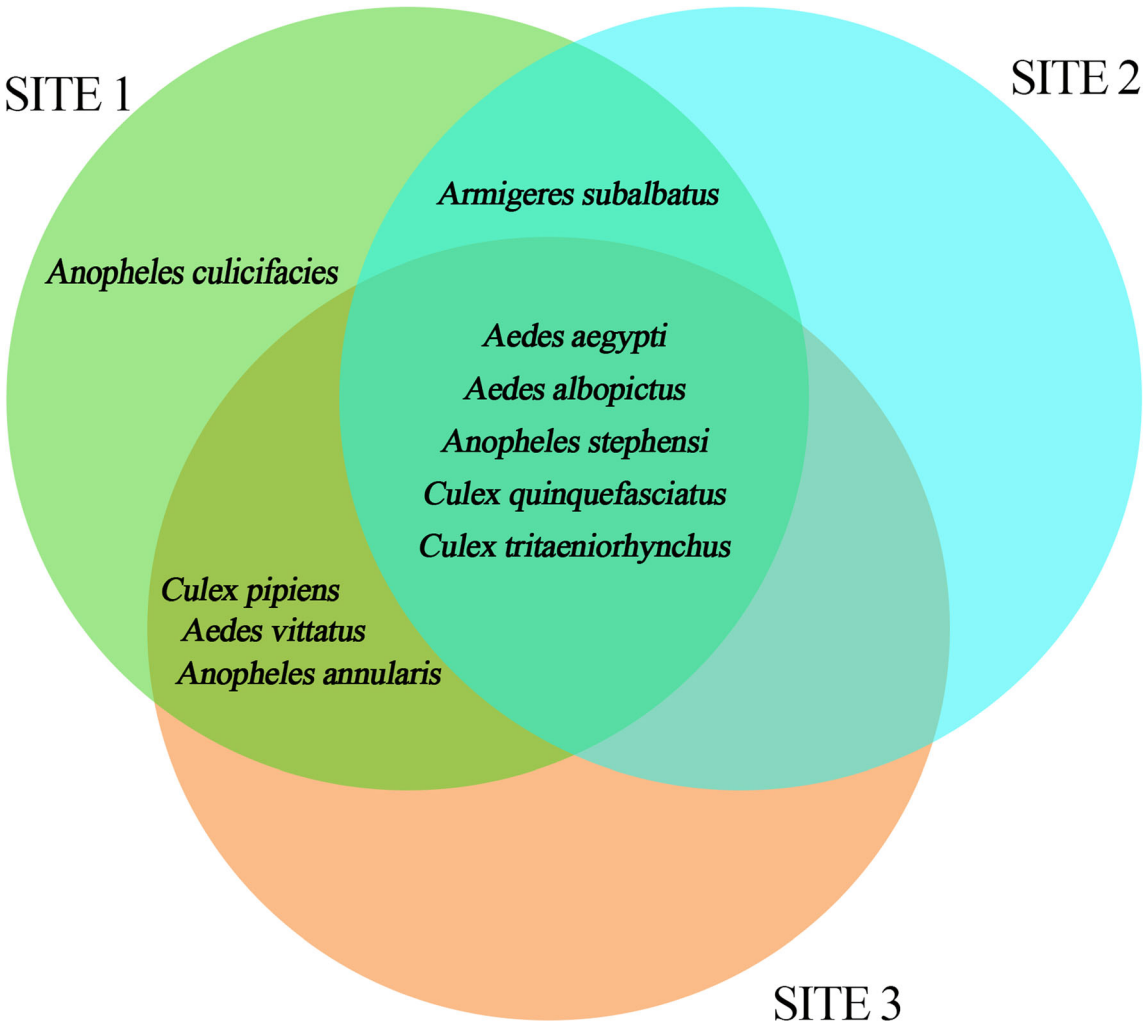


Fig. 6 Venn diagram of mosquito species in Nelliyampathy, Palakkad district of Kerala, India. Diagram shows the occurrence of mosquito species in the study sites: site 1 (green), site 2 (blue), site 3 (orange)

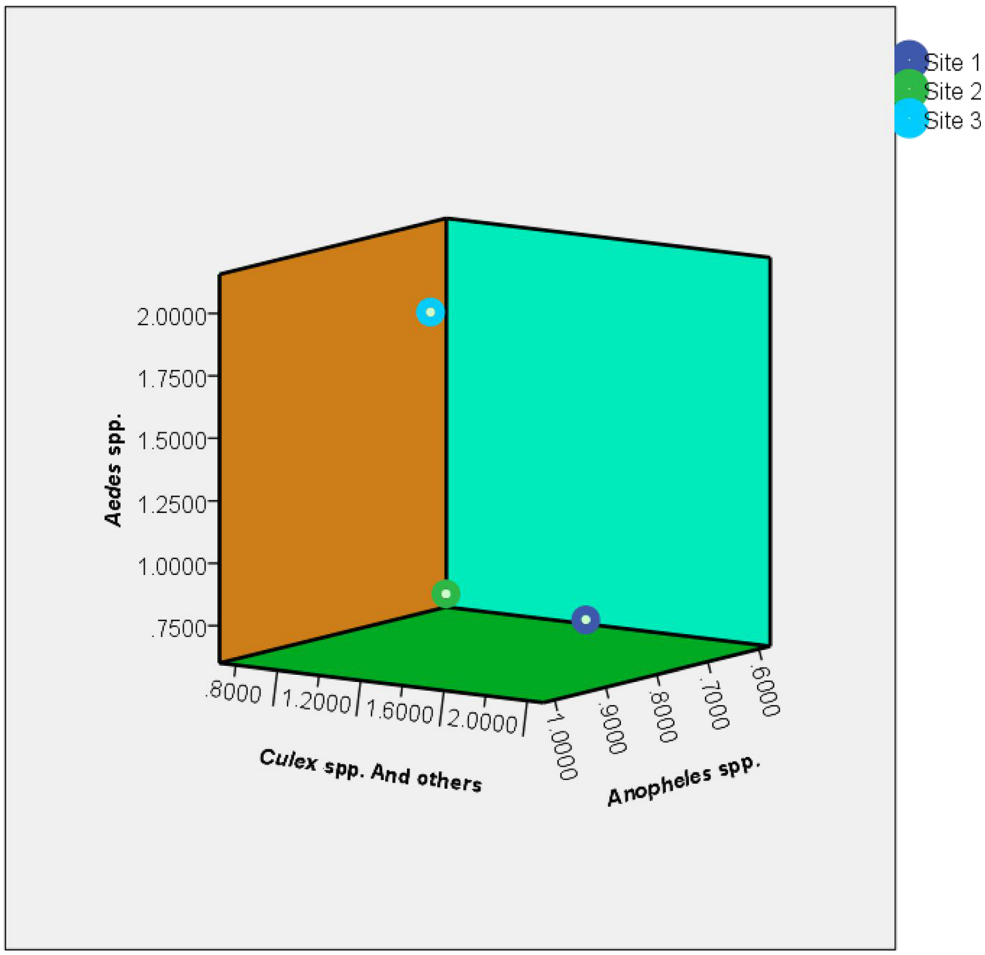


Fig. 7 Shannon index of *Aedes*, *Anopheles*, *Culex,* and *Armigeres* species

of the statistical consistency (Goldstein and DeSalle 2011). The results from the COI gene-based species identification through the NJ phylogenetic tree analysis strongly approved the results of morphological identification. Based on the similarity of COI sequences, the mosquitoes were grouped into species, with strong bootstrap values in the NJ phylogenetic tree approving the results of conventional morphological identification. Our study analyzed and confirmed the COI sequences of ten epidemiologically important mosquitoes (Table 1). As per the NJ phylogenetic tree analysis, the morphologically identified *Aedes*, *Culex*, *Anopheles* and *Armigeres* species were supported by strong bootstrap values.

Table 3 Shannon index of *Aedes*, *Anopheles*, *Culex* and other species

Site	Shannon index value		
	Aedes	*Culex* and others	*Anopheles*
Site 1	0.8602	2.1846	0.9011
Site 2	0.6774	0.8018	0.6108
Site 3	1.8599	0.9291	0.6931

The *Aedes*, *Culex*, and *Anopheles*, species analyzed in this study are epidemiologically important since they transmit several severe human disease agents in India, specifically in Kerala (Anoopkumar et al. 2017; Vijayan 2010). In addition, the significance of mitochondrial gene analysis in taxonomy is clear at circumstances where one member of the sibling species complex is responsible for the transmission of several diseases while the other is not. Precisely, *An. annularis* A is responsible for the transmission of malaria in India, but *An. annularis* B does not transmit this disease (Atrie et al. 1999).

Apart from facilitating the differentiation of sibling taxa, the use of mitochondrial COI gene in taxonomic studies can unveil the significant genetic divergence between single species, which might have disease transmission implications (Beebe et al. 2005). Several previous studies revealed significant genetic diversities in both *Ae. aegypti* and *Ae. albopictus* just as we noted in our study. This offers substantial evidence on the mosquito population origin and relationships and insights into the role of these mosquito species in disease transmission (Mendes dos Santos et al. 2011). Our study revealed that both species exhibited considerable genetic differences.

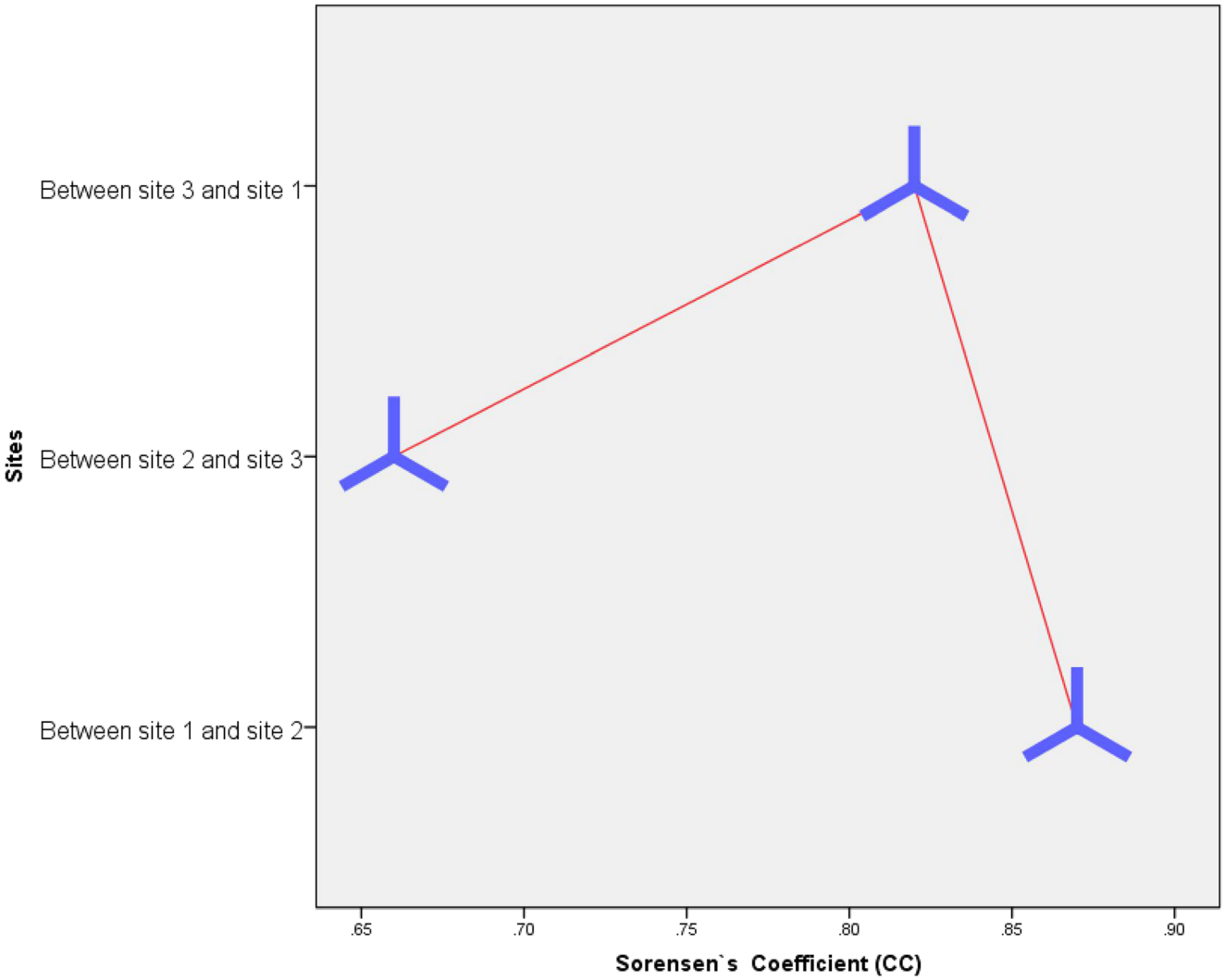


Fig. 8 Sorensen's Coefficient values for the comparison of mosquito species composition between three study sites

Aedes albopictus is the vector of nearly 22 arboviruses, including chikungunya, West Nile virus (WNV), dengue, and possibly zika virus (Aliota et al. 2016; Chouin-Carneiro et al. 2016).

Several previous studies reported that *Culex* spp. might be undergoing speciation together with microevolution (Harbach et al. 2012), resulting in species complexes including the formation of hybrid mosquitoes from sibling species mating (Medeiros-Sousa et al. 2015). These species complexes are adapted to live in both temperate and tropical climate regions in Brazil since they are formed from *Cx. quinquefasciatus* and *Cx. pipiens* (Wilke et al. 2016).

In the present investigation, we identified the epidemiologically significant *Culex* spp., particularly *Cx. quinquefasciatus*, a key vector of WNV. The variability of COI gene sequences between *Cx. quinquefasciatus* and *Cx. pipiens* have been reported as low (Shaikevich et al. 2016). Therefore, the gradually narrowing siphon (Fig. 9) and difference in the number of branches in the siphon seta of *Cx. pipiens* has been recognized as an added advantage in the distinguishing of *Cx. pipiens* from *Cx. quinquefasciatus*. *Culex quinquefasciatus* has a wide shaped siphon in the middle and the numbers of branches in the siphon seta significantly differ from other *Culex* spp. Additional taxonomic literature regarding the differences in the morphological characters between *Cx. pipiens* and *Cx. quinquefasciatus* has been discussed by Dehghan et al. (2016).

To our knowledge, this is the first and foremost report on the molecular analysis of selected field-collected mosquitoes from Nelliyampathy. In addition, our study also described the diversity of *Aedes*, *Culex*, *Anopheles*, and *Armigeres* adults. Species diversity of *Aedes* spp. and *Anopheles* spp. was high in sites 3 and site 1, respectively. The diversity of *Culex* spp. and other species was very high in site 1. The transmission of mosquito-borne diseases encompasses a complex interaction between pathogens and arthropod vectors, the host organism, and their environment. Approximately 30% of emerging vector-borne disease events was triggered by mosquito-borne pathogens of wildlife origins (Ganser and Wisely

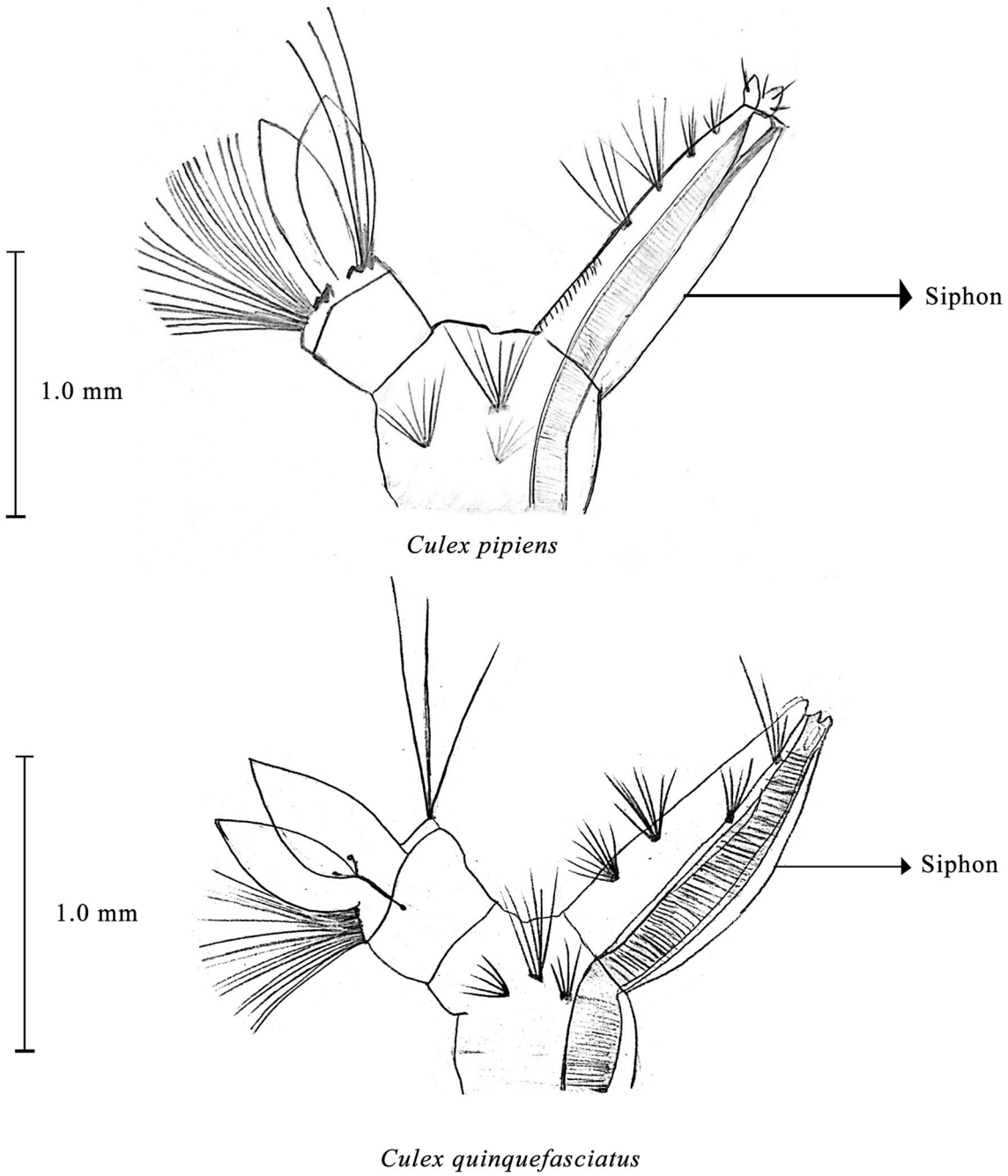


Fig. 9 Morphological identification of fourth instar larvae of *Culex pipiens* and *Cx. quinquefaciatus*

2013). Thus, the present study also examined similarities between mosquito communities in three sites using Sorensen's Coefficient analysis and indicated great similarities between them. The analysis of similarities between two communities offer a better understanding of the mosquito population that may lead to ecologically efficient strategies in vector control programmes (YAN and Zhong 2005). The results gathered from the present study will be economically as well medically beneficial to humans. Hence, investigation on the species diversity, genetic divergence and similarities between the mosquito populations with special reference to the mosquito-borne disease epidemics is essential to develop effective mosquito vector control strategies.

Conclusion

The findings conveyed in this study using COI gene analysis of mosquito specimens have facilitated the determination of molecular taxonomic status and species diversity of ten

mosquito species from three selected sites in Nelliyampathy. The high number of foreigners and tourist visitors in the study area and its specific nature as an ecotone urgently necessitate the molecular characterization of vector mosquitoes with special reference to phylogenetic analysis together with species-specific similarity determination and population diversity indices. As the foremost comprehensive molecular analysis of these epidemiologically important mosquitoes, our findings offer a base for future research which is necessary for the better understanding of novel vector control approaches. The reliability, versatility, and accuracy of the aforesaid strategies as a tool for mosquito species identification, determination of genetic divergence, species-specific similarity and population diversity make them an indispensable approach in vector control surveillance programmes. The results can also be used to develop significant recommendations and suggestions for public health policy regarding vector-borne diseases and their transmission in two different ecosystems, specifically in an ecotone habitat.

Acknowledgments The authors would like to thank Principal, St. Joseph's College, Irinjalakuda for the laboratory facilities provided. The author Embalil Mathachan Aneesh thanks University Grants Commission, Government of India (F.NO.42-609/2013 SR and UGC Research Award - F30-6/20-16(SA-II)) for funding.

Funding UGC- University Grants Commission, Government of India (F.NO.42–609/2013 SR).
UGC Research Award - F30–6/20–16(SA-II).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

References

Aivazi A-A, Vijayan V (2010) Efficacy of *Ruta graveolens* extract and its synergistic effect with cypermethrin against *Anopheles stephensi* Liston larvae. Toxicol Environ Chem 92:893–901. https://doi.org/10.1080/02772240903092306

Aliota MT, Peinado SA, Osorio JE, Bartholomay LC (2016) *Culex pipiens* and *Aedes triseriatus* mosquito susceptibility to Zika virus. Emerg Infect Dis 22:1857. https://doi.org/10.3201/eid2210.161082

Anoopkumar A, Puthur S, Varghese P, Rebello S, Aneesh EM (2017) Life cycle, bio-ecology and DNA barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). J Commun Dis 49. https://doi.org/10.24321/0019.5138.201719

Apote N (1947) Pharmaceutical abstracts-section I. New Engl J Med 237: 149. https://doi.org/10.1002/jps.3030371303

Atrie B, Subbarao SK, Pillai M, Rao S, Sharma V (1999) Population cytogenetic evidence for sibling species in *Anopheles annularis* (Diptera: Culicidae). Ann Entomol Soc Am 92:243–249. https://doi.org/10.1093/aesa/92.2.243

Barraud CP (1934) The fauna of British India including Ceylon and Burma. Taylor And Francis, Red Lion Court, London

Batovska J, Blacket MJ, Brown K, Lynch SE (2016) Molecular identification of mosquitoes (Diptera: Culicidae) in southeastern Australia. Ecol Evol 6:3001–3011. https://doi.org/10.1002/ece3.2095

Beebe NW, Whelan P, van den Hurk A, Ritchie SA, Cooper RD (2005) Genetic diversity of the dengue vector *Aedes aegypti* in Australia and implications for future surveillance and mainland incursion monitoring. Commun Dis Intell Q Rep 29(3):299–304

Börstler J, Lühken R, Rudolf M, Steinke S, Melaun C, Becker S, Garms R, Krüger A (2014) The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*. J Vector Ecol 39:204–212. https://doi.org/10.1111/j.1948-7134.2014.12088.x

Chan A, Chiang LP, Hapuarachchi HC, Tan CH, Pang SC, Lee R, Lee KS, Ng LC, Lam-Phua SG (2014) DNA barcoding: complementing morphological identification of mosquito species in Singapore. Parasit Vectors 7:569. https://doi.org/10.1186/s13071-014-0569-4

Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, Dupont-Rouzeyrol M, Lourenco-de-Oliveira R, Failloux AB (2016) Differential susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika virus. PLoS Negl Trop Dis 10: e0004543. https://doi.org/10.1371/journal.pntd.0004543

Christophers SR (1933) Family Culicidæ: Tribe Anophelini, vol 4. Taylor & Francis, London

Clark-Gil S, Darsie R (1983) The mosquitoes of Guatemala, their identification, distribution and bionomics, with keys to adult females and larvae. Mosquito Systematics 15:151–284

Cywinska A, Hunter F, Hebert PD (2006) Identifying Canadian mosquito species through DNA barcodes. Med Vet Entomol 20:413–424. https://doi.org/10.1111/j.1365-2915.2006.00653.x

Dehghan H, Sadraei J, Moosa-Kazemi SH, Abolghasemi E, Solimani H, Nodoshan AJ, Najafi MH (2016) A pictorial key for *Culex pipiens* complex (Diptera: Culicidae) in Iran. J Arthropod Borne Dis 10:291

Do TTT, Martens P, Luu NH, Wright P, Choisy M (2014) Climatic-driven seasonality of emerging dengue fever in Hanoi, Vietnam. BMC Public Health 14:1078. https://doi.org/10.1186/1471-2458-14-1078

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Heled J, Kearse M, Moir R, StonesHavas S, Sturrock S, Thierer T, Wilson A (2010) Geneious v. 5.1, available from 485 http://www.geneious.com. Accessed 24 April 2012

Fansiri T, Fontaine A, Diancourt L, Caro V, Thaisomboonsuk B, Richardson JH, Jarman RG, Ponlawat A, Lambrechts L (2013) Genetic mapping of specific interactions between *Aedes aegypti* mosquitoes and dengue viruses. PLoS Genet 9:e1003621. https://doi.org/10.1371/journal.pgen.1003621

Ganser C, Wisely SM (2013) Patterns of spatio-temporal distribution, abundance, and diversity in a mosquito community from the eastern Smoky Hills of Kansas. J Vector Ecol 38:229–236. https://doi.org/10.1111/j.1948-7134.2013.12035.x

Goldstein PZ, DeSalle R (2011) Integrating DNA barcode data and taxonomic practice: determination, discovery, and description. Bioessays 33:135–147. https://doi.org/10.1002/bies.201000036

Gunay F, Alten B, Simsek F, Aldemir A, Linton Y-M (2015) Barcoding Turkish *Culex* mosquitoes to facilitate arbovirus vector incrimination studies reveals hidden diversity and new potential vectors. Acta Trop 143:112–120. https://doi.org/10.1016/j.actatropica.2014.10.013

Harbach RE, Kitching IJ, Culverwell CL, Dubois J, Linton YM (2012) Phylogeny of mosquitoes of tribe Culicini (Diptera: Culicidae) based on morphological diversity. Zool Scr 41:499–514. https://doi.org/10.1111/j.1463-6409.2012.00546.x

Hayat M, Subba Rao B (1981) A systematic catalogue of Encyrtidae (Hymenoptera: Chalcidoidea) from the Indian subcontinent. Colemania 1:103–125

Higa Y, Toma T, Tsuda Y, Miyagi I (2010) A multiplex PCR-based molecular identification of five morphologically related, medically

important subgenus *Stegomyia* mosquitoes from the genus *Aedes* (Diptera: Culicidae) found in the Ryukyu archipelago, Japan. Jpn J Infect Dis 63:312–316

Kang D, Sim C (2013) Identification of *Culex* complex species using SNP markers based on high-resolution melting analysis. Mol Ecol Resour 13:369–376. https://doi.org/10.1111/1755-0998.12083

Kumar NP, Rajavel A, Natarajan R, Jambulingam P (2007) DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). J Med Entomol 44:1–7. https://doi.org/10.1093/jmedent/41.5.01

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874. https://doi.org/10.1093/molbev/msw054

Laurito M, Almirón WR, Ludueña-Almeida FF (2015) Discrimination of four *Culex* (*Culex*) species from the Neotropics based on geometric morphometrics. Zoomorphology 134:447–455. https://doi.org/10.1093/molbev/msw054

Low VL, Lim PE, Chen CD, Lim YA, Tan TK, Norma-Rashid Y, Lee HL, Sofian-Azirun M (2014) Mitochondrial DNA analyses reveal low genetic diversity in *Culex quinquefasciatus* from residential areas in Malaysia. Med Vet Entomol 28:157–168. https://doi.org/10.1111/mve.12022

Medeiros-Sousa AR, Ceretti-Júnior W, de Carvalho GC, Nardi MS, Araujo AB, Vendrami DP, Marrelli MT (2015) Diversity and abundance of mosquitoes (Diptera: Culicidae) in an urban park: larval habitats and temporal variation. Acta Trop 150:200–209. https://doi.org/10.1016/j.actatropica.2015.08.002

Mendes dos Santos JM, Fraga EC, Maia JF, Tadei WP (2011) Genetic diversity in dengue mosquito, *Aedes aegypti* (Diptera: Culicidae) from Amazon region: comparative analysis with isozymes and RAPD loci. Open Trop Med J 4:11–20. https://doi.org/10.2174/1874315301104010011

Mwangangi JM et al (2013) Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. Malar J 12:13. https://doi.org/10.1186/1475-2875-12-13

Otranto D, Capelli G, Genchi C (2009) Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. Parasit Vectors 2(suppl 1):S2. https://doi.org/10.1186/1756-3305-2-S1-S2

Puthur S, Anoopkumar A, Rebello S, Aneesh EM (2018) *Hypericum japonicum*: a double-headed sword to combat vector control and cancer. Appl Biochem Biotechnol 186:1–11. https://doi.org/10.1007/s12010-018-2713-7

Ratnasingham S, Hebert PD (2007) BOLD: The Barcode of Life Data System. Mol Ecol Resour 7:355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Reinert JF, Harbach RE (2005) Generic and subgeneric status of aedine mosquito species (Diptera: Culicidae: Aedini) occurring in the Australasian region. Zootaxa 887:1–10. https://doi.org/10.11646/zootaxa.887.1.1

Rozo-Lopez P, Mengual X (2015) Mosquito species (Diptera, Culicidae) in three ecosystems from the Colombian Andes: identification through DNA barcoding and adult morphology. ZooKeys 513:39–64. https://doi.org/10.3897/zookeys.513.9561

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Shaikevich EV, Vinogradova EB, Bouattour A, De Almeida APG (2016) Genetic diversity of *Culex pipiens* mosquitoes in distinct populations from Europe: contribution of *Cx. quinquefasciatus* in Mediterranean populations. Parasit Vectors 9:47. https://doi.org/10.1186/s13071-016-1333-8

Singh O et al (2004) Differentiation of members of the *Anopheles fluviatilis* species complex by an allele-specific polymerase chain reaction based on 28S ribosomal DNA sequences. Am J Trop Med Hyg 70:27–32. https://doi.org/10.4269/ajtmh.2004.70.27

Stone A, Knight KL, Starcke H (1959) A synoptic catalog of the mosquitoes of the world (Diptera, Culicidae). Entomological Society of America, Washington, D.C.

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035. https://doi.org/10.1073/pnas.0404206101

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Thongsripong P, Green A, Kittayapong P, Kapan D, Wilcox B, Bennett S (2013) Mosquito vector diversity across habitats in Central Thailand endemic for dengue and other arthropod-borne diseases. PLoS Negl Trop Dis 7:e2507. https://doi.org/10.1371/journal.pntd.0002507

Tipayamongkholgul M, Fang C-T, Klinchan S, Liu C-M, King C-C (2009) Effects of the El Niño-southern oscillation on dengue epidemics in Thailand, 1996-2005. BMC Public Health 9:422. https://doi.org/10.1186/1471-2458-9-422

Vijayan V (2010) Laboratory selection of carbofuran tolerant line of *Culex quinquefasciatus* say, the filarial vector at Mysore. J Commun Dis 42:201–207

Ward RD (2005) Identification and geographical distribution of the mosquitoes of North America. In: Darsie RF Jr, Ward RA (ed) North of Mexico, 2nd edn. University Press of Florida, USA. Parasitology 131:580. https://doi.org/10.1017/S0031182005228834

WHO WH (2011) First WHO global forum on medical devices: context, outcomes, and future actions. WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland

Wilke ABB, de Oliveira Christe R, Multini LC, Vidal PO, Wilk-da-Silva R, de Carvalho GC, Marrelli MT (2016) Morphometric wing characters as a tool for mosquito identification. PLoS One 11:e0161643. https://doi.org/10.1371/journal.pone.0161643

Yan ZC, Zhong H (2005) Comparison of adult mosquito community structure on various habitats. J Insect Sci 12:193–197. https://doi.org/10.1111/j.1005-295X.2005.00024.x

Environmental Chemistry for a Sustainable World 51

Inamuddin
Mohd Imran Ahamed
Eric Lichtfouse
Abdullah M. Asiri *Editors*

Methods for Bioremediation of Water and Wastewater Pollution

Springer

Environmental Chemistry for a Sustainable World

Volume 51

Other Publications by the Editors

Books
Environmental Chemistry
http://www.springer.com/978-3-540-22860-8

Organic Contaminants in Riverine and Groundwater Systems
http://www.springer.com/978-3-540-31169-0

Sustainable Agriculture
Volume 1: http://www.springer.com/978-90-481-2665-1
Volume 2: http://www.springer.com/978-94-007-0393-3

Book series
Environmental Chemistry for a Sustainable World
http://www.springer.com/series/11480

Sustainable Agriculture Reviews
http://www.springer.com/series/8380

Journals
Environmental Chemistry Letters
http://www.springer.com/10311

More information about this series at http://www.springer.com/series/11480

Inamuddin • Mohd Imran Ahamed
Eric Lichtfouse • Abdullah M. Asiri
Editors

Methods for Bioremediation of Water and Wastewater Pollution

Springer

Editors
Inamuddin
Department of Chemistry
King Abdulaziz University
Jeddah, Saudi Arabia

Department of Applied Chemistry
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Eric Lichtfouse
Aix-Marseille University, CNRS,
IRD, INRAE, Coll France, CEREGE
Aix-en-Provence, France

Mohd Imran Ahamed
Department of Chemistry
Aligarh Muslim University
Aligarh, Uttar Pradesh, India

Abdullah M. Asiri
Department of Chemistry
King Abdulaziz University
Jeddah, Saudi Arabia

ISSN 2213-7114 ISSN 2213-7122 (electronic)
Environmental Chemistry for a Sustainable World
ISBN 978-3-030-48984-7 ISBN 978-3-030-48985-4 (eBook)
https://doi.org/10.1007/978-3-030-48985-4

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Chapter 12
Phytoextraction of Heavy Metals

A. N. Anoopkumar, Sharrel Rebello, Elsa Devassy, K. Kavya Raj, Sreedev Puthur, Embalil Mathachan Aneesh, Raveendran Sindhu, Parameswaran Binod, and Ashok Pandey

Contents

Abstract Heavy metals are considered as the major classes of a contaminant in nature. Heavy metal contamination from fertilizers, metal mining, and industrial activities leads to toxic effects on humans and other organisms. Although the toxic effects of these elements have been recognized for a long time, exposure to these elements continues. The toxic effects induced by them can lead to death in humans. Several advanced strategies have primarily employed to tidy up the surrounding from toxicants; however, most of the strategies are considered as problematic when getting results. The current concerns regarding the contamination due to heavy metal deposition have introduced the novel advanced technologies to detect the presence of them from soil and wastewater. Several plants have been recognized as potent herbs since they are able to absorb these toxicants. The phytoremediation techniques

A. N. Anoopkumar
Department of Zoology, Christ College, University of Calicut, Irinjalakuda, Kerala, India

Communicable Disease Research Laboratory (CDRL), St. Joseph's College, Irinjalakuda, Kerala, India

S. Rebello · E. Devassy · K. Kavya Raj · S. Puthur · E. M. Aneesh
Communicable Disease Research Laboratory (CDRL), St. Joseph's College, Irinjalakuda, Kerala, India

R. Sindhu (✉) · P. Binod
Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum, Kerala, India

A. Pandey
CSIR-Indian Institute for Toxicology Research (CSIR-IITR), Lucknow, Uttar Pradesh, India

Inamuddin et al. (eds.), *Methods for Bioremediation of Water and Wastewater Pollution*, Environmental Chemistry for a Sustainable World 51, https://doi.org/10.1007/978-3-030-48985-4_12

usually uptake the heavy metals from the soil and wastewater and recognized as a well-established approach to remediate the toxic effects induced by them. The plant-based techniques have some advantages over the conventional strategies. Therefore, the present research has focused on the various technologies used to remove the metal pollutants from natural resources especially from water and soil. These affordable and effective technologies are potentially cost-effective and environmental friendly.

Keywords Contaminants · Heavy metals · Phytoextraction · Remediation

12.1 Introduction

Environmental pollution by the deposition of toxic heavy metals in natural resources including water and soil has become a serious threat over the globe in recent years. The disturbance of natural biogeochemical cycles and industrialization together with urbanization has instigated the toxic impacts (Srivastava 2016). Industrial actions including metal fabrication shops, textile factories, service stations, waste disposal areas, and chemical works followed by intensive cultivation are specifically awkward of contaminating the environment (Freitas et al. 2004; Wong 2003). The enhancing reliance on synthetic fertilizers in the soil for the agricultural purpose has enforced a long-term threat over the environment (Barla et al. 2017; McLaughlin et al. 1999; Rebello et al. 2019). Emission of toxic elements has principally regulated in industrialized countries, whereas in the case of developing countries, hasty population explosion and industrial development coupled with worst pollution control strategies that have ensued in wide heavy metal pollution over natural resources (Ji 2000). The entry of heavy metals into the food chain consequently develops a severe impact to animal and human health (Sarwar et al. 2017). The heavy metals usually have a high atomic number, high density, and mass (Alloway 2012). These elements also accumulated in the living organisms, referred to as bioaccumulation, thereby increasing their concentrations in higher trophic levels by biomagnification.

The major heavy metals include copper (Cu), manganese (Mn), iron (Fe), chromium (Cr), and nickel (Ni). The excessive accumulation of these elements in plant cells has induced toxic effects even at low concentrations by adversely affecting photosynthetic and respiratory processes, plant growth, membrane integrity, deoxyribonucleic acid structure, and functionality and enzymatic activities (Lajayer et al. 2017b). In addition, certain redundant compounds including lead (Pb), mercury (Hg), cadmium (Cd), and arsenic (As) are considered as highly toxic towards various biochemical and physiological processes take place in plants (Lajayer et al. 2017a). The conventional strategies for indemnification of heavy metals tainted water and other natural resources might not be feasible in most of all the situations since they are expensive and inefficient (Lajayer et al. 2017a). This chapter thereby determines

the various strategies and concepts and risks and effectiveness involved in the phytoextraction of heavy metals associated with environmental contamination and natural resources.

12.2 Heavy Metals: Sources, Contamination, and Effects in the Environment

Current disquiets concerning the heavy metal-induced environmental damage have extended the need of developing appropriate novel and versatile technologies to find out its presence in natural resources. Instead of numerous contaminants, the effluent from various industrial firms including paper mill is recognized as a rich source of nitrogen, calcium, phosphorus, and magnesium together with trace elements (Jain et al. 2005). Pollution due to the accumulation of metal elements cadmium, chromium, copper, silver, lead, and zinc induces a hazardous effect on biological systems since most of them do not endure biodegradation (Table 12.1) (Pehlivan et al. 2009).

Table 12.1 Major plants those are able to accumulate heavy metals

Sl. no.	Metal	Plant species that accumulate heavy metals
1.	Nickel	*Alyssum serpyllifolium*
		Bornmuellera kiyakii
		Sebertia acuminata
		Berkheya coddii
2.	Cobalt	*Crotalaria cobalticola*
3.	Zinc	*Picris divaricata*
		Arabis gemmifera
		Sedum alfredii
		Arabidopsis halleri
4.	Copper	*Crassula helmsii*
		Ipomea alpina
5.	Lead	*Hemidesmus indicus*
		Plantago orbignyana
		Sesbania drummondii
6.	Selenium	*Stanleya pinnata*
7.	Cadmium	*Arabidopsis halleri*
		Bidens pilosa
8.	Manganese	*Virotianeurophylla*
		Austromyrtus bidwillii
		Maytenus founieri
		Phytolacca americana
9.	Chromium	*Leersia hexandra*
		Gynura pseudochina
		Salsola kali

Hence, these toxic heavy metals need to be transformed into non-toxic compounds using various physic-chemical strategies (Gaur and Adholeya 2004; Rebello et al. 2018).

The anthropogenic sources such as pesticides, energy, power, construction, steel manufacturing, waste incineration, food processing waste disposal, smelting, and mining followed by coal combustion and military operations are primarily considered as important sources of environmental contamination. This has indicated that the enhanced anthropogenic activities have influenced the environment directly through food chain since heavy metal contamination in vegetables causes potential health threats and safety issues (Ugya et al. 2019). Irrigation of agricultural fields using wastewater frequently has resulted elevated level of heavy metals in several crops (Pan et al. 2016; Rana et al. 2014). In addition, the poor agricultural practices, misuse of the soil, and urban waste disposal, followed by the regular use of the chemical have resulted in soil pollution (Li et al. 2016; Mahmoud and Ghoneim 2016).

It is well known that the frequent consumption of food sources tainted with the aforesaid elements has cause severe risk to human health. One of the reasons for this condition is the accumulation of heavy metals at an elevated level. The frequent intake of a hazardous range of heavy metals through food resources has resulted in chronic accumulation in the liver and kidney of humans followed by triggering disruption of several biological and physiological processes including kidney, cardiovascular, bone, and nervous-associated diseases (Mahmood and Malik 2014). Several cleanup inventions are developed for the detoxification of contaminated natural resources; however, few technologies stand efficient to decontaminate the resources. This has enhanced the need for using plants and plants allied microorganisms to degrade, inactive, or remove the toxic environmental contaminants and to invigorate contaminated regions which are receiving much more care and consideration (Vaikosen and Alade 2017).

12.3 Deposition of Toxic Elements

Several toxic elements including nickel, manganese, copper, and zinc are important micronutrients required for the completion of plants' life cycle. Hence, the concept concerning the use of the plant as a phytoremediation technique has gained much more interest in in the past few eons (Number et al. 1997; Shiowatana et al. 2001). The important heavy metals influence the economic balances by reducing the crop production and induce the risk over and groundwater contamination. Research based on basic chemistry, ecological impacts, and linked health disorders caused by heavy metals are essential to find out their bioavailability and remedial preferences together with speciation. The speciation and chemical nature of the element has also influenced the fate and spread. The initial fast reactions of heavy metal contamination normally require minutes to hours, whereas its slow adsorption necessitates days and years. This indicates their diverse chemical form with toxicity, and

bioavailability has principally involved in the redistribution of toxic elements (Buekers 2007). The distribution of the aforesaid metals is supposed to be controlled by various processes induced by metals from the resources. They include (i) dissolution and precipitation of minerals; (ii) ion exchange; (iii) adsorption, desorption, and aqueous complexation; and (iv) biological immobilization followed by plant uptake.

The accumulation of lead in the kidneys, central nervous system, and gastrointestinal tract may lead to severe threat including poisoning, hyperactivity, decreased reaction time, weakness of the joints, anorexia, loss of memory, lower intelligence quotient, mental deterioration, insomnia, shortened attention span, and impaired development even death. It is well reported that its toxic effects have been much more broadly studied than any other heavy metals in trace amounts since it can generate severe bruise to the kidneys, neuron system, and brain together with a vast range of toxic effects in biological systems. Direct ingestion is primarily renowned as the one of the major sources of lead exposure from the soil. Previous studies have reported its toxic effects; however, they also revealed that it is never accumulated in the fruit crops (strawberries, beans, tomatoes, corn, apples, and squash) in large amounts. Its high concentrations are principal to be found on the exterior of leafy vegetables (Wuana and Okieimen 2011).

Arsenic is one of the heavy metals responsible for environmental contamination which always induced notorious toxic effects to humans and other living organisms. The various species of these heavy metals determine its level of toxicity to the living organisms. The inorganic and organic form of arsenic is strongly influenced by the following factors such composition of minerals in surrounding environment, the pH, and activities executed by microbial communities. The two mineral species of arsenic, namely, arsenite and arsenate, are recognized as the foremost one in the mainstream of the environment (Yusof and Malek 2009). These two aforementioned elements are known as highly toxic since the other forms are less toxic.

The naturally occurring mercury is present silver–white, odorless liquid and shiny, and is present in various forms. It usually combines with various other elements including oxygen, chlorine, and sulfur and results in the formation of white powders or crystals containing inorganic mercury compounds. Due to its easy vaporization and low boiling point, mercury is always gained much more significance in numerous industrial products. Like any other element, mercury has been found as several forms in soil. Mainly three forms of silver have been found to be present in the soils, and they include (1) the most reduced Hg^0 (2), ionic of mercurous ion Hg^2 (3), and mercuric ion Hg^{2+}. The major binding force for the adsorption of mercury includes electrostatic forces, precipitated as sulfide, hydroxide, phosphate, and hydroxide. Conventionally, certain anaerobic bacteria have been used for the methylation, a major way for the decontamination of mercury from the contaminated resource (Rodriguez et al. 2005). The aforementioned concepts and statements have verified that the organomercury compounds, especially the mercury salts, are among the major poisonous elements in our surroundings. In addition, the degree of toxicity and mechanisms of their action over the surroundings and

organisms has strongly interlinked with the redox state and type of compound (Wagner-Döbler).

The phosphate fertilizers, refined petroleum products, and detergents are the principal source of cadmium in environmental pollution. The acidification of soils by acid rain has enhanced the geochemical transport of cadmium. It is a biopersistent; however, it has exhibited only a few toxicological concerns, once ingested in the body of an organism, leftovers resident for a long period of time. Within the body, it seriously affects many enzymes and proteins and causes renal damage followed by proteinuria. Precisely, they generate harmful effects towards various important enzymes that are essential for the resorption of proteins and hormones. It is as well capable of diminishing the action of alcohol dehydrogenase, delta-aminolevulinic acid synthetase, and lipoamide dehydrogenase, followed by arylsulfatase; simultaneously it augments the activity of pyruvate decarboxylase, delta-aminolevulinic acid dehydratase, and pyruvate dehydrogenase.

Copper is an important element indispensable for both plants (as a micronutrient) and hominids (essential for the production of blood hemoglobin) in several ways. At a minimal level, copper plays an important role in disease resistance and seed production together with regulation of water whereas its high concentration has resulted in kidney damage, anemia, stomach, and intestinal disorders and liver damage. It normally ingested into the human body through the drinking water from copper pipes. The contaminated soils have exhibited certain effects that are either direct or indirect. The direct effects comprise the negative effects on crop growth and profit, whereas the indirect concerns include the entry of toxic components into the food chain with potential adverse impact on the human population. The drastic reduction in crop production could also lead to economic loss for the long term.

Nickel is a heavy metal element; it can generate toxic effects when the concentration has reached above the threshold level. It can cause various genetic disorders including cancer in animals. The major source of nickel is the steel manufacturing companies, plating industries, and industries. It may also be released into the atmosphere by trash incinerators and power plants. Most of all nickel compounds will adsorb to sediment when they are released into the environment. Nickel becomes leaches down to the neighboring groundwater in acidic soils and also induces adverse effects to microorganisms and thereby diminishes their population size. However, the microorganisms generally grow resistance against nickel.

12.4 Metal Accumulating Plants: The Notable Natural Resource for Phytoextraction

Most of the plants are able to accrue high levels of heavy metals in their tissues. In this regard, mainly three technologies such as phytoextraction and phytostabilization, followed by phytovolatilization, have been used for

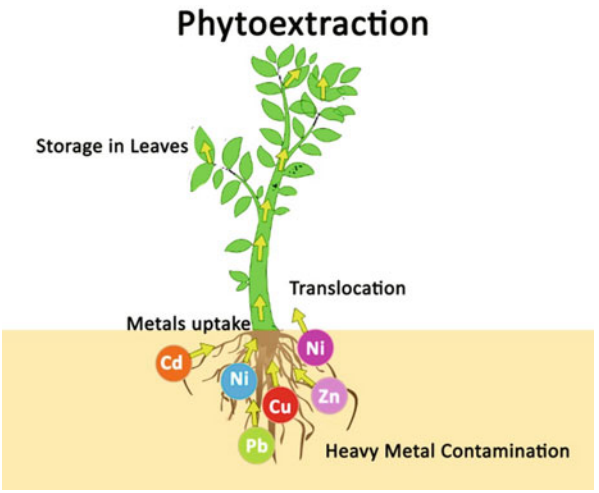


Fig. 12.1 Phytoextraction of heavy metals – a graphical representation. The heavy metals such as cadmium, nickel, copper, zinc, and lead from soil have been transferred to the shoots of the plants through roots. After the successful uptake of heavy metals by the root, it will penetrate through the different parts inside the plant and finally stored these compounds at the leaves

phytoremediation. The phytoextraction uses plants to decontaminate the elements from soil, and the phytostabilization reduces the mobility of metals, while the phytovolatilization converts metals to volatile chemical species. By virtue of this notable property, phytoextraction could be considered as economically viable and is alternative to the high cost of conventional remediation strategies (Wither and Brooks 1977). The process of heavy metals accumulation in plants involves a series of steps comprising the mobilization of soil, its uptake by roots, transport to shoot through the xylem, and distribution followed by storage in the tissues (Clemens et al. 2002). The uptake of elements by plant species strongly depends on diverse contributing aspects such as the total quantity of the available elements in soil, usually referred as quantity factor, the reaction kinetics, and the intensity factor (Brümmer 1986).

Phytoextraction in a natural way is principally troubled by low biomass of heavy metals, and it has required a long period of time to reach environmentally permissible levels. Taking into the applications of phytoextraction, several authors have verified the highly specific and efficient mechanisms of plants to get vital micronutrients, even at low ppm levels. The phytoextraction feats the capability of herbs to uptake the metals from natural resources (Fig. 12.1) (Raskin et al. 1994). However, the phytoextraction has several limits that are arising from threatened metal obtainability and troubles in uptake by roots; and needs great energy as well as xylem loading and symplastic mobility (Meagher 2000). Plants exhibit diverse responses to metal contamination, and most of the morphophysiological responses are sensitive to very minute concentrations. The metal accumulation is usually expressed by "plant to-soil metal concentration ratio." In addition, both the translocation factor and bioconcentration factor clearly influence the phytoextraction.

Tolerant herb species have a habit of less accumulation of heavy metals since they restrict transfer between soil to root together with root to shoot. Usually, the plants exhibit greater than 1 BAC (biological absorption coefficient) can be used for

phytoextraction; for phytostabilization, plants with higher than 1 bioconcentration factor and lower than 1 transcription factor value can be used (Brümmer 1986). The effectiveness of phytoextractions depends on several desirable properties such as (1) high biomass and rapid growth, (2) well-developed rot system for uptaking large volumes of elements, (3) effective tolerance to metals with high concentrations, (4) great translocation factor, and (5) adaptability against specific area. The phytoextraction of heavy metal-contaminated natural resources especially soil follows on the utilization of herbs to translocate the elements to their specific harvestable regions.

Principal focus of phytoextraction is diminishing the concentration if heavy metals in contaminating resources within a short time frame (Nascimento and Xing 2006). The engineering calculations regarding the concept of phytoextraction suggested that effective plant-based decontamination of natural resources would normally necessitate crops capable to concentrate toxic elements in high. Deposition of heavy metals at high concentration would definitely destruct the non-accumulator plant within short time. However, biotechnology has now been productively employed to assess heavy metal uptake through plants. For instance, the expression of binding proteins that are specific against heavy metals and mammalian metallothionein has resulted in increased metal tolerance in *Nicotiana tabacum* (Lefebvre et al. 1987).

Accumulation of heavy metals in natural resources is considered as an interesting area of research that, excepting to major commercial uses, should be responsible to provide answers to certain important questions of phytochemistry, nutrition, and environmental hazards followed by heavy metal accumulation. Phytoextraction of heavy metals at the industrial level, although still in its early stages, may 1 day grow into a well-known cleanup strategy. An integrated multidisciplinary study that links plant biochemistry, agricultural applications, soil microbiology, soil chemistry, and environmental engineering is essential for the development of phytoremediation techniques. It is imperative to note that plants that deposit toxic elements can be grown intended for economic benefits, leaving the water or soil including various natural resources with a diminished level of heavy metal contamination. Environmental cleanup using plants may guarantee a cleaner and greener earth for all of us.

Acknowledgment Raveendran Sindhu acknowledges DST for sanctioning a project under DST WOS-B scheme.

References

Alloway BJ (2012) Heavy metals in soils: trace metals and metalloids in soils and their bioavailability, vol 22. Springer. https://doi.org/10.1007/978-94-007-4470-7_2

Barla A, Shrivastava A, Majumdar A, Upadhyay MK, Bose S (2017) Heavy metal dispersion in water saturated and water unsaturated soil of Bengal delta region, India. Chemosphere 168:807–816. https://doi.org/10.1016/j.chemosphere.2016.10.132

Brümmer G (1986) Heavy metal species, mobility and availability in soils. In: The importance of chemical "speciation" in environmental processes. Springer, pp 169–192. https://doi.org/10.1007/978-3-642-70441-3_11

Buekers J (2007) Fixation of cadmium, copper, nickel and zinc in soil: kinetics, mechanisms and its effect on metal bioavailability PhD, Department of Microbial and Molecular Systems, Katholieke Universiteit Leuven, Flanders, Belgium 1

Clemens S, Palmgren MG, Krämer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci 7:309–315. https://doi.org/10.1016/S1360-1385(02)02295-1

Freitas H, Prasad M, Pratas J (2004) Plant community tolerant to trace elements growing on the degraded soils of Sao Domingos mine in the south east of Portugal: environmental implications. Environ Int 30:65–72. https://doi.org/10.1016/S0160-4120(03)00149-1

Gaur A, Adholeya A (2004) Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Curr Sci 86:528–534

Jain N, Bhatia A, Kaushik R, Kumar S, Joshi H, Pathak H (2005) Impact of post-methanation distillery effluent irrigation on groundwater quality. Environ Monit Assess 110:243–255. https://doi.org/10.1007/s10661-005-7695-6

Ji G (2000) Environmental problems in soil and groundwater induced by acid rain and management strategies in China soils and groundwater pollution and remediation

Lajayer BA, Ghorbanpour M, Nikabadi S (2017a) Heavy metals in contaminated environment: destiny of secondary metabolite biosynthesis, oxidative status and phytoextraction in medicinal plants. Ecotoxicol Environ Saf 145:377–390. https://doi.org/10.1016/j.ecoenv.2017.07.035

Lajayer HA, Savaghebi G, Hadian J, Hatami M, Pezhmanmehr M (2017b) Comparison of copper and zinc effects on growth, micro-and macronutrients status and essential oil constituents in pennyroyal (Mentha pulegium L.). Braz J Bot 40:379–388. https://doi.org/10.1007/s40415-016-0353-0

Lefebvre DD, Miki BL, Laliberté J-F (1987) Mammalian metallothionein functions in plants. Nat Biotechnol 5:1053. https://doi.org/10.1038/nbt1087-1053

Li L, Hu T, Li X, Mu S, Cheng Z, Ge W, Gao J (2016) Genome-wide analysis of shoot growth-associated alternative splicing in moso bamboo. Mol Genet Genomics 291:1695–1714. https://doi.org/10.1007/s00438-016-1212-1

Mahmood A, Malik RN (2014) Human health risk assessment of heavy metals via consumption of contaminated vegetables collected from different irrigation sources in Lahore, Pakistan. Arab J Chem 7:91–99. https://doi.org/10.1016/j.arabjc.2013.07.002

Mahmoud EK, Ghoneim AM (2016) Effect of polluted water on soil and plant contamination by heavy metals in El-Mahalla El-Kobra, Egypt. Solid Earth 7:703–711. https://doi.org/10.5194/se-7-703-2016

McLaughlin MJ, Parker D, Clarke J (1999) Metals and micronutrients–food safety issues. Field Crop Res 60:143–163. https://doi.org/10.1016/S0378-4290(98)00137-3

Meagher RB (2000) Phytoremediation of toxic elemental and organic pollutants. Curr Opin Plant Biol 3:153–162. https://doi.org/10.1016/S1369-5266(99)00054-0

Nascimento CWAD, Xing B (2006) Phytoextraction: a review on enhanced metal availability and plant accumulation. Sci Agric 63:299–311. https://doi.org/10.1590/S0103-90162006000300014

Number WA, Ma C, Kingscott J, Evans M (1997) Recent developments for in situ treatment of metal contaminated soils. EPA Office of Solid Waste and Emergency Response, Technology, Washington, DC

Pan X-D, Wu P-G, Jiang X-G (2016) Levels and potential health risk of heavy metals in marketed vegetables in Zhejiang, China. Sci Rep 6:20317. https://doi.org/10.1038/srep20317

Pehlivan E, Özkan AM, Dinç S, Parlayici Ş (2009) Adsorption of Cu2+ and Pb2+ ion on dolomite powder. J Hazard Mater 167:1044–1049. https://doi.org/10.1016/j.jhazmat.2009.01.096

Rana M, Farooq A, Adnan S, Osama S, Abdul W, Muhammad F, Maqsood A (2014) Assessment of heavy metals in vegetables cultivated in land irrigated from hudiara drain. J Environ Sci Comput Sci Eng Technol 3:1218–1227

Raskin I, Kumar N, Dushenkov S, Salt D (1994) Bioconcentration of heavy metals by plants. Curr Opin Biotechnol 5:285–290. https://doi.org/10.1016/0958-1669(94)90030-2

Rebello S, Anoopkumar A, Puthur S, Sindhu R, Binod P, Pandey A, Aneesh EM (2018) Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour Technol Rep 3:1–6. https://doi.org/10.1016/j.biteb.2018.05.007

Rebello S, Balakrishnan D, Anoopkumar A, Sindhu R, Binod P, Pandey A, Aneesh EM (2019) Industrial enzymes as feed supplements—advantages to nutrition and global environment. In: Green bio-processes. Springer, pp 293–304. https://doi.org/10.1007/978-981-13-3263-0_15

Rodriguez L, Lopez-Bellido F, Carnicer A, Recreo F, Tallos A, Monteagudo J (2005) Mercury recovery from soils by phytoremediation. In: Environmental chemistry. Springer, pp 197–204. https://doi.org/10.1007/3-540-26531-7_18

Sarwar N et al (2017) Phytoremediation strategies for soils contaminated with heavy metals: modifications and future perspectives. Chemosphere 171:710–721. https://doi.org/10.1016/j.chemosphere.2016.12.116

Shiowatana J, McLaren RG, Chanmekha N, Samphao A (2001) Fractionation of arsenic in soil by a continuous-flow sequential extraction method. J Environ Qual 30:1940–1949. https://doi.org/10.2134/jeq2001.1940

Srivastava N (2016) Phytoremediation of heavy metals contaminated soils through transgenic plants. In: Phytoremediation. Springer, pp 345–391. https://doi.org/10.1007/978-3-319-40148-5_12

Ugya A, Ahmad A, Adamu H, Giwa S, Imam T (2019) Phytoextraction of heavy metals and risk associated with vegetables grown from soil irrigated with refinery wastewater. J Appl Biol Biotechnol 7:14–19. https://doi.org/10.7324/JABB.2019.70203

Vaikosen EN, Alade GO (2017) Determination of heavy metals in medicinal plants from the wild and cultivated garden in Wilberforce Island, Niger Delta region, Nigeria. J Pharm Pharm Res 5:129–143

Wagner-Döbler I. Microbiological treatment of mercury loaded waste water—the biological mercury-decontamination-system. German Res Cent Biotechnol

Wither E, Brooks R (1977) Hyperaccumulation of nickel by some plants of Southeast Asia. J Geochem Explor 8:579–583. https://doi.org/10.1016/0375-6742(77)90100-5

Wong MH (2003) Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50:775–780. https://doi.org/10.1016/S0045-6535(02)00232-1

Wuana RA, Okieimen FE (2011) Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. Isrn Ecol 2011:1–20. https://doi.org/10.5402/2011/402647

Yusof AM, Malek NANN (2009) Removal of Cr (VI) and As (V) from aqueous solutions by HDTMA-modified zeolite Y. J Hazard Mater 162:1019–1024. https://doi.org/10.1016/j.jhazmat.2008.05.134

Research Article

Life Cycle, Bio-ecology and DNA Barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse)

A N Anoopkumar[1], Sreedev Puthur[2], Paulson Varghese[3], Sharrel Rebello[4], Embalil Mathachan Aneesh[5]

Abstract

Mosquito borne diseases remain as the world's most severe insect-borne disease with excessive rates of morbidity and mortality. Mosquitoes transmit various severe diseases such as dengue, malaria, filariasis, viral encephalitis, chickungunya and zika virus infections causing millions of deaths worldwide; and no part of the world is liberated from mosquito borne diseases. *Aedes aegypti* and *Aedes albopictus* represent the two important mosquito vectors for dengue virus transmission in America and Asia. According to the Integrated Disease Surveillance Project (ISDP), 8888 confirmed dengue infections were reported from January 1st to June 30th 2017 in Kerala state, India with 409 confirmed dengue infections reported from Thrissur district of Kerala including Irinjalakuda Municipality (the area of this study). Additionally 15 confirmed and 56 suspected dengue fever deaths were also reported from Kerala state, India. Current epidemics of dengue and severe mosquito borne diseases from Kerala have exposed the need for more comprehensive understanding of the mosquito species types, their vectorial capacity and the habitat characteristics that offer them for proper breeding environment in the study area. The present study also explored the applicability of CO1-based DNA bar coding as an alternative approach to identify mosquito species such as *A. aegypti and A. albopictus*.

Keywords: *Aedes aegypti, Aedes albopictus,* CO1 barcoding, vectors

Introduction

Mosquitoes are significant groups of arthropods that inhabit aquatic habitats. They are probably adverse arthropods, which transmits wide range of pathogens that cause drastic deadly diseases such as human malaria, dengue, filariasis and viral encephalitis[1]. Zika virus, another mosquito borne infection was first reported in Brazil by Pan American Health Organization in 2015 and since then the virus has spread tremendously all around America. Zika infection begins with mild symptoms lasting up to a week after being bitten by vector mosquito; but an infection during pregnancy may cause certain neurological anomalies like microcephaly and several other brain defects[2]. Thus control of mosquitoes becomes the need of the hour to prevent wide epidemic infections. However, it is difficult to control and prevent severe consequences created by mosquito species[3].

Currently a total of 3549 recognized mosquito species belonging to subfamily Culicinae are reported all over the world[4];

[1]Department of Zoology, Christ College Irinjalakkuda, University of Calicut.

[2,4,5]Communicable Disease Research Laboratory, Department of Zoology, St. Josephs College, Irinjalakkuda.

[3]Trivandrum Medical College.

Correspondence: Dr. Embalil Mathachan Aneesh, Communicable Disease Research Laboratory, Department of Zoology, St. Josephs College, Irinjalakkuda.

E-mail Id: aneeshembalil@gmail.com

Orcid Id: http://orcid.org/0000-0001-8140-3888

How to cite this article: Anoopkumar A.N, Puthur S, Varghese P et al. Life Cycle, Bio-ecology and DNA Barcoding of mosquitoes *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). *J Commun Dis* 2017; 49 (3): 32-41.

Digital Object Identifier (DOI): 10.24321/0019.5138.201719

E ISSN: 0019-5138 **l P ISSN:** 2394-7047

with more than hundred species of mosquitoes capable of disease transmission to humans and other animals[1]. One-third of this subfamily is represented by the Genus *Aedes* (Suleman *et al*. 1996) of which members of *A. aegypti and A. albopictus* representing the two important mosquito vectors for dengue virus transmission in America and Asia[5].

Urbanization significantly made modifications in the *Aedes* mosquito ecology by certain environmental changes[6]. The mosquito borne diseases such as dengue, zika, and yellow fever are primarily transmitted to the humans by the mosquito species *A. aegypti* and vector control is significant to restrain the transmission of these drastic viral diseases[7]. Studies indicate that 390 million people in the world face dengue infections per year with 96 million infections confirmed clinically[8]. Additionally, the rapid spread of mosquito borne diseases is vitalized by global freight transportation and international travel[9, 10]. *A. albopictus* are vigorous throughout the year in tropical and subtropical habitats. Over winter they lay eggs on the sides of water-filled containers such as bird baths, flowerpots, animal watering dishes, natural holes in vegetation and tires[11]. Destruction of these mosquito breeding habitat is an efficient method for mosquito vector control[12].

For the proper development, the larvae and pupae of mosquitoes necessitate an aquatic environment with standing or flowing water. Larvae of the majority mosquito species generally filter out and feed organic matter, other microorganisms from water[13]. Heterotrophic microorganisms such as bacteria, fungi and protozoans from detritous surfaces or containers are significant for the larval diet[14]. Freshwater swamps, rice fields, borrow pits, marshes, puddles, water-filled tracks, ditches, gulleys and drains are tremendous source of mosquito larval habitats. Wide range of 'natural container- habitats' such as, rock-pools, water-filled bamboo stumps, tree holes filled with water, leaf axis in banana, snail shell and coconut husks offer enough requirements for mosquito larval habitat. Man-made container habitats including water storage jars, tin cans, motor vehicle tyres and discarded kitchen utensils also contribute space for breeding of mosquitoes[15].

After the larvae have accomplished their fourth larval molt they develop into pupae (called tumblers). Pupae don't require food and be alive for 1-3 days before the adult form. Male adult mosquitoes primarily feed nectar from plants to get sugar while the female mosquitoes imbibe the blood meal to generate viable eggs[13]. Female mosquitoes usually nourish every 3-5days. *A. albopictus* females are diurnal feeders; they not only give preference to attack large mammals but also imbibe blood meals from birds[16].

A conventional method to identify mosquito species is morphological identification, which is critically based on external characters and they may differ for different species. Taxonomic keys such as Bram, Harrison and Scanlon, Rattanarithikul are significantly used for morphological identification of individual mosquito species[17-22], Apart from the requirement of much expertise, morphological identification is exceedingly time-consuming and imperfect identification may result when significant morphological features such as bristles and scales are impaired[23]. Prompt and perfect species identification with immense accuracy can be achieved through molecular approach[24].

DNA barcoding has been encouraged as a consistent method for the species identification of both invertebrate and vertebrate taxa[25]. DNA based molecular techniques and approaches for species identification, molecular phylogeny, and genetic diversity of mosquito acquire attention in recent years[26-31]. The present study aimed to evaluate the molecular diversity, vectorial capacity and habitat characteristics of *Aedes* mosquito species in the study area.

Materials and Methods

Sample collection

Samples *of A. aegypti* and *A. albopictus* were collected from Irinjalakkuda municipality which belongs to Thrissur district of Kerala state, India (10.33 N 76.23 E) (Fig. 1). The study area is rich in diverse topography and provides habitat for different kinds of mosquito species. Different spots were randomly selected with an intention to cover entire topography of the study area.

Figure 1.Study area

E ISSN: 0019-5138 | P ISSN: 2394-7047

Mosquito larval collections were carried out from different habitats including both natural and artificial using dippers (12cm diameter and of 300 ml capacity), pippets and plankton nets. Dippers and plankton nets were used in open sources for sampling while pippets were used in tree holes. Collected larvae were carefully transferred alive to the insectary and allowed to emerge as adults. Identification of the collected specimen was authenticated at CDRL, St. Joseph's College, Irinjalakuda.

Insect rearing

A. aegypti and *A. albopictus* larave were reared in plastic trays (36x 24x7) containing dechlorinated water. The larvae were fed on powdered dog biscuits and yeast in the ratio of 3:1. The insectary was maintained at 27± 2°C and 75–85% relative humidity, with 12:12 light and dark photoperiod cycle for larval growth. Pupae were transferred to a bowl containing dechlorinated water and were maintained in insectary (30x30x30) where adults emerged. Adult mosquitoes were fed with water soaked grapes. On day 6, an immobilized young chick was kept inside the cage to provide blood meal for female mosquitoes. Plastic bowl (12 cm diameter) containing dechlorinated water with a lining of partially immersed filter paper was kept inside the cage to allow the female mosquitoes to lay their eggs.

DNA isolation, PCR amplification and sequencing

CTAB method was used to isolate Genomic DNA from insects (Hunt, 1997). The mitochondrial gene of the mosquito species was amplified by CO1 PCR with forward primer 5'-TATTATTAGACAAGAATCTGGTAAA-3' and reverse primer: 5'- AGGAAATGTTGA GGGAAGAAAGTAA-3'. PCR amplification was performed in 25µl volumes containing 15ng of DNA, 30 pmol of each primer, Taq DNA polymerase reaction buffer with $MgCl_2$, 2.5 mM dNTPs and 1 unit of Taq DNA polymerase mixed in the PCR buffer (Sigma). Amplification parameters in the Mastercycler were as follows: An initial denaturaton of 95°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds. Annealing and extension were performed at 54°C for 30 seconds and 72°C for 1 minute respectively. The final extension was accomplished at 72°C for 10 minute. PCR product was purified with llustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), according to the manufactures instructions and recommendations. Sequencing was performed using Big Dye Terminator cycle sequencing chemistry for ABI Bioprism (Applied Biosystems). The sequences generated were deposited in the NCBI gene bank data base (http://www.ncbi.nlm.nih.gov).

Data analysis

Significant variation in the birth rate and duration of development of *A. aegypti* and *A. albopictus* was illustrated as pie diagram using IBM SPSS Version 24.

Phylogenetic analysis

Neighbor-Joining method was used to investigate the evolutionary relationships[32]. The sum of branch length of tree is equal to 1.23192720. Maximum Composite Likelihood method was used to calculate evolutionary distance[33]. Eight nucleotide sequences were involved in analysis. 1st+2nd+3rd+Noncoding positions were included. Position showing gaps and missing data were excluded. A total of 371 positions were appeared in the final data set. Evolutionary analyses were performed through the software MEGA6 (Molecular Evolutionary Genetics Analysis, version 3.1)[34]. Species discrimination using DNA barcode was performed by comparing the nucleotide sequences on NCBI gene bank data base.

Results

Life cycle and bio-ecology of *A. aegypti* and *A. albopictus*

The data obtained on the vectorial capacity of the collected mosquito species were compiled in table I. Continuous observations were made for both the species in respect of their fecundity and other ecological aspects for three generations. Thus a comparative analysis on the life cycle (Table II and Fig.2) of *A. aegypti* and *A. albopictus* was possible. The results from the present study revealed certain variation in birth rate and duration of development (in terms of number of days). *A. aegypti* took 28.5 days to complete its life cycle while *A. albopictus* use only 22.5 days as depicted in fig 3. Both of them took 8.5 days to attain pupal stage; and there was no variation observed in this aspect. However, there was significant difference in the life expectancy of adult mosquitoes of *A. aegypti* and *A. albopictus* which was calculated to be twenty and fourteen respectively. This variation was evident from the stage of number of eggs obtained from *A. aegypti* (=114) and *A. albopictus* (= 65) as shown in table III. However, the percentage of egg hatching (85.9 and 86.32) and percentage of adult emergence (80 and 81.5) remained almost same for *A. aegypti* and *A. albopictus.*

Table 1.Vector status of Aedes aegypti and Aedes albopictus

Vector status of *Aedes aegypti* and *Aedes albopictus*	
Aedes aegypti	***Aedes albopictus***
A. aegypti is the formost Zika vector[45].	Capable of transmitting Zika virus[45].
Dengue vector [46].	Dengue vector (Beebe et al. 2005), Viral pathogens including chikungunya, several filarial Nematodes namely Dirofilaria immitis[47].
Bites mainly humans (anthropophilic)[45].	Primarily attack wild and domestic animals (zoophilic) but also humans[45].
Typically feeds several times per cycle of egg production[41].	Usually feeds single per cycle of egg production[41].
Adapts well to human urban settlements[41].	Inhabits rural and urban areas[41].

Table 2.Characteristics of common mosquito vectors *Aedes aegypti* and *Aedes albopictus*

Characteristics of common mosquito vectors *Aedes aegypti* and *Aedes albopictus*	
Aedes aegypti	***Aedes albopictus***
A. Eggs	
• Eggs are laid on damp surfaces in areas[48].	• Water-holding containers such as tyres, animal watering dishes, birdbaths, flowerpots and natural holes in vegetation are the excellent space for egg laying[11].
• Commonly eggs are laid at varying distances above the water line[49].	• Eggs are typically elongate and / or ovoid in shape and are smooth[49].
• Eggs are smooth, long, and ovoid in shape[49].	• Eggs do not encompass floats and micropylar collars[50].
• Eggs are usually one millimeter long and encompass the presence of very prominent micropylar collars[50].	• Eggs are black and having a length of 0.5 mm[11].
B. Laravae	
• The larvae pass through four instars. And Temperature significantly influences larval development[49].	• Larvae are vigorous feeders[49].
• Males develop quicker than females[51].	• They feed particulate organic matter from water[51].
C. Pupae	
• After the fourth larval molt, it enters the pupal stage[51].	• Pupae are active[51].
• pupae may react to stimuli[49].	• They do not require food but can move about[51].
D. Adult	
• Adult having just about 4 to 7 mm[52].	• Adult having approximately 2.0 to 10.0 mm. males are on average 20% smaller than females)[51].
• Adults posses white scales on the dorsal (top) surface of the thorax[52].	• Adult posses bold black shiny scales and distinct silver white scales on the palpus and tarsi[11].
• The abdomen is usually dark brown to black and also showed white scales[52].	• Each tarsal segment bear white basal scale and legs are black in color[51].
• Males are smaller than females[49].	• Males are approximately 20% smaller than females[51].
• Males encompass[49].	• The abdomen narrows into a point is the characteristic of the genus *Aedes*[51].

E ISSN: 0019-5138 | P ISSN: 2394-7047

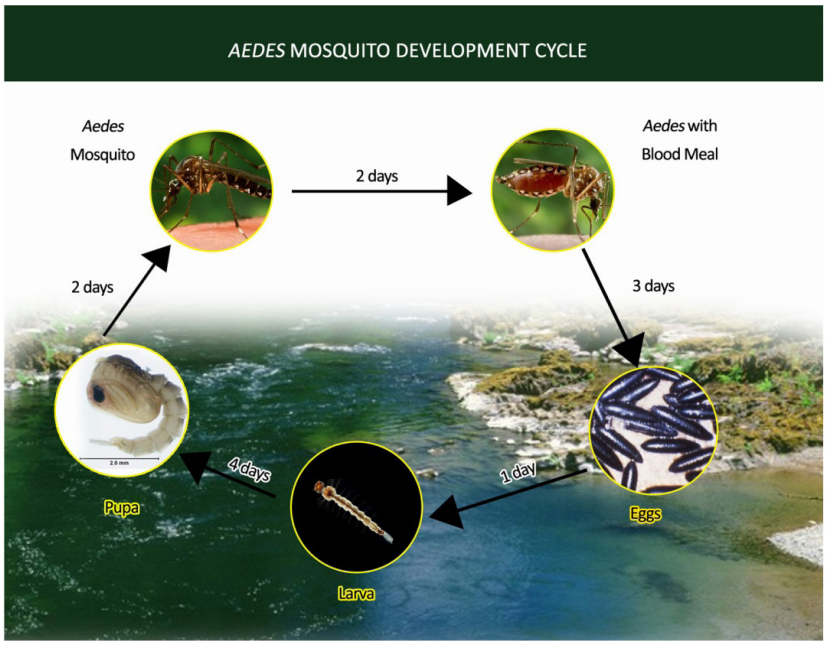
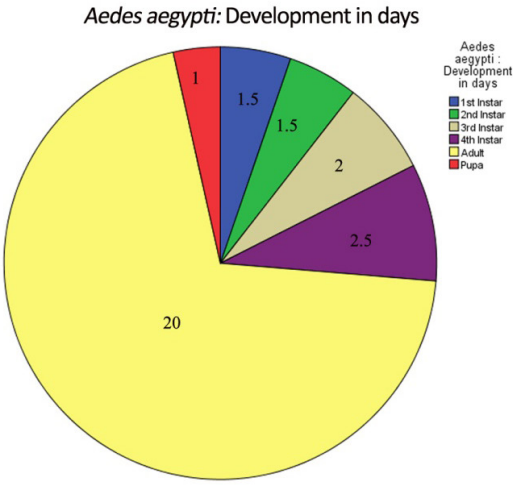


Figure 2.*Aedes* Mosquito Development Cycle

a



b

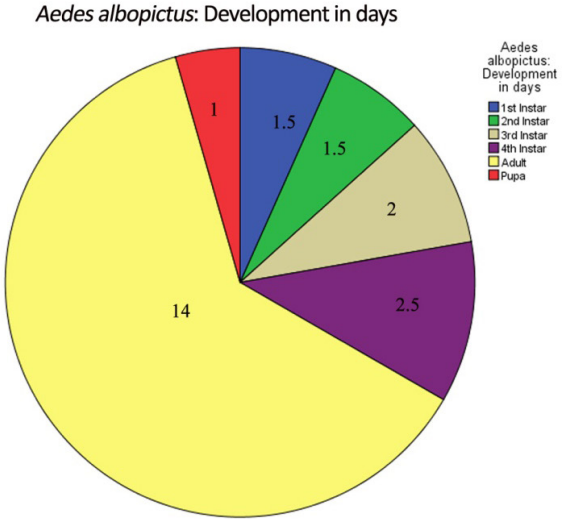


Figure 3.Development in days (a) *Aedes aegypti* (b) *Aedes albopictus*

Table 3.Fecundity of *Aedes aegypti* and *Aedes albopictus*

Fecundity of *Aedes aegypti* and *Aedes albopictus*		
	Aedes aegypti	***Aedes albopictus***
No. of. Eggs	114	65
Hatching	98	56
Percentage of hatching	85.9	86.32
4th instar larvae	95	54
Pupae	93	53
Adult	92	53
Percentage of adult emergence	80	81.5

The study indicated that manmade materials were predominantly found to be a habitat for mosquitoes rather than natural counterparts. Tree hole, cemented tanks, stream pools, tyres, bowls and containers are the six different kinds of habitats populated by both the species *A. aegypti* and *A. albopictus* (Table IV*)*. Out of the 14 habitats, 57 % of mosquitoes prefer artificial means of habitat selection.Analysis on different habitats occupied by mosquitoes revealed that out of the 11 habitats of *A. aegypti* was only four were natural habitats. Similarly only four of the 9 habitats used by *A. albopictus* were natural habitats. Out of the total 14 habitats populated by mosquito species *A. aegypti* and *A. albopictus*, six were inhabited by both the species.

Table 4.Habitat preference of different *Aedes* species in the study area

Habitat preference of different *Aedes* species in the study area		
Habitat	*Aedes aegypti*	*Aedes albopictus*
Tree hole	+	+
Cemented tanks	+	+
Stream pools	+	+
Mud pot	+	-
Plastic containers	+	-
Tyers	+	+
Plant pot	+	-
Nuts	+	-
Tin	-	+
Bowls	+	+
Coconut shell	+	-
Leaf axils	-	+
Bamboo	-	+
Containers	+	+

CO1-based DNA barcoding and phylogenetic analysis

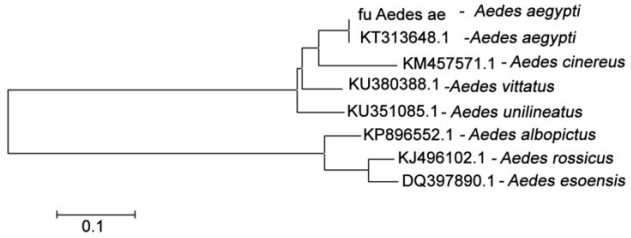
The morphological features of the test specimens were matched with molecular characterization while comparing theCO 1 genes with NCBI genbank. Thus it confirms that the test specimens were *A. aegypti* and *A. albopictus.* CO1-based phylogenetic analyses (Table V*)* showed that *A. aegypti* strain was congregated with *A. aegypti* KT313648.1 (Fig. 4a) and *A. albopictus* strain was clustered with *A. albopictus* KP896552.1 (Fig. 4b).

E ISSN: 0019-5138 | P ISSN: 2394-7047

Table 5.Accession id with species retrieved from phylogenetic analysis

Accession id with species retrieved from phylogenetic analysis		
Number	Species	GenBank ID
1	*Aedes aegypti*	Fu Aedes ae
2	*Aedes aegypti*	KT313648.1
3	*Aedes cinereus*	KM457571.1
4	*Aedes albopictus*	KP896552.1
5	*Aedes rossicus*	KJ496102.1
6	*Aedes vittatus*	KU380388.1
7	*Aedes esoensis*	DQ397890.1
8	*Aedes albopictus*	SNA-
9	*Aedes albopictus*	KP896552.1
10	*Aedes rossicus*	KJ496102.1
11	*Aedes esoensis*	DQ397890.1
12	*Aedes unilineatus*	KU351085.1
13	*Aedes vittatus*	*KU380388.1*
14	*Aedes aegypti*	*KT313648.1*
15	*Aedes cinereus*	*KM457571.1*

(a) *Aedes aegypti*



(b) *Aedes albopitus.*

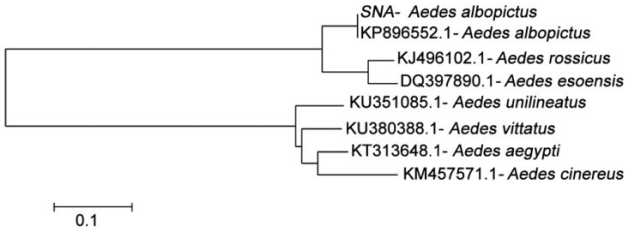


Figure 4.Phylogenetic analysis against other reported mosquito species

Discussion

A. aegypti is well adapted to live urban areas and they preferentially feed humans[35]. Agro industrial modifications of habitats of mosquitoes including climatic discrepancy significantly influence outbreak of mosquito borne diseases. Hence the disease situation can be tackled by improving the knowledge about the mosquito vectors and mosquito

borne- diseases. The information should explain the risk of diseases transmitted, and planning of control measures to support disease control programs. In the light of their economic importance, the present study was made to monitor and asses the mosquito species types in the study area, their vectorial capacity and the habitat characteristics that offer them proper breeding environment. The present study reported the life expectancy of adult *A. aegypti* and *A. albopictus* ranged from 22.5 to 28.5 days. This report goes in accordance to findings of Maricopa in 2006[36], who found the above mosquito species utilized around two weeks to a month depending upon environmental condition to complete its life cycle.

A number of *Aedes* mosquito species are responsible for the transmission of arboviral diseases such as dengue and yellow fever[6, 37, 38]. Mosquito-borne diseases critically posses economic impact, including defeat in commercial; and all part of the world were infected by vector-borne diseases[39]. The incidence of dengue has grown dramatically worldwide. The World Health Organization reported that 50–100 million people infected by dengue fever every year[40, 41].

According to the Integrated Disease Surveillance Project (ISDP), 8888 confirmed dengue infections were reported form January 1st to June 30th 2017 in Kerala state, India (Table VI). Of which 409 confirmed dengue infections were reported from Thrissur district, Kerala (Table VI). 15 confirmed dengue fever deaths were also observed from Thiruvananthapuram (6), Kollam (5), Palakkad (1), Kozikkode (3) districts of Kerala state, India[42].

Table 6.Month wise on Data Communicable Diseases from January 1st to June 30th, ISDP 2017

Month wise on Data Communicable Diseases from January 1st to June 30th (KERALA STATE), ISDP 2017			
Sl No.	Month	Dengue fever	
		Confirmed	Death
1	January	435	-
2	February	302	1
3	March	463	-
4	April	1066	1
5	May	2477	7
6	June	4145	6
	Total	8888	15

Immature mosquitoes use a variety of aquatic habitats such as ponds, ditches, streams, marshes, temporary and permanent pools, plant containers (leaves, tree holes, and bamboo nodes), artificial containers (tires, cans, flower vases, bird feeders) habitats[43, 44]. Similarly, the important habitats chosen by *Aedes* species in the study area includes tree hole, cemented tanks, stream pools, fountains, ditches, mud pot, plastic containers, tyres, plant pot, rocky pool, pods, nuts, tin, flower bracts, interriodes, latex collecting containers, bowls, duck weed ponds, coconut shell, leaf axils, temporary pools, fishponds, bamboo and containers (Table II).The present study reported that 57% of "mosquitoes prefer artificial means of habitat selection". These results drastically alarming the health care systems. *A. aegypti* increases arbovirus transmission in urban areas while *A. albopictus* in rural areas[40].

In addition, we also explored the applicability of advanced DNA- based molecular approaches such as DNA barcoding for taxonomical identification of mosquito species. Comparison with data in Genbank revealed that the test DNA sequences were close sequence matches against *A. albopictus* and *A. aegypti*. The phylogenetic investigation was achieved through MEGA 6. The results from MEGA 6 authenticated that *A. albopictus* strain was congregated with *A. albopictus* KP896552.1. While *A. aegypti* strain was clusterd with *A. aegypti* KT313648. Similarly, Abigail Chan and co workers in 2016 used DNA barcoding as an alternative, universally applicable method to support the existing methods for mosquito identification. Conclusively, the presence of diverse habitats (including artificial and natural) and the progressing history of disease outbreak by *Aedes* species in the study area inspire an intensification of the vector surveillance activities.

Acknowledgements

We thank Principal, St. Joseph's College, Irinjalakuda for the laboratory facilities provided. The authors thank University Grants Commission, Government of India (F.NO.42-609/2013 SR), Kerala State Council for Science, Technology and Environment (KSCSTE) (No.27/SRSLS/2013/CSTE).

Conflict of Interest: None

References

1. Rasool, V., S. Rasool, and S. Mushtaq, *Viral encephalitis and its management through advanced molecular*

diagnostic methods: a review. Clinical pediatrics 2014. 53(2): p. 118-120.
2. Plourde, A.R. and E.M. Bloch, *A literature review of Zika virus.* Emerging infectious diseases 2016. 22(7): p. 1185.
3. Aneesh, E. and V. Vijayan, *Laboratory selection of carbofuran tolerant line of Culex quinquefasciatus Say, the filarial vector at Mysore.* J Commun Dis 2010. 42(3): 201-07.
4. Harbach, R.E. and I.J. Kitching, *The phylogeny of Anophelinae revisited: inferences about the origin and classification of Anopheles (Diptera: Culicidae).* Zoologica Scripta 2016. 45(1): p. 34-47.
5. Freitas, M., et al., *Novel DNA extraction assay for molecular identification of Aedes spp eggs.* Genetics and Molecular Research 2014. 13(4): p. 8776-8782.
6. Li, Y., et al., *Urbanization increases Aedes albopictus larval habitats and accelerates mosquito development and survivorship.* PLoS neglected tropical diseases, 2014. 8(11): p. e3301.
7. Souza, V.M. *Identifying Aedes aegypti Mosquitoes by Sensors and One-Class Classifiers*. in *Iberoamerican Congress on Pattern Recognition* 2016. Springer.
8. Bhatt, S., et al., *The global distribution and burden of dengue.* Nature 2013. 496(7446): p. 504.
9. Straetemans, M. and E.C.G.o.V.-R.R.f.C.V.T.i. Europe, *Vector-related risk mapping of the introduction and establishment of Aedes albopictus in Europe.* Euro surveillance: bulletin Européen sur les maladies transmissibles= European communicable disease bulletin 2008. 13(7).
10. Medlock, J.M., et al., *A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options.* Vector-borne and zoonotic diseases 2012. 12(6): p. 435-447.
11. Hawley, W.A., *The biology of Aedes albopictus.* Journal of the American Mosquito Control Association. Supplement, 1988. 1: p. 1-39.
12. Dame, D. and T. Fasulo, *Mosquitoes.* Public Health Pesticide Applicator Training Manual 2003.
13. Rueda, L.M., *Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission* 2004, Walter Reed Army Inst Of Research Washington Dc Department Of Entomology.
14. Merritt, R., R. Dadd, and E. Walker, *Feeding behavior, natural food, and nutritional relationships of larval mosquitoes.* Annual review of entomology 1992. 37(1): p. 349-374.
15. Service, M., *Medical entomology for students* 2008: Cambridge University Press.
16. Savage, H., et al., *Host-feeding patterns of Aedes albopictus (Diptera: Culicidae) at a temperate North American site.* Journal of Medical Entomology 1993. 30(1): p. 27-34.
17. Bram, R.A., *Contributions to the mosquito fauna of southeast Asia-II. The genus Culex in Thailand (Diptera: Culicidae)* 1967, SMITHSONIAN INSTITUTION WASHINGTON DC.
18. Harrison, B.A. and J.E. Scanlon, *Medical Entomology Studies-II. The Subgenus Anopheles in Thailand (Diptera: Culicidae)(Contributions of the American Entomological Institute. Volume 12, Number 1)* 1975, WALTER REED ARMY INST OF RESEARCH WASHINGTON DC DEPARTMENT OF ENTOMOLOGY.
19. Rattanarithikul, R., et al., *Illustrated keys to the mosquitoes of Thailand I. Background; geographic distribution; lists of genera, subgenera, and species; and a key to the genera.* Southeast Asian Journal of Tropical Medicine and Public Health, 2005. 36(S1): p. 1.
20. Panthusiri, P., *ILLUSTRATED KEYS TO THE MOSQUITOES OF THAILAND IV. ANOPHELES.* The Southeast Asian Journal of Tropical Medicine and Public Health 2006. 37: p. 2.
21. Rattanarithikul, R., et al., *Illustrated keys to the mosquitoes of Thailand V. Genera Orthopodomyia, Kimia, Malaya, Topomyia, Tripteroides, and Toxorhynchites.* The Southeast Asian journal of tropical medicine and public health 2007. 38: p. 1-65.
22. Panthusiri, P., *ILLUSTRATED KEYS TO THE MOSQUITOES OF THAILAND VI. TRIBE AEDINI.* The Southeast Asian Journal of Tropical Medicine and Public Health 2010. 41: p. 1.
23. Jinbo, U., T. Kato, and M. Ito, *Current progress in DNA barcoding and future implications for entomology.* Entomological Science 2011. 14(2): p. 107-124.
24. Cywinska, A., F. Hunter, and P.D. Hebert, *Identifying Canadian mosquito species through DNA barcodes.* Medical and veterinary entomology 2006. 20(4): p. 413-424.
25. Hebert, P.D., A. Cywinska, and S.L. Ball, *Biological identifications through DNA barcodes.* Proceedings of the Royal Society of London B: Biological Sciences 2003. 270(1512): p. 313-321.
26. Manonmani, A., et al., *rDNA-ITS2 polymerase chain reaction assay for the sibling species of Anopheles fluviatilis.* Acta tropica 2001. 78(1): p. 3-9.
27. Singh, O., et al., *Differentiation of members of the Anopheles fluviatilis species complex by an allele-specific polymerase chain reaction based on 28S ribosomal DNA sequences.* The American journal of tropical medicine and hygiene 2004. 70(1): p. 27-32.
28. Kang, D. and C. Sim, *Identification of Culex complex species using SNP markers based on high-resolution melting analysis.* Molecular ecology resources 2013. 13(3): p. 369-376.
29. Pfeiler, E., et al., *Genetic diversity and population genetics of mosquitoes (Diptera: Culicidae: Culex spp.) from the Sonoran Desert of North America.* The Scientific World Journal 2013. 2013.
30. Low, V., et al., *Mitochondrial DNA analyses reveal low genetic diversity in Culex quinquefasciatus from residential areas in Malaysia.* Medical and veterinary entomology 2014. 28(2): p. 157-168.

31. Shepard, J.J., T.G. Andreadis, and C.R. Vossbrinck, *Molecular phylogeny and evolutionary relationships among mosquitoes (Diptera: Culicidae) from the northeastern United States based on small subunit ribosomal DNA (18S rDNA) sequences.* Journal of medical entomology 2006. 43(3): p. 443-454.
32. Saitou, N. and M. Nei, *The neighbor-joining method: a new method for reconstructing phylogenetic trees.* Molecular biology and evolution 1987. 4(4): p. 406-425.
33. Tamura, K., M. Nei, and S. Kumar, *Prospects for inferring very large phylogenies by using the neighbor-joining method.* Proceedings of the National Academy of Sciences of the United States of America 2004. 101(30): p. 11030-11035.
34. Tamura, K., et al., *MEGA6: molecular evolutionary genetics analysis version 6.0.* Molecular biology and evolution 2013. 30(12): p. 2725-2729.
35. Medeiros-Sousa, A.R., et al., *Diversity and abundance of mosquitoes (Diptera: Culicidae) in an urban park: Larval habitats and temporal variation.* Acta tropica 2015. 150: p. 200-209.
36. Maricopa, *Life cycle and information on Aedes aegypti mosquitoes* 2008.
37. Gubler, D.J., *Dengue, urbanization and globalization: the unholy trinity of the 21st century.* Tropical medicine and health 2011. 39(4SUPPLEMENT): p. S3-S11.
38. Murray, C.J., et al., *Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010.* The lancet, 2012. 380(9859): p. 2197-2223.
39. Fradin, M.S. and J.F. Day, *Comparative efficacy of insect repellents against mosquito bites.* New England Journal of Medicine 2002. 347(1): p. 13-18
40. Van den Berg, H., R. Velayudhan, and M. Ejov, *Regional framework for surveillance and control of invasive mosquito vectors and Re-emerging vector-borne diseases 2014–2020.* World Health Organization, 2013. 26.
41. Organization, W.H., *Yellow fever: rapid field entomological assessment during yellow fever outbreaks in Africa: handbook: methodological field approaches for scientists with a basic background in entomology* 2014.
42. ISDP, *Integrated Disease Surveillance Project Kerala State.* 2017. Directorate of Health service, District wise daily report (24-06-2017, 28-06-2107).
43. Laird, M., *The natural history of larval mosquito habitats* 1988: Academic Press Ltd.
44. Rueda, L.M., *Global diversity of mosquitoes (Insecta: Diptera: Culicidae) in freshwater.* Hydrobiologia 2008. 595(1): p. 477-487.
45. Organization, W.H., *Zika virus, microcephaly and Guillain-Barré syndrome situation report* 2016.
46. Ashfaq, M., et al., *Analyzing mosquito (Diptera: Culicidae) diversity in Pakistan by DNA barcoding.* PLoS One, 2014. 9(5): p. e97268.
47. Scholte, E.-J., W. Takken, and B.G. Knols, *Infection of adult Aedes aegypti and Ae. albopictus mosquitoes with the entomopathogenic fungus Metarhizium anisopliae.* Acta tropica 2007. 102(3): p. 151-158.
48. Jia, P., et al., *How does the dengue vector mosquito Aedes albopictus respond to global warming?* Parasites & vectors 2017. 10(1): p. 140.
49. Zettel, C. and P. Kaufman, *Yellow fever mosquito Aedes aegypti (Linnaeus)(Insecta: Diptera: Culicidae)* 2012, Citeseer.
50. Bova, J., S. Paulson, and G. Paulson, *Morphological Differentiation of the Eggs of North American Container-Inhabiting Aedes Mosquitoes.* Journal of the American Mosquito Control Association 2016. 32(3): p. 244-246.
51. Rios, L. and J.E. Maruniak, *Asian Tiger Mosquito, Aedes albopictus (Skuse)(Insecta: Diptera: Culicidae)* 2004, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.
52. Farajollahi, A. and D.C. Price, *A rapid identification guide for larvae of the most common North American container-inhabiting Aedes species of medical importance.* Journal of the American Mosquito Control Association 2013. 29(3): p. 203-221.

Date of Submission: 2017-08-11
Date of Acceptance: 2017-09-07

Symbiosis

www.symbiosisonline.org
www.symbiosisonlinepublishing.com

Research Article | SOJ Microbiology and Infectious Diseases | Open Access

Screening of a Few traditionally used Medicinal Plants for their Larvicidal Efficacy against *Aedes aegypti* Linn (Diptera: Culicidae), a Dengue Fever Vector

Anoopkumar AN[1], Sreedev Puthur[2], Sharrel Rebello[2] and Embalil Mathachan Aneesh[2*]

[1]*Department of Zoology, Christ College, Irinjalakuda, University of Calicut*

[2]*Communicable Disease Research Laboratory, Department of Zoology, St Joseph's college, Irinjalakuda*

Received: 21 July, 2017; Accepted: 30 August, 2017; Published: 06 September, 2017

***Corresponding author:** Embalil Mathachan Aneesh, Communicable Disease Research Laboratory, Department of Zoology, St Joseph's college, Irinjalakuda. Tel: +91 9400741861; Email: aneeshembalil@gmail.com*

Abstract

Mosquitoes are the significant group of insects as they cause morbidity and mortality to human beings by severe diseases, such as zika, malaria, filariasis, dengue fever, Japanese encephalitis, West Nile virus and chikungunya. According to World Health Organization draft on global vector control response, 2017 more than 80 percentage of global human populations were significantly infected by at least one vector-borne disease. This vector - borne diseases may induce social and economic burden over the globe. Herbal products with larvicidal properties have been used as natural insecticides for eco-friendly vector control programme. The larvicidal efficacy of nine medicinal plants collected from Wayanad district of Kerala, a part of Western Ghats, India was assessed against fourth instar larvae of *Aedes aegypti*. The larvicidal bio assay had been carried out according to WHO standard protocol with acetone, methanol, petroleum ether and water as extracts. Out of nine traditionally used medicinal plants screened, *Persicaria hydropiper* and *Plectranthus hadiensis* have significant larvicidal efficacy with LC_{50} 489.278 Mg/ L and LC_{50} 411.746 Mg/ L respectively against the fourth instar larvae of this dengue fever vector. Hence the aforesaid plants can be used as a potential natural insecticide against mosquitoes thus, to control mosquito borne diseases.

Keywords: *Aedes aegypti*; Vector mosquitoes; medicinal plant; larvicidal efficacy; plant extracts

Introduction

Mosquitoes (Diptera: Culicidae) have significant impact on human health, principally because they act as vectors for ruinous pathogens and parasites causing many severe infectious diseases such as dengue, chikungunya, malaria, West Nile virus and filariasis [1- 3]. Dengue fever recognized as the most severe mosquito-borne disease, responsible for medical and economic burden together with defeat in commercial [4-11]. The World Health Organization in 2017 reported that roughly 65 to 136 million people were infected by dengue fever in every year [12]. The genus *Aedes* are liable to transmit various arboviral diseases including dengue fever all over the world [13]. *Aedes aegypti* has been reported as the principal vector of chikungunya and dengue in the United States and other regions of tropical and sub tropical countries [14]. There are only a small number of vaccines are available to treat the pathogens transmitted by mosquitoes and the scientific community is yet to discover the vaccines for severe mosquito borne diseases including dengue [15]. However, fight against mosquito transmitting diseases is a challenging problem to public health [3,16].

Mosquito control – targeting its larvae remains the most effective approach to prevent various mosquito borne diseases [17]. Control of such ailments is ending up progressively troublesome be-reason for expanding resistance in mosquitoes to synthetic insecticides [18]. Plant derived products are safer than synthetic insecticides [19].

Several people use either synthetic or plant based repellents to protect them from mosquito transmitting diseases. Bed nets treated with insecticide and indoor residual spraying may also be used for preventing mosquito-borne diseases [3,17,20-23]. Active, frequent use of synthetic insecticides in farming and health programs may leads to various harmful results such as ecosystem destabilization, environmental pollution, hazardous effects to human beings and non target organisms [24,25].

Use of plant based insecticides against mosquitoes becoming a significant approach for the prevention of various mosquito transmitted diseases because of a number of advantages rather than artificial repellents [26,27].

Plant derived products with insecticidal activity have been used in the recent years to control different types of vectors [28].Various methods have been implicated to control mosquito population. One of the methods to prevent mosquito transmitting disease is by killing its larvae at larval stage. The modern mosquito control method is based on artificial insecticides [29].

World Health Organization in 2008 reported that 80 percentage of population of some countries in Asia and Africa may use traditional medicines to cure various diseases due to monetary and environmental constrain. Traditional medicines

 * **Corresponding author email:** *aneeshembalil@gmail.com*

are used to maintain health by preventing various severe diseases based on knowledge, experience and practice [30].

Extracts of plants constitute various bio active phyto compounds; hence they can be used as alternative approach to mosquito larval control. Many scientific studies have proven that the plant extracts or plant derived products can be used as an alternate approach to control mosquito population [31-33].

Various vector borne diseases can be prevented by means of traditionally used medicinal plants. Hence, the demand for traditional medicines is enhancing as they are usually recognized to be bio degradable, natural, safer than synthetic drugs [34]. Thus, searching for natural insecticides is of greatest significance in vector control. This study focused on screening of few traditionally used medicinal plants for their larvicidal efficacy against dengue fever vector Aedes aegypti to develop an efficient, natural, biodegradable insecticide of plant origin.

Methods

Plant Material

Fresh leaves of nine traditionally used medicinal plants were collected from different regions of Thirunelly (11°53'N, 76°0'E), Wayanad a part of Western Ghats, Kerala, India. The collection was performed during their dynamic growing season, June to September (monsoon season).

Preparation of Extracts

Fresh leaves of nine medicinal plants were collected and shade dried (Table 1). The dried plant materials were ground to fine powder using a mechanical grinder and proceeded for soxhlet extraction using different solvents such as petroleum ether, methanol acetone and water. The extracts obtained were evaporated and used for further study.

Table 1: List of plants collected for bioassay from Thirunelly,Western Ghats, Kerala, India

S No.	Plant Name	Family	Local Name	Parts used	GPS	
					Latitude	Longitude
1	***Sphaernthus indicus***	Asteraceae	Adakkamaniyan	Seed	11°53’29.20”N	76° 0’59.00”E
2	***Hydrocotyle javanica***	Apiaceae	Eranga	Leaf	11°53’29.50”N	76° 0’34.20”E
3	***Deris trifoliate Lour***	Fabaceae	Thuduthuduppankayi	Leaf	11°53’29.40”N	76° 0’34.20”E
4	***Persicaria hydropiper***	Polygonaceae	Kovvanenji	Leaf	1°53’25.00”N	76° 0’50.40”E
5	***Acanthospermum hispidum***	Asteraceae	Nherinjil	Leaf	11°53’27.80”N	76° 0’47.80”E
6	***Drymaria cordata***	Caryophyllaceae	Odivally	Leaf	11°53’21.14”N	76° 0’59.64”E
7	***Toddelia asiatica***	Rutaceae	Narinarakam	Leaf	11°53’7.30”N	76° 0’38.90”E
8	***Plectranthus hadiensis***	Lamiacea	Bhaya	Leaf	11°53’23.00”N	76° 1’1.90”E
9	***Triumfetta rhomboidea***	Malvaceae	Kodithoova	Leaf	11°53’27.64”N	76° 0’47.59”E

Mosquito’s Culture

Aedes aegypti were reared in the Communicable Disease Research Laboratory, Department of Zoology, St Josephs College, Irinjalakuda. The larvae were maintained and fed with dog biscuit and yeast in the ratio 3:1. Adult mosquitoes were provided with 10% sucrose solution and young chick was kept within the cage to offer blood meal. Mosquitoes were held at 27 ± 2°C and 75–85% relative humidity, with 12:12 Light and dark photoperiod cycle.

Larvicidal Bioassay

Larvicidal bioassay of plant extracts were tested against fourth instar larvae of *Aedes aegypti*. The tests were conducted in glass beakers with WHO standard protocol. Larvicidal efficacy was tested against fourth instar larvae of *Aedes aegypti* using petroleum ether, methanol, acetone and water extracts of the plant material (1000 mg/L). A set of control groups were included for each test. 1 ml of petroleum ether, acetone, methanol and water was mixed separately with 249ml of distilled water for control groups. Twenty five healthy larvae were released in each glass beaker and mortality was observed after 24 hours of exposure. The dead larvae in replicates were pooled and percentage of larval moratlity was calculated. The larvicidal bioassay was performed at 27 ± 2°C and 75–85% relative humidity, with 12:12 Light and dark photoperiod cycle.

Statistical Analysis

Larval mortality was calculated in percentage and if the control mortality was ranged from 5-20%, it was corrected using Abbott’s formula [35].

$$corrected\ mortality = \frac{\%\ test\ \ mortality - \%\ control\ mortality}{100 - \%\ control\ mortality} \times 100$$

LC_{50} and LC_{90} values for different solvent extract such as petroleum ether, methanol, acetone and water was calculated by using IBM SPPSS Version.24.

Results

Results for screening of the nine traditionally used medicinal plants against *Aedes aegypti* fourth instar larvae were shown in Table 2. The highest mortality was observed in leaf extract of two plant species such as *Persicaria hydropiper* and *Plectranthus hadiensis*. The petroleum ether extract of *Plectranthus hadiensis* showed promising larvicidal efficacy with LC_{50} - 411.746 mg/L against fourth instar larvae of *Aedes aegypti* (Table 3). However, methanol, acetone and water extracts of the same may also showed considerable larval mortality. Methanol extract of *Persicaria hydropiper* also showed significant larvicidal property with LC_{50}-489.278 mg/L against *Aedes aegypti* (Table 3). Besides this, petroleum ether, acetone and water extracts of *Persicaria hydropiper* exhibit substantial larvicidal property against fourth instar larvae of *Aedes aegypti.* The medicinal plant *Sphaernthus indicus* showed less mortality in Petroleum ether, methanol, Acetone and Water extract against *Aedes aegypti*. Similarly the other medicinal plant species such as *Sphaernthus indicus*, *Hydrocotyle javanica, Deris trifoliate Lour, Acanthospermum hispidum Drymaria cordata, Toddelia Asiatic* and *Triumfetta rhomboide* showed relativiely less mortality against *Aedes aegypti* fourth instar larvae. Among the nine plants investigated, maximum larval mortality was obtained from the petroleum ether and methanol extract of *Plectranthus hadiensis and Persicaria hydropiper* respectively.

Table 2: Screening of plant extracts for larvicidal activity against *Aedes aegypti* at 1000 mg/L concentration

S No.	Plants tested	Petroleum ether	Acetone	Methanol	Water
1	***Sphaernthus indicus***	-	-	-	-
2	***Hydrocotyle javanica***	-	-	-	-
3	***Deris trifoliate Lour.***	-	-	-	-
4	***Persicaria hydropiper***	+	+	+	+
5	***Acanthospermum hispidum***	-	-	-	-
6	***Drymaria cordata***	-	-	-	-
7	***Toddelia asiatica***	-	-	-	-
8	***Plectranthus hadiensis***	+	+	+	+
9	***Triumfetta rhomboidea***	-	-	-	-

+ above 50 percent mortality at 24 hr, - No larval mortality

Table 3: Larvicidal activity of plant extracts against fourth instar larvae of *Aedes aegypti* after 24 h exposure (mg/L)

Plants	Acetone		Methanol		Petroleum ether		Water	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Persicaria hydropiper	658.005 (422.174-1625.366)	5572.689 (2027.987-143992.195)	489.278 (316.218-962.289)	4121.964 (1651.772-68018.777)	750.326 (494.333-1763.542)	5023.5 (2013.139-72991.339)	773.69 (469.252-2767.500)	8120.726 (2434.857-759740.251)
Plectranthus hadiensis	432.238 (262.951-874.953)	4555.766 (1662.711-148706.043)	500.655 (336.181-903.638)	3452.934 (1544.054-31689.036)	411.746 (285.842-633.040)	2250.134 (1194.474-10160.482)	485.791 (295.213-1112.471)	5512.748 (1857.242-303616.247)

Discussion

Natural insecticides are safer than synthetic ones as they posses little chance of developing insecticide resistance [1]. Vector control at its larval stage is the significant option as they are slow mobile and their habitats can easily be recognized [36]. However, frequent use of synthetic insecticides for mosquito control may leads to various harmful effects to human beings and non target organisms [24,25]. Hence, the demand for plant based products is enhancing as they are usually recognized to be bio degradable, natural, and safer than synthetic drugs [34].

Extracts of plants contain various active phyto compounds; hence they can be used as alternative approach to mosquito larval control. Many scientific studies have proven that plant derived products can be used as an alternate approach to control mosquito population [31,32].

The finding of the present investigation indicated that *Persicaria hydropiper* can be used as an alternative to synthetic insecticides to control mosquito transmitting diseases. The

petroleum ether, methanol, acetone and water extract of *Persicaria hydropiper* showed highest mortality against fourth instar larvae of *Aedes aegypti*. Similarly *Plectranthus hadiensis* showed potential mortality againt fourth instar larvae of *Aedes aegypti.* The remaining plant species such as *Sphaernthus indicus, Hydrocotyle javanica, Deris trifoliate Lour, Acanthospermum hispidum Drymaria cordata, Toddelia asiatica, Triumfetta rhomboidea* comparitvely showed less mortality than the other two plant species.

The present investigation obviously proved that the leaf extracts of *Plectranthus hadiensis* and *Persicaria hydropiper* has remarkable larvicidal property against *Aedes aegypti* vector mosquitoes. The flora vegetation of India has prosperous aromatic plant diversity; hence they can be used for the development of natural insecticides for controlling mosquito population and to prevent mosquito transmitting diseases. The results from the present investigation might encourage the search for novel, natural insecticides offering an alternative to synthetic insecticeds from traditionally used medicinal plants. The leaf extracts of *Plectranthus hadiensis* and *Persicaria hydropiper* have the potential to be used as an ideal approach for the mosquito control programmes.

Acknowledgements

We thank Principal, St. Joseph's College, Irinjalakuda for the laboratory facilities provided. We are greatly thankful to University Grants Commission (UGC), New Delhi for providing funds under UGC Major Research Project (F.NO.42-609/2013 (SR). We also thank Kerala State Council for Science, Technology and Environment (KSCSTE) for funds under Major Research Project (No.27/SRSLS/2013/CSTE).

References

1. Ghosh A, Chowdhury N, Chandra G. Plant extracts as potential mosquito larvicides. Indian J Med Res. 2012;135(5):581-598.
2. Mehlhorn H. Encyclopedia of parasitology, 3rd edn. Springer, Heidelberg; 2008.
3. Mehlhorn H, Al Rasheid KAS, Al-Quraishy S, Abdel-Ghaffar F. Research and increase of expertise in arachno-entomology are urgently needed. Parasitol Res. 2012;110(1):259-265. doi: 10.1007/s00436-011-2480-7
4. Anders KL, Nguyet NM, Chau NV, Hung NT, Thuy TT, Lien le B, et al. Epidemiological factors associated with dengue shock syndrome and mortality in hospitalized dengue patients in Ho Chi Minh City, Vietnam. Am J Trop Med Hyg. 2011;84:127-134. doi:10.4269/ajtmh.2011.10-0476
5. Coudeville L, Shepard DS, Zambrano B, Dayan G. Dengue economic burden in the Americas: estimates from dengue illness. Am J Trop Med Hyg. 2009;81:127-127.
6. Beaute J, Vong S. Cost and disease burden of dengue in Cambodia. BMC Public Health. 2010;10:521. doi: 10.1186/1471-2458-10-521
7. Shepard DS, Coudeville L, Halasa YA, Zambrano B, Dayan GH. Economic impact of dengue illness in the Americans. Am J Trop Med Hyg. 2011;84(2):200-207. doi: 10.4269/ajtmh.2011.10-0503
8. Halasa YA, Shepard DS, ZengW. Economic cost of dengue in Puerto Rico. Am J Trop Med Hyg. 2012;86(5):745-752. doi: 10.4269/ajtmh.2012.11-0784
9. Mogasale V, Maskery B, Lim J, Thiem VD, Tho LH, Anh DD, et al. Economic evi- dence for policy makers in Vietnam: dengue fever economic burden of disease and household willingness to pay for dengue vaccines. Int J Infect Dis.2012;16:e310. doi:10.1016/j.ijid.2012.05.1005
10. Wettstein ZS, Fleming M, Chang AY, Copenhaver DJ, Wateska AR, Bartsch SM, et al. Total economic cost and burden of dengue in Nicaragua: 1996-2010. Am J Trop Med Hyg. 2012;87(4):616-622. doi: 10.4269/ajtmh.2012.12-0146
11. Ali KM, Asha A V, Aneesh EM. Bioecology and Vectorial Capacity of Aedes mosquitoes (Diptera : Culicidae) in Irinjalakuda Municipality , Kerala , India in Relation to Disease Transmission. Int J Curr Res Acad Rev. 2014;2(4):43-49.
12. WHO. Global Vector Control Response - Background document to inform deliberations during the 70th session of the World Health Assembly. 2017.
13. Division of Vector-Borne Disease. Dengue and Aedes aegypti mosquito. San Juan. 2012.
14. Surveillance and Control of Aedes aegypti and Aedes albopictus in the United States. Division of Vector-Borne Diseases: Centers for Disease Control. 2016.
15. Farag SA, Osama H, Mohamed R, Mohamed H. Development of longer-lasting repellence cellulosic based curtain fabrics. Material Sciences and Applications. 2011;2(3):200-208. doi:10.4236/msa.2011.23025
16. Jensen M, Mehlhorn H. Seventy-five years of Resochin in the fight against malaria. Parasitol Res. 2009;105(3):609-627. doi: 10.1007/s00436-009-1524-8
17. Amer A, Mehlhorn H. Larvicidal effects of various essential oils against Aedes, Anopheles,and Culex larvae (Diptera, Culicidae). Parasitol Res. 2006;99(4):466-472.
18. Ranson H, Rossiter L, Ortelli F, Jensen B, Wang X, Roth CW, et al. Identification of a novel class oil of insect glutathione S- transferase involved in resistance to DDT in the malaria vector Anopheles gambiae. Biochm J. 2001;359:295-304.
19. Bowers WS. Biorational approaches for insect control. Korean J Appl Entomol. 1992;31(3):289-303.
20. Mehlhorn H, Schmahl G, Schmidt J. Extract of the seeds of the plant Vitex agnus castus proven to be highly efficacious as a repellent against ticks, fleas,mosquitoes and biting flies. ParasitolRes. 2005;95(5):363-365.
21. Amer A, Mehlhorn H. The sensilla of Aedes and Anopheles mosquitoes and their importance in repellency. Parasitol Res. 2006;99(4):491-499.
22. Lees RS, Knols B, Bellini R, Benedict MQ, Bheecarry A, Bossin HC et al. Review: improving our knowledge of male mosquito biology in relation to genetic control programmes. Acta Trop. 2014 Apr;132 Suppl:S2-11. doi: 10.1016/j.actatropica.2013.11.005
23. Benelli G .Research inmosquito control: current challenges for a brighter future. Parasitol Res. 2015;114(8):2801-2805. doi: 10.1007/s00436-015-4586-9

24. Jirakanjanakit N, Rongnoparut P, Saengtharatip S, Chareonviriyaphap T, Duchon S, Bellec C, et al. Insecticide susceptible/resistance status in Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus (Diptera: Culicidae) in Thailand during 2003–2005. J Econ Entomol. 2007;100(2):545-550.

25. Sarwar M, Ahmad N,Toufiq M. Host plant resistance relationships in chickpea (Cicer arietinum L) against gram pod borer (Helicoverpa armigera Hubner). Pak J Bot. 2009;41:3047-3052.

26. Moore SJ, Lenglet AD. An overview of plants used as insect repellents. In Traditional Medicinal Plants and Malaria. CRC Press. 2004;344-363.

27. Maia MF, Moore SJ. Plant-based insect repellents: a review of their efficacy, development and testing. Malar J. 2011;10 Suppl 1:S11. doi: 10.1186/1475-2875-10-S1-S11

28. Sreedev P, Aneesh EM. A Comparative Analysis Of Larvicidal Efficacy Of Piperlongum And Ruta Graveolens Against Aedes Aegypti A Dengue Virus Vector. Asia Pac j res. 2016;1(36):200-205.

29. Liu H, Xu Q, Zhang L, Liu N. Chlorpyrifos resistance in mosquito Culex quinquefasciatus J Med Entomol. 2005;42(5):815-820.

30. WHO World Health Organization. Traditional medicine. Fact Sheet. 2008.

31. Sharma P, Mohan L, Srivastava CN. Phytoextract-induced developmental deformities in malaria vector. Bioresour Technol. 2006;97(14):1599-1604.

32. Amer A, Mehlhorn H. Larvicidal effects of various essential oils against Aedes, Anopheles,and Culex larvae (Diptera, Culicidae). Parasitol Res. 2006;99(4):466-472.

33. Vinu Rajan PK, Sreedev P, Misvar Ali K, Aneesh EM. Larvicidal Efficacy of Piper Longum Fruit Extract against Aedes Aegypti, a Dengue Fever Vector. 2015;4(1):123-127.

34. Abd Kadir SL, Yaakob H, Mohamed Zulkifli R. Potential anti-dengue medicinal plants: A review. J Nat Med. 2013;67(4):677-689. doi: 10.1007/s11418-013-0767-y

35. Abbott WS. A method of computing of the effectiveness of an insecticide. J Econ Entomol. 1925;8(2):265-267.

36. Ghosh A, Chowdhury N, Chandra G. Plant extracts as potential mosquito larvicides. Indian J Med Res. 2012;135(5):581-598.

Refining Biomass Residues for Sustainable Energy and Bioproducts

Technology, Advances, Life Cycle Assessment, and Economics

Edited by
R. Praveen Kumar
Edgard Gnansounou
Jegannathan Kenthorai Raman
Gurunathan Baskar

Refining Biomass Residues for Sustainable Energy and Bioproducts

Technology, Advances, Life Cycle Assessment, and Economics

Edited by

R. Praveen Kumar

Department of Biotechnology, Arunai Engineering College, Tiruvannamalai, India

Edgard Gnansounou

Bioenergy and Energy Planning Research Group, Swiss Federal Institute of Technology Lausanne, Lausanne, Switzerland

Jegannathan Kenthorai Raman

Bioenergy and Energy Planning Research Group, Swiss Federal Institute of Technology Lausanne, Lausanne, Switzerland

Gurunathan Baskar

Department of Biotechnology, St. Joseph's College of Engineering, Chennai, India

ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

Notices
Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-818996-2

Publisher: Brian Romer
Acquisition Editor: Raquel Zanol
Editorial Project Manager: Michelle W. Fisher
Production Project Manager: Nirmala Arumugam
Cover Designer: Greg Harris

Typeset by MPS Limited, Chennai, India

Comparative life-cycle analysis of synthetic detergents and biosurfactants—an overview

23

Sharrel Rebello[1], *A.N Anoopkumar*[1,2], *Raveendran Sindhu*[3], *Parameswaran Binod*[3], *Ashok Pandey*[4] *and Embalil Mathachan Aneesh*[1]
[1]Communicable Disease Research Laboratory, St Joseph's College, Irinjalakuda, India, [2]Department of Zoology, Christ College, University of Calicut, Irinjalakuda, India, [3]Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum, India, [4]Centre for Innovation and Translational Research, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, India

23.1 Introduction

The realm of surfactants has marked its presence in every aspect of human life right from household, cosmetics, chemical industries, agriculture, biotechnology, petrochemicals, pharmaceuticals, etc. (Rebello et al., 2014; Rodrigues et al., 2006). Their range of utility extended from the soft hygiene-oriented toothpastes and soaps to harsh and toxic pesticides with a myriad of varieties, brands, and activity levels. As per statistical reports, the surfactant market is expected to reach a targeted economy of $44.9 billion by 2022 (https://www.prnewswire.com/news-releases/the-global-surfactant-chemical-and-material-market-should-reach-449-billion-by-2022300580229.html).

Irrespective of its wide utility in human life, surfactants have also gained more attention on the impacts it leaves off during synthesis, use, and its disposal. A critical analysis of published reports indicates that surfactants have greatly depleted and harmed the macro as well as microbiota of the aquatic and terrestrial environment (Rebello et al., 2014; Susmi et al., 2010; Cserhati et al., 2002; Azizullah et al., 2012). The high level of toxicity of surfactants is always associated with its unwise disposal to adjoining water bodies, thereby ascertain the need of pretreatment of surfactant effluents before their disposal (Ivanković and Hrenović, 2010; Bandala et al., 2008). Apart from the environment, humans are also seriously affected by the aftermaths of surfactant pollution (Enomoto et al., 2007; Hrabak et al., 1982); but they are often unaware of the hidden consequences due to ignorance or anesthetic agents that mask the exact level of impacts (Azizullah et al., 2012; Vian et al., 1995).

The complexity and disposal strategy of each kind of surfactant varies and depends on its exact chemistry (Pletnev et al., 2001). In such a scenario of toxicity

Refining Biomass Residues for Sustainable Energy and Bioproducts. DOI: https://doi.org/10.1016/B978-0-12-818996-2.00023-5

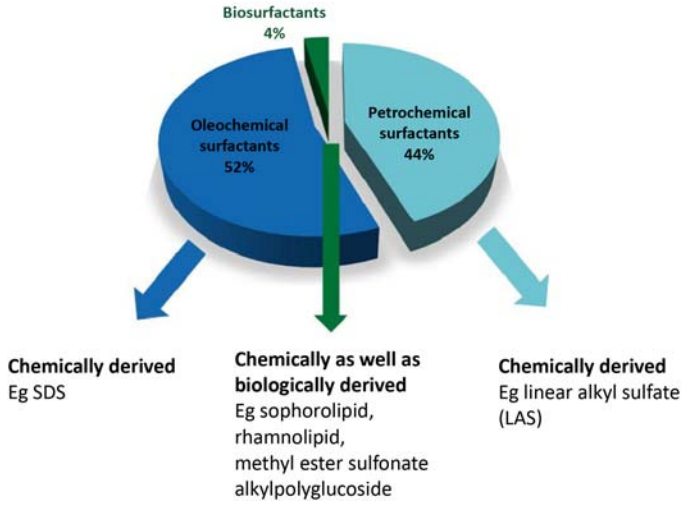


Figure 23.1 An overview of surfactants used in the market based on their origin.

induced by synthetic detergents the use of eco-friendly, green, and biologically derived biosurfactants have been promoted (Marchant and Banat, 2012). Generally, surfactants can be generated by one of the three routes, namely, petrochemicals (from petroleum products), oleochemicals (from plant oils), or biosurfactants (plant or microbial) as depicted in Fig. 23.1. The use of petrochemical- and oleochemical-derived surfactants has gained predominance over biosurfactants due to their economic feasibility compared to biosurfactants. However, the cost of environmental pollution induced by the former two processes in causing increased greenhouse gas emission, global warming, ozone depletion, etc. is highly significant. The current chapter thus targets the life-cycle analysis (LCA) of different surfactant systems and outlays the process of LCA at a whole.

23.2 Life-cycle analysis—an overview

LCA of any process plays a significant role in assessing the entire process of any synthetic as well as nonsynthetic production right from the raw materials, stages of production, and finally the aftermaths of the production on the environment as depicted in Fig. 23.2. In a true sense, all the effects and impacts that can be associated with all factors contributing to the birth of a product, distribution, and its recycling can be assessed (Frischknecht and Krewitt, 2007). The technical frame or steps to be obeyed in conducting an LCA analysis has been critically reviewed by the environmental protection agencies to get a clear idea on different essential steps in the better evaluation of any production process (Madsen et al., 2001; Union, 2010).

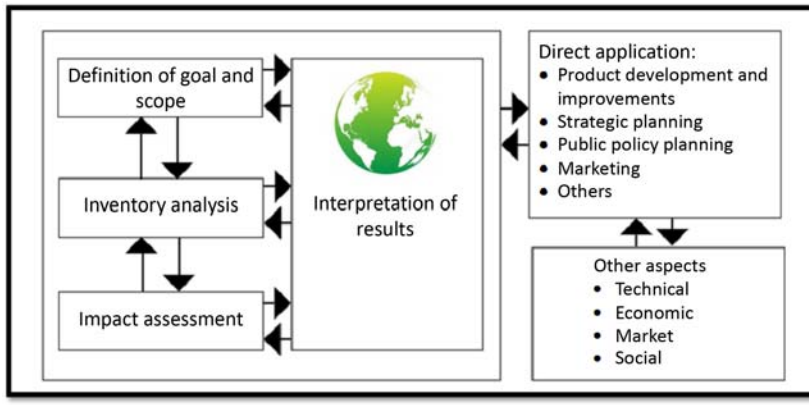


Figure 23.2 Life-cycle analysis, an overview of different phases.

However, all of them follow the standard methodologies specified by the International Organization for Standardization (ISO) in ISO 14040 and 14044 (Finkbeiner et al., 2011).

Generally, the LCA of any process includes defining the goal of the analysis, inventory analysis, impact analysis, and improvement analysis (Rebitzer et al., 2004). The first and foremost step of defining the goal explains the necessity to conduct an LCA for a process or product, the involved functional units, boundaries, and depth of data provided in the study (Curran, 2017). This would span the various stages of raw material acquisition, their bulk processing, product fabrication; packaging as well as transported distribution of the finished product; and the final stage of reuse, recycle, or disposal of associated products. The impact analysis of various processes includes different categories, such as climate change, ozone layer depletion, eutrophication, acidification, toxicology on human health and ecosystems, exhaustion of resources, water use, land use, and noise (Rebitzer et al., 2004). As a whole, LCA involves the total effect of different inputs or raw materials used in the process (even if they are synthesized in different locations), the effects induced by the process as well as the consequences till the disposal of the product or its component (https://prezi.com/ihie2hajsseo/life-cycle-analysis-for-laundry-detergent/).

23.3 Merits and demerits of life-cycle analysis

LCA, sometimes nicknamed the "cradle to grave" and sometimes restricted to "cradle to gate" analytical approach of production systems, broadens the boundaries of environmental impact assessment (La Rosa, 2016; Laurent et al., 2016). The basic step of representing laying down every small step in the forms of figures and flowcharts, enlisting the energy costs, associated emissions, generated by-products as well as risk factors throughout the process enables to critically evaluate the economic and environmental viability of a process.

The essentiality of LCA to identify, evaluate, and overcome the aftermaths of different production systems—chemical, physical and biological—has gained relevance with the increasing environmental awareness and ever increasing instances of natural disasters (Kellenberger and Althaus, 2009). The UN Earth Summit held at 1992 envisaged the significance of critically evaluating every input and output factor of a process to lay down the ups and downs associated with every industrial process (Jensen, 1992). Such an analysis would enable to identify the critical risk factors linked with any procedure and compare the suitability of different techniques. Moreover, such an analysis could also direct our efforts and research to the improvement and development of alternate eco-friendly and sometimes cost-effective strategies leading to productive results (Seppälä et al., 2001; Miettinen and Hamalainen, 1997).

Lack of expertise in LCA, unreliability of data due to quality as well as regional variations, high cost of LCA, restricted or unfriendly access to different LCA methods, and inadequate provision to address unexpected risk events apart from normal operative conditions often nullify or diminish the advantages of LCA (DEAT, 2004; Finnveden, 2000). The environmental conditions in different continents vary and thus generalization of any process would be unwise. As noted in a study on LCA of household detergents in Europe, the high-energy requirement attributed to heat water during washing is not found in tropical countries. Therefore the association of increased risk factors with use rather than the production of detergents could be neglected in a different country. Furthermore, drastic environmental depleting effects of various products used in the production and disposal of xenobiotics would thus gain light than the factors, such as increased energy requirement. It is also true that even secondary factors, such as washing machines, could drastically contribute to the LCA (Cullen and Allwood, 2009). Another drawback associated with LCA is the inconsistency in the data provided in various LCA, making it difficult to compare the data of various studies even in a particular geographical continent (Curran, 2014).

23.4 Industrial production and life-cycle analysis of synthetic surfactants

The industrial synthesis of surfactants mostly depends on the chemical synthesis or modification of different petroleum derived by-products to surface active molecules. The individual surfactants are generally classified as anionic, cationic, nonionic, and amphoteric surfactants based on their overall charge. The synthesis of individual surfactants might include simple steps such as that of triglyceride with NaOH as observed in soaps, or multistep mediated as observed in the synthesis of linear alkylbenzene sulfonate (Huang, 2008). The latter is synthesized by the addition of benzene on chlorinated paraffin in presence of hydrochloric acid and further sulfonation with sulfur trioxide or sulfuric acid. Table 23.1 summarizes the steps involved in the chemical synthesis of common synthetic detergents. The most

Table 23.1 Chemical synthesis of surfactants an overview.

Name of the surfactant	Type of surfactant	Process in brief
Alcohol sulfate (FAS)	Anionic	Sulfation of alcohol
Alcohol ether sulfate	Anionic	Fatty acid alcohol ethoxylated and sulfated
Alkylbenzene sulfonate	Anionic	Chlorinated paraffin reacted with benzene in HCl and sulfonated
SAS	Anionic	Paraffin sulfonated with sulfur trioxide, olefin obtained by ethylene polymerization
α-Olefin sulfonates	Anionic	α-Olefin sulfonated with sulfur trioxide
MES	Anionic	Methylester of a fatty acid sulfonated
Isothiocyanates	Anionic	Fatty acid reacted with sodium isothiocyanate
AEO	Nonionic	Alcohol (fossil or renewable) reacted with ethene oxide
APEO	Nonionic	Olefin reacted with phenol and then ethane oxide
APG	Nonionic	Saccharide with fatty acid
Fatty acid ethoxylate	Nonionic	Reaction of fatty acid with ethane oxide or polyethylene glycol
Quaternary ammonium salts	Cationic	Reaction of tertiary amine and methyl chloride or dimethylsulfate
Betaine	Amphoteric	Reaction between tertiary amine and monochloroacetic acid together with sodium hydroxide
Sulfobetain	Amphoteric	Addition of epichlorohydrin to tertiary amine followed by sulfation

SAS, Secondary alkane sulfonates; *MES*, methyl ester sulfonate; *AEO*, alcohol ethoxylate; *APEO*, alkylphenolethoxilate; *APG*, alkylpolyglucoside.

commonly used laundry detergents are often produced as a mixture or combinations of a wide array of surfactant molecules along with granular enzymes to increase their cleaning efficiency. Fig. 23.3 depicts LCA in the synthesis of linear alkylbenzene sulfonate (LAS).

LCA of six different household detergents used in Europe was done exempting environmental and human toxicity impacts from the study (Golsteijn et al., 2015), thereby questioning the true impact of the LCA in a true sense. As per the study, the use of detergent and the source of ingredients are found to be major environmental contributors than processes of manufacture, transport, and disposal of detergents in terms the energy used and water used for processing (Saouter and Hoof, 2002). The high-energy demand of detergent use is due to the necessity to increase the heat of the water in European countries and almost 98% of the biological oxygen demand is contributed by the disposal.

Table 23.2 enlists some of the LCAs conducted on surfactants. The LCA of detergent builders, such as sodium tripolyphosfate (STPP) and zeolites, indicate that their effect on the environment is less in relation to the benefits they provide to different sectors, also discouraging environment harmful detergent builders, such as nitrilotriacetic acid (Morse et al., 1995). However, STPP the major phosphate-based compound in detergents greatly contributes to eutrophication characterized by large-scale algal development, leading to a ban in their use in detergents at least in some countries (Singh et al., 2014). Thus the choice of less toxic and degradable inputs for detergent synthesis also is relevant, thereby leading to the development of various phosphate-free environment-friendly surfactant candidates. The LCA of LAS, one of the predominantly used synthetic anionic surfactants, has been found to drastically affect the production of fossil fuels, alter the extent of land usage, and contribute generation of respiratory inorganics (Thannimalay and Yusoff, 2014).

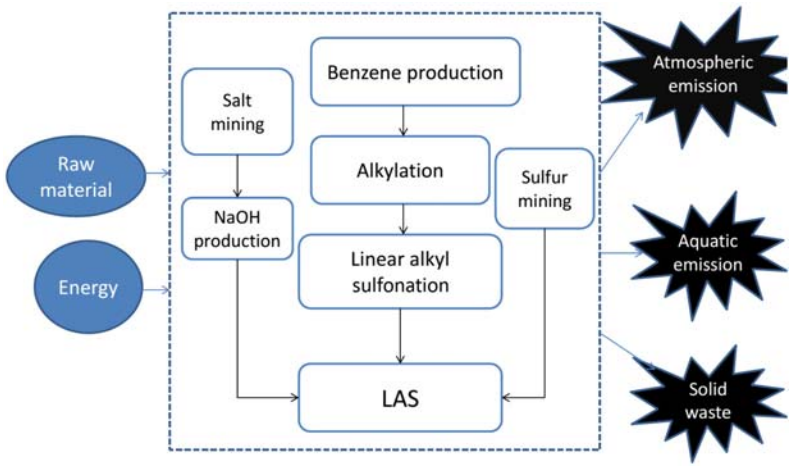


Figure 23.3 LCA inventory for synthesis of synthetic detergent linear alkylbenzene sulfonate. *LAS*, Linear alkylbenzene sulfonate; *LCA*, life-cycle analysis.

Table 23.2 List of life-cycle analysis conducted on surfactants as examples.

Name of the surfactant	Type of surfactant	Reference
STPP, Zeolite, NTT	Detergent builders	Morse et al. (1995)
Zeolite A	(detergent builder)	Fawer et al. (1998)
Laundry detergents	Database of different detergents	Saouter and Hoof (2002)
Laundry detergents	Both petrochemical-based and oil-based detergents	Schowanek et al. (2018)
Methyl ester sulfonates	Palm oil based	Zolkarnain et al. (2016)
Alkylpolyglucoside	Plant oil-based biosurfactant	Guilbot et al. (2013)

The use of gas to liquid-based methods for linear alkylbenzene synthesis compared to conventional methods is found to emit considerably fewer quantities of greenhouse gases thereby reducing its effect on global warming (Forman et al., 2014).

23.5 Industrial production and life-cycle analysis of biosurfactants

Biosurfactants are regarded as the green surfactants owing to their eco-friendliness, biodegradability, and least toxicity compared to synthetic surfactants. Biosurfactants can be divided as two, namely, the ones produced using the plant-derived fatty acid and microbially derived biosurfactants. Biosurfactant synthesis involves various steps as shown in Fig. 23.4.

A study on the synthesis of palm oil−based methyl ester sulfonates (MES) indicated that most impact categories include fossil fuel, respiratory organic compounds, and climate change and they were found to be comparatively less when compared to the impact caused by conventional petroleum-based production techniques (Zolkarnain et al., 2016). In a similar context, the comparative LCA of LAS (synthetic detergent) and MES revealed that the latter was found to be more eco-friendly in terms of significant risk factors associated with its production (Thannimalay and Yusoff, 2014).

A relative analysis of different biosurfactants, such as rhamnolipids and sophorolipids, indicates that the major contributors to environmental impact include air emissions, electricity, and thermal requirements (Kopsahelis et al., 2018). The production of acetylated sophorolipids is found to be much more efficient than sophorolipids in terms of the toxicity associated with it (Baccile et al., 2016). The LCA on the green synthesis of oil-based alkylpolyglucoside indicated that phases of its formulation and terminal use played a critical role in its environmental impact contribution of 15% and 51%, respectively (Guilbot et al., 2013). The study also laid forward the environmental benefits of replacing glass bottles instead of plastic.

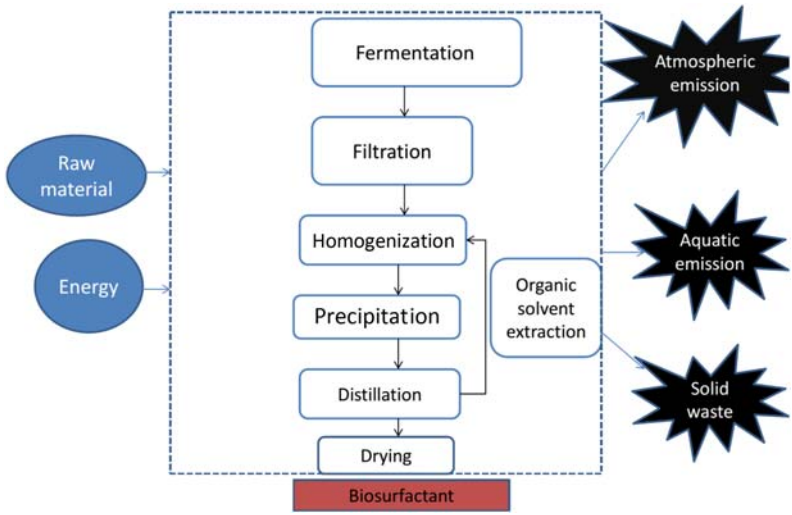


Figure 23.4 LCA inventory for synthesis of biosurfactant from a microbe. *LCA*, Life-cycle analysis.

The use of microbial consortium of *Azotobacter vinelandii* and *Pseudomonas* sp. in biosurfactant production indicated that evolved 4545 ± 817.93 g CO_2 in terms of g CO_2/1000 kg biosurfactant, a global warming potential of 0.046 t/1000 kg biosurfactant and a eutrophication potentials of 0.008 t/1000 kg and 0.0014 tonnes/1000 kg of biosurfactant (Aru and Onwurah, 2018). The biosynthesis of biosurfactant alkanolamide over conventional sodium methylate−based alkanolamide revealed that the former was more energy efficient (Adlercreutz et al., 2010). However, the former was 1.4 times costlier than conventional alkanolamide synthesis mainly attributed due to the use of immobilized lipases in the biosynthetic process.

23.6 Conclusion

The relevance of LCA obtaining a clear and close look at the impact of a process is well evident from concurrent research. However, the reliability of such analysis greatly relies on the effective inclusion of both environmental and human toxicity impact factors. The choice of suitable proxy data and efficient LCA analytical expertise becomes very essential. The chapter came to a conclusion that LCA favored biosurfactant production than the synthetic detergents. Thus the choice of better and eco-friendly solutions to surfactant synthesis should be adopted for the proper environmental sustainability.

Acknowledgment

Raveendran Sindhu acknowledges the Department of Science and Technology for sanctioning a project under DST WOS-B scheme.

References

Adlercreutz, D., et al., 2010. Alkanolamide biosurfactants: techno-economic evaluation of biocatalytic versus chemical production. Ind. Biotechnol. 6 (4), 204−211.

Aru, O.O., Onwurah, I.N., 2018. Life cycle assessment of the environmental impact of biosurfactant production from oil waste by a diculture of *Azotobacter vinelandii* and *Pseudomonas* sp. J. Bioremediat. Biodegrad. 9, 435.

Azizullah, A., et al., 2012. Chronic toxicity of a laundry detergent to the freshwater flagellate *Euglena gracilis*. Ecotoxicology 1−8.

Baccile, N., et al., 2016. Development of a cradle-to-grave approach for acetylated acidic sophorolipid biosurfactants. ACS Sustain. Chem. Eng. 5 (1), 1186−1198.

Bandala, E.R., Velasco, Y., Torres, L.G., 2008. Decontamination of soil washing wastewater using solar driven advanced oxidation processes. J. Hazard. Mater. 160 (2−3), 402−407.

Cserhati, T., Forgacs, E., Oros, G., 2002. Biological activity and environmental impact of anionic surfactants. Environ. Int. 28 (5), 337−348.

Cullen, J.M., Allwood, J.M., 2009. The role of washing machines in life cycle assessment studies: the dangers of using LCA for prioritization. J. Ind. Ecol. 13 (1), 27−37.

Curran, M.A., 2014. Strengths and limitations of life cycle assessment. Background and Future Prospects in Life Cycle Assessment. Springer, pp. 189−206.

Curran, M.A., 2017. Overview of goal and scope definition in life cycle assessment. Goal and Scope Definition in Life Cycle Assessment. Springer, pp. 1−62.

DEAT, 2004. Life Cycle Assessment, Integrated Environmental Management, in: Information Series 9, Department of Environmental Affairs and Tourism (DEAT), Pretoria.

Enomoto, R., et al., 2007. Cationic surfactants induce apoptosis in normal and cancer cells. Ann. N.Y. Acad. Sci. 1095 (1), 1−6.

European Union, Framework and requirements for life cycle impact assessment models and indicators, in: International Reference Life Cycle Data System (ILCD) Handbook, first ed., European Union, Luxembourg, 2010.

Fawer, M., Postlethwaite, D., Klüppel, H.-J., 1998. Life cycle inventory for the production of zeolite a for detergents. Int. J. Life Cycle Assess. 3 (2), 71−74.

M. Finkbeiner, R. Tan, M. Raimbault. Life cycle assessment (ISO 14040/44)as basis for environmental declarations and carbon footprint of products, in: ISO Technical Committee 207 Workshop, Norway, 2011.

Finnveden, G., 2000. On the limitations of life cycle assessment and environmental systems analysis tools in general. Int. J. Life Cycle Assess. 5 (4), 229.

Forman, G.S., Hauser, A.B., Adda, S.M., 2014. Life cycle analysis of gas to liquids (GTL) derived linear alkyl benzene. J. Cleaner Prod. 80, 30−37.

Frischknecht, R., Krewitt, W., 2007. Meeting the NEEDS of European environmental sustainability assessment. Energy 3, 4.

Golsteijn, L., et al., 2015. A compilation of life cycle studies for six household detergent product categories in Europe: the basis for product-specific AISE Charter Advanced Sustainability Profiles. Environ. Sci. Eur. 27 (1), 23.

Guilbot, J., et al., 2013. Life cycle assessment of surfactants: the case of an alkyl polyglucoside used as a self emulsifier in cosmetics. Green Chem. 15 (12), 3337–3354.

Hrabak, A., Antoni, F., Szabo, M.T., 1982. Damaging effect of detergents on human lymphocytes. Bull. Environ. Contam. Toxicol. 28 (4), 504–511.

Huang, H., 2008. Life Cycle Assessment Comparison Between Pepfactant® and Chemical Surfactant Production.

Ivanković, T., Hrenović, J., 2010. Surfactants in the environment. Arch. Ind. Hyg. Toxicol. 61 (1), 95–110.

Jensen, A., 1992. Life cycle assessment. A Guide to Approaches, Experiences and Information Sources. 1997. European Environment Agency, p. 121.

Kellenberger, D., Althaus, H.-J., 2009. Relevance of simplifications in LCA of building components. Build. Environ. 44 (4), 818–825.

Kopsahelis, A., et al., 2018. Gate-to-gate life cycle assessment of biosurfactants and bioplasticizers production via biotechnological exploitation of fats and waste oils. J. Chem. Technol. Biotechnol.

La Rosa, A.D., 2016. Life cycle assessment of biopolymers. In: Pacheco-Torgal, F., et al., (Eds.), Biopolymers and Biotech Admixtures for Eco-Efficient Construction Materials. Woodhead Publishing, pp. 57–78.

Laurent, A.-B., et al., 2016. Cradle-to-gate environmental life cycle assessment of the portfolio of an innovative forest products manufacturing unit. BioResources 11 (4), 8981–9001.

Madsen, T., et al., 2001. Environmental and health assessment of substances in household detergents and cosmetic detergent products. Environ. Project 615 (2001), 221.

Marchant, R., Banat, I.M., 2012. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol. Lett. 1–9.

Miettinen, P., Hamalainen, R.P., 1997. How to benefit from decision analysis in environmental life cycle assessment (LCA). Eur. J. Oper. Res. 102 (2), 279–294.

Morse, G., Perry, R., Lester, J., 1995. The life-cycle environmental impact of key detergent builder systems in the EU. Sci. Total Environ. 166 (1-3), 179–192.

Pletnev, M.Y., Fainerman, D.M.B.V.B., Miller, R., 2001. Chemistry of surfactants. Studies in Interface Science. Elsevier, pp. 1–97.

Rebello, S., et al., 2014. Surfactants: toxicity, remediation and green surfactants. Environ. Chem. Lett. 12 (2), 275–287.

Rebitzer, G., et al., 2004. Life cycle assessment: Part 1: Framework, goal and scope definition, inventory analysis, and applications. Environ. Int. 30 (5), 701–720.

Rodrigues, L., et al., 2006. Biosurfactants: potential applications in medicine. J. Antimicrob. Chemother. 57 (4), 609–618.

Saouter, E., Hoof, G. Van, 2002. A database for the life-cycle assessment of Procter & Gamble laundry detergents. Int. J. Life Cycle Assess. 7 (2), 103–114.

Schowanek, D., et al., 2018. New and updated life cycle inventories for surfactants used in European detergents: summary of the ERASM surfactant life cycle and ecofootprinting project. Int. J. Life Cycle Assess. 23 (4), 867–886.

Seppälä, J., Basson, L., Norris, G.A., 2001. Decision analysis frameworks for life-cycle impact assessment. J. Ind. Ecol. 5 (4), 45–68.

Singh, J.S., et al., 2014. A critical analytical study on certain popular Indian detergent powders with respect to the related BIS specifications and their environmental impact. Universal J. Environ. Res. Technol. 4 (4).

Susmi, T., et al., 2010. Toxic effects of sodium dodecyl sulphate on grass carp *Ctenopharyngodon idella*. Fish. Technol. 47 (2), 145.

Thannimalay, L., Yusoff, S., 2014. Comparative analysis of environmental evaluation of LAS and MES in detergent—a Malaysian case study. World Appl. Sci. J. 31 (9), 1635−1647.

Vian, L., et al., 1995. Comparison of three in vitro cytotoxicity assays for estimating surfactant ocular irritation. Toxicol. In Vitro 9 (2), 185−190.

Zolkarnain, N., et al., 2016. Environmental performance of palm-based methyl ester sulphonates production using life cycle approach. J. Oil Palm Res. 28 (1), 104−113.

Bioresource Technology 301 (2020) 122678

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Review

Sustainability and life cycle assessments of lignocellulosic and algal pretreatments

Sharrel Rebello[a,*], A.N. Anoopkumar[a,b], Embalil Mathachan Aneesh[a], Raveendran Sindhu[c], Parameswaran Binod[c], Ashok Pandey[d]

[a] Communicable Disease Research Laboratory, St Joseph's College, Irinjalakuda, Kerala, India
[b] Department of Zoology, Christ College, Irinjalakuda, University of Calicut, Kerala, India
[c] Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum 695 019, India
[d] Centre for Innovation and Translational Research, CSIR- Indian Institute of Toxicology Research (CSIR-IITR), 31 MG Marg, Lucknow 226 001, India

ARTICLE INFO

Keywords:
Life cycle analysis
Lignocelluloses
Algae
Pretreatment

ABSTRACT

Bioenergy and Bioproducts have gained augmented relevance in the wake of depleting fossil fuels and escalating environmental problems induced by anthropogenic activities. The paper outlays the various applications of biomass and their significance in various processes. The prospects of lignocelluloses and algal raw materials to biofuel production are well established; however the life cycle analysis of every bioprocess becomes essential for its technical feasibility. The paper mainly targets the life cycle analysis of various pretreatment strategies adopted in the generation of biofuels. Biomass pretreatment- accounts to a major cost contributory factor in the entire production process and thus the identification of alternate cost effective strategies is of much significance. The LCA analysis identifies biofuel superior to petroleum chemicals based on its environmental effects, however better results are expected to be achieved by depending on methods using solar based energy sources for limiting fossil fuels even in processes of biofuel production.

1. Introduction

The upsurge in human population growth coupled with the developments in society has made an extensive demand for energy and food (Ahorsu et al., 2018). The overexploitation of energy and the depletion of current energy reserves including fossil fuels the demands to explore cost effective alternative resources (Muduli, 2010). Still there is a copious source of fossil fuels at reasonable cost; however this is expected to change in the future scenario, but more censoriously an extensive exploitation of fossil fuels is suspected to be sustainable in the lifelong due to the endorsed upsurge in the emission of greenhouse gases such as such as carbon dioxide, methane, and nitrous oxide from the frequent use of fuels and the environmental impact induced by them on the global warming (Hill et al., 2006; Solomon et al., 2007). Therefore, the need for interest in pinpointing alternative approaches of renewable resources is increased in the current scenario (Demirbas, 2009).Table 1.

With the ever-increasing research knowledge, global environmental perceptions including economic aspects, removal of by-products and contaminants, from the past periods, there has been a prominent increase in scientific knowledge in the value of algae, lignocellulose-based biomass (Arevalo-Gallegos et al., 2017). The significant properties like renewability, ease of availability throughout the year over the globe; recyclability together with natural abundance, all the aforementioned benefits make lignocellulose and algae as an eco-attractive candidate (Arevalo-Gallegos et al., 2017; Yang et al., 2011). Research is in progress over the world on 'greener' technologies based concepts. The intervention of green chemistry based on green agenda concerns has either redirected or directed the present search against the development of value-added eco-attractive recyclable materials. Based on the aforementioned concepts, the deviation from petroleum-based non-renewable resources to the renewable biomass-based resources is positioned at the center of interest in world-wide industrial research (Bilal et al., 2017; Iqbal, 2015). Herein, concepts based on bio-refinery aspects have disputes on eco-pollution, thus they can be recognized as the evolution of concerns identical to green chemistry (Gruber et al., 2006). Yet the concepts on refinery is not a novel, however, in the current scenario, the integrated bio-refineries including research on lignocellulose and algae, are perceived as a significant route to find out our intentions for safeguarding nature and sustained prosperity (Arevalo-Gallegos et al., 2017). Therefore the effort has been shifted to the aforesaid concepts to address the rising environmental impacts where fossil fuels based energy are and resources are unsustainable.

* Corresponding author.
E-mail address: sharrel.rebello@gmail.com (S. Rebello).

https://doi.org/10.1016/j.biortech.2019.122678
Received 31 October 2019; Received in revised form 17 December 2019; Accepted 22 December 2019
Available online 26 December 2019
0960-8524/

Table 1
Important Algae Based Companies in World.

Sl. No.	Company	Head quarter	Technology
1	Algenol	Florida	a. Production of ethanol from algae b. Production of gasoline, diesel and jet with involvement of algae, carbon dioxide, salty water and sunlight.
2	SolixAlgredients	Colorado	Production of Solasta ®, Astaxanthin, Solmega® and DHA omega-3
3	Sapphire Energy	San Diego, California, United States	Production of biofuel green crude
4	TerraVia Holdings, Inc. (formerly Solazyme)	United States	Production of high value oils from low cost plant sugars
5	Aurora Biofuels	California	Excellent algae derived products with extensive application in nutrition, pharmaceutical, and fuel market.
6	Earthwise *Spirulina*	California	high-quality *Spirulina* production
7	Nutress	Europe	Production of value added products to feed, pharmacy and food sector.
8	Shibin Chlorella	India	Production of *Chlorella*
9	Herbal hills	India	Production of *Spirulina* tablets
10	Parry Nutraceuticals	Chennai, India	Microalgal health supplements
11	Spiruzan™		Combination of *Spirulina* and Astaxanthin
12	Taiwan Chlorella Manufacturing Company (TCMC)	Taiwan	*Chlorella* production
13	Nutrex-Hawaii Hawaiian	Kailua-Kona	Popular brands of *spirulina*
14	Bulk Supplements	China	Pure *Spirulina* production
15	Far East Bio-Tec Co., Ltd. (ALGAPHARMA BIOTECH CORP.)	Taiwan	*Spirulina* production
16	FEBICO SOROKINA®	Taiwan	PPAR dietary supplements

Of the various renewable resources available as sustainable and cost effective feed stocks for biofuel production, lignocelluloses biomass as well as algae has been explored to a great extent. As natural elements that could replace the use of extensive fossil fuels, both lignocelluloses biomass and algae, gains equal priority to combined efforts to sustainable energy production. Though they represent two different types of feedstocks, the main utility of both these sources are mainly targeted to a common aim of improved biofuel yield, apart from its various other uses. While, plant derived lignocelluloses demands land for extensive cultivation, algal based strategies can be overcome by reactors demanding comparatively less space. Irrespective, of their cultivation strategies, both lignocelluloses biomass as well as algae is utilized only after pretreatment of these are conducted effectively. The current review paper thus outlays the various uses of lignocelluloses and algal biomass, the LCA analysis of the different pretreatment strategies and the challenges that need to be addressed to yield sustainable energy resources.

2. Lignocellulosic & algal biomass as sustainable alternative

2.1. Lignocellulose biomass & byproducts

The Lignocellulosic biomass (LCB) is recognized as the most copiously accessible eco-attractive resource constituting to about 1.3 billion tons of global yield every year. During the past few years, the scientific community had confirmed significant development in Lignocellulose associated processes which triggered a wide range of biotechnological applications concerned with the purification and immobilization of ligninolytic enzymes to express their potential benefits (Asgher et al., 2017; Iqbal et al., 2011). The hydrolysis of Lignocellulosic biomass has led to the release of several reducing sugars. These sugars are greatly esteemed for the production of biogas, phenols, bioethanol, and aldehydes. The biological polymers such as hemicellulose, lignin, and cellulose are the major components of the LCB. They are strongly linked with each other by hydrogen and covalent bond thereby forms a highly recalcitrant structure. In addition to this, previous studies have validated that the production of acids, xylitol, saccharides, cellulose acetate, saccharides, and biogasoline/biodiesel are recognized as the promising applications of LCB (Behera et al., 2014). Except for biofuels, other products such as biofertilizer/manure as a good value-added eco-attractive deliverable has been made from the residues of biomethane and bioethanol (Kumar et al., 2009; Limayem and Ricke, 2012).

The role of cellulases, laccase, and hemicellulases is important in lignin degradation into hexose and pentose formation (Balan et al., 2013). After the successful production of ethanol and biomethane, the formed solid residue can be efficiently used for the development of biofertilizer. The formed residue can also act as an important source of essential nutrients for plant growth (Balan et al., 2013; Sylvia et al., 2005). Biobutanol has possessed several benefits than the traditional biofuel, and are i) higher energy content, ii) can be mixed with diesel in pure or mixed form, iii) can be used in automobile engines without any modifications, iv) lower vapor pressure, v) less susceptible to separation in water (Bankar et al., 2013; Prakash et al., 2017). However, certain drawbacks and challenges such as the excess cost of saccharification, pretreatment, and fermentation have made this strategy not a popular one. In addition, the lignocellulosic biomass has to exhibit enormous potential on the bioethanol and lactic acid production (Balan, 2014; Isikgor and Becer, 2015).

The forest biomass as lignocelluloses characterizes an effective reservoir of carbon-rich material. The wood hydrolysate is found to be potentially renewable feedstock and inexpensive that has been made through dilute acid or enzymatic hydrolysis of either hemicellulose or cellulose to fermentable forms of sugars like mannose, galactose, glucose and xylose (Cara et al., 2008; Rebello et al., 2018).

The natural fibers of lignocellulosic materials constitute several functional groups. These make them fit for appropriate alternatives for a wide range of applications in various industries associated with filter materials, biocomposites and technical textiles. Though, their deprived mechanical properties together with high biodegradability limit the use of natural fibers (Manna et al., 2017; Marin et al., 2013; Reddy and Yang, 2005). Kenaf, jute, sisal fibers and banana have been principally exploited for the preparation of natural fiber-reinforced combinations. Moreover, the lignocellulosic biomass has been used for water treatment as an innovative technology for the past few years. However, the various factors such as capacity, specificity, affinity together with their physicochemical nature have influenced their efficiency (Basso et al., 2002; Kristensen et al., 2007).

2.2. Microalgae: sustainable and renewable applications

Algae are primarily recognized as the photosynthetic organisms that have been grown in a wide range of aquatic habitats such as rivers, lakes, oceans, wastewater, and ponds. They are also able to tolerate the

various environmental factors like extreme pH, a wide range of temperatures, different light intensities followed by salinities (Barsanti et al., 2008). Based on the size of them, two kinds of algae have been classified; i) macroalgae (comparatively large in size) 2) microalgae (small in size). The macroalgae are large-size algae, multicellular and visible with the naked eye whereas, microalgae are microscopic, prokaryotic and sometimes eukaryotic, and shows resemblance towards the Chloroxybacteria.

Due to the presence of large amounts of carbon compounds, the microalgae have been primarily utilized in health supplements, cosmetics, biofuels, and pharmaceuticals. They also exhibit significant applications in atmospheric CO_2 mitigation and wastewater treatment. Various kinds of bioproducts such as lipids, proteins, bioactive compounds, antioxidants, and polysaccharides are produced by them (Brennan and Owende, 2010; Zhang et al., 2017). The concern in algae as an eco-attractive and sustainable feedstock for various bio-products has opened a new way in the biorefinery. The applications of genetic engineering coupled with growth enhancement techniques may extend their potential benefits for future research concerning renewable bioproducts. The production of biofuels from industrially cultivated microalgae has been dramatically upsurge in the past few years. The excess quantity of algae is directly sold as food and nutrient supplements, whereas their processed elements are used in cosmetics and biopharmaceuticals (Borowitzka, 2013; Pulz and Gross, 2004).

The microalgae also produce several other value-added elements including carotenoids, vitamins, bioactive compounds, polyunsaturated fatty acids (PUFAs) and pigments (da Silva Vaz et al., 2016). Derwenskus et al. 2019 reported that wet algae such as *Chlorella vulgaris* and *Phaeodactylum tricornutum* are known for their potential to produce carotenoids and mono- and polyunsaturated fatty acids (PUFA) (Derwenskus et al., 2019). In addition, the freshwater algae *Haematococcus pluvialis* is recognized as an important source of astaxanthin pigment. Similarly, *Chlorella vulgaris* is has been used as a food supplement while the and *Dunaliella* species is specific for β-carotene production (Sharma and Sharma, 2017). The thermochemical conversion or thermal liquefaction converts the algal biomass into bio-oils, which contains several organic elements deposited as lipids, carbohydrates, and proteins (Peng et al., 2000; Peng et al., 2001; Sawayama et al., 1999). The diversity of resources for bioenergy and biofuel has become a serious issue in the current scenario. Therefore, today, significant attention has been paid against bio-hydrogen production. However, the industrial production of bio-hydrogen is not feasible since the various processes for the production is recognized as expensive and it provides only low biomass concentration. Recent research trends reported that some of the algae are able to trigger considerable quantity of hydrogen gas. However, the technology for the aforementioned concept is still in its initial stage and processes need to be improved at higher level (Saifuddin and Priatharsini, 2016).

Due to the presence of sulfur-containing polysaccharide in the biomass from *Spirulina platensis*, the entry of human pathogenic viruses including measles virus, Herpes simplex, and Human cytomegalo viruses has been blocked (Ayehunie et al., 1998; Hayashi et al., 1996). Similarly, the antiviral activity of red alga *Porphyridium* has been enhanced due to sulfur containing polysaccharides, which prevents the entry of HSV-1, HSV-2 (Huleihel et al., 2001). An effective metabolite (cryptophycin) which is isolated from *Nostoc* ATCC 53789 was found to be exhibit higher anticancer activity (Schwartz et al., 1990).

The numerous bioactive elements from both macro- and microalgae are capable of promoting plant growth through seed germination followed by leaf, stem growth, and flowering. They also provide protection against plant diseases. This has indicated that the research on both macro and microalgae has gained extensive significance in the current scenario. For most of the aforementioned benefits, the industry is still emerging and the practical applications of them have extended into new regions. With the growth of sophisticated instrumentation and approaches, the microalgal biotechnology can now meet the maximum demands of the pharmaceutical, cosmetics and food industries.

3. Life cycle analysis and its significance

Life cycle analysis or often referred to cradle to grave analysis is of great relevance to evaluate the impact of any production process, irrespective of its aim of production. The process of life cycle assessment of any process involves the evaluation of the various environmental impacts of it on the ecosystem. Four different principles of goal definition, inventory analysis, impact assessment and interpretation are done as the process. The various steps or inventories in the production including from raw materials, every treatment process, purification step, energy utilisation/generation rate, waste generated amidst the process etc till its final product generation are considered as inventories and are analysed to do a life cycle analysis. It is an environmental management tool employed to examine the life cycle of a product based on a framework given by ISO 14040 and 14044 (Finkbeiner, 2013; Schau and Fet, 2008). Moreover, the inclusion of guidelines to various algal based biotechnological interventions would validate their utility (Morales et al., 2019). Such analysis can also be applied to the process of bioenergy generation to evaluate its effectiveness asn environmental impact.

4. Life cycle analysis of lignocellulose biotreatment

For the large scale production of ethanol in developing countries, the inexpensive and abundantly available LCB can be used. The three major steps such as delignification, saccharification followed by fermentation are renowned as the three principal steps for bioethanol production from LCB. The major organic materials included in the LCB are i) agricultural crops, ii) grass, iii) algae, iv) agrowastes, v) wood and certain other renewable resources. In addition, for the past few years, the LCB such as rice straw, timothy grass, wheat straw, sugarcane bagasse, woody raw materials, paper pulps, barley, softwoods, and forest residues have been extensively explored for biofuel production (Akhtar et al., 2016; Loow et al., 2015).

The production of renewable energy provides the potential to offer a prominent source of low carbon energy to reduce the chance of toxicity in the environment. The sustainable use of environmental elements including lignocellulosic biomass is now acknowledged to be a significant natural resource for bioenergy production, since carbon neutral process is associated with several environmental impacts such as growth of feedstock, system production and transportation issues (Borrion et al., 2012). The lignocellulosic biomass is often recognized to be a carbon neutral, however, specific amount of GHG have formed and released during the life cycle. The growth of feedstock at farming level followed by the conversion of biofuel and transportation are noted as the significant steps involved in the LCA study of biofuel.

4.1. Hurdles to lignocelluloses based biofuels

Wide ranges of lignocellulosic biomass such as food based feed stock (corn, wheat, sugarcane, sorghum etc) and non food based feedstock (sugarcane bagasse, corn stover, wheat grass etc) are attributed to the generation of First generation and Second generation biofuels. Such lignocellulose based biofuel generation are found to be less harsh and ecofriendly compared to fossil fuel based production strategies in terms of direct GHG emissions; however concerns do exist on indirect emissions due to plant growth as well as conversion of food land to land for fuel production (Zhang, 2019). The use of various grass like species such as Miscanthus have also been attempted in various industries (Lask et al., 2019), yet the extent of success of these industries are still a question mark (Zhang, 2019). Moreover, large amount of fossil fuels are also consumed in downstream pretreatment processes, which need to be resolved. The green house gas emissions from nitrogen fertilisers as well as requirement of large hectares of land for energy generation was

cited as drawbacks in the LCA of ricestraw based ethanol production (Kumar and Murthy, 2012). The disadavantage of high electricity consumed during the process can be overcome with the use of renewable energy sources instead of fossil fuels.

4.2. LCA based solutions to pretreatment

Several pre-treatments such as biological, chemical, physical and physicochemical approaches are principally applied for lignin degradation (Kuila and Sharma, 2017). Mn-dependent peroxidase, lignin peroxidase (LiP) and Laccase- facilitated pre-treatment has found their significance in the pre-treatment stage since they have offered meagre inhibitor formation, substrate specificity, less necessity for water, followed by maximum efficiency at mild conditions. The action of xylanase and cellulase during the saccharification process has transformed the hemicellulose and cellulose into glucose and xylose (Galbe and Zacchi, 2012; Singhvi and Gokhale, 2013). The breakdown of the lignin barrier has accompanied the delignification stage (Kuila and Sharma, 2017; Petersson et al., 2007). Previous studies have introduced several pre-treatment strategies ranging from mechanical (irradiation, physical, steam explosion, thermal and extrusion), biological (consortium, bacteria, enzymatic, fungal) and combined (Van Fan et al., 2018). Pretreatments posseses significant role in improving the various processes by increasing the substrate accessibility to obtain high yield of various biobased products including biogas and biofuels as shown in Fig. 1.

The LCA analysis of pretreatment involving Liquid hot water (LHW), steam, dilute acid treatment and organosolvents indicate that LHW using pressurised deioinised water alone for pretreatment was quite effective in reducing the GHG and yielding more sugar for fermentation to biofuel production (Prasad et al., 2016). In another study using rice straw steam explosion pretreatments gave better results compared to dilute acid, dilute alkali, hot water pretreatments; however the results of energy yield was promising compared to gasoline alternatives (Kumar and Murthy, 2012).

A comprehensive review on the utility and LCA of thermochemical processes in biomass treatment process gives an updated idea on the topic and help to identify suitable pretreatment strategy for the biomass of choice (Ubando et al., 2019). Sugarcane is found to be pretreated effectively by combinatorial approach involving hydrothermal strategies, NaOH (84%) and acid + NaOH (86%) yielding better sugars, but the Life cycle analysis of the process is not provided to evaluate the reliability of the process based on its impact to the environment (Candido et al., 2019).

The environmental impact of various chemicals used for pretreatment suggest that methanol had the least effect on global warming potential (GWP), eutrophication, acidification, photochemical oxidation demand and marine and human ecotoxicity; whereas use of sodium hydroxide posed the highest bad effects to the environment (Smullen et al., 2019). A combinatorial approach involving chemical treatment using alkaline peroxide, mechanical treatment and enzymatic hydrolysis of sugarcane bagasse is found to reduce the energy consumption to 65% and waste generation compared to conventional chemical methods (Chuetor et al., 2019).

5. Life cycle analysis of algal biomass and its pretreatment

Algal based biofuel production has gained much significance in the scenario of excessive depletion of fossil fuels, increased green house gas emission, toxic effects to the environment, global warming and resultant climate change (Roux et al., 2017). However, the more energy intensive production of algal biofuels as compared to conventional fuels has practically reduced or not made feasible prevalent use of algal biofuels (Shirvani et al., 2011). The effective utility of algae against natural gas (a representative of fossil fuel) for the production of methane is appreciated in terms of the benefits of three reduced components viz, green house gas emissions, ozone depletion and eutrophication index (Langlois et al., 2012). The presence of large lipid contents, rapid rate of growth, no requirement of cultivable land are the main attributes of algae that adds to its utility in biofuel generation (Sharma et al., 2018). Furthermore, the absence of lignin, 3 D structures and complex recalcitrant structures in algae also extends its candidature as an ideal biomass for energy production compared to terrestrial plants. These fastest growing plants are grown worldwide in open or closed ponds, raceways as well as photobioreactors industrially to produce algal biofuels as enlisted in table 2.

The biofuel production from microalgae involves two major steps such as downstream and upstream processes. The downstream step plants highlight the sustainable production and harvesting patterns of biofuel; while, the upstream step possesses diverse cultivation aspects that are required to maximize the biomass (Medipally et al., 2015). Biofuels from algae are economically viable, require slight water use, mitigate CO_2, and require no additional plots. Although commercial production of biodiesel from algae-based strategies are not practical, owing to its expensive downstream processes and low biomass concentration.

Irrespective of its acceptance as an ecofriendly method for energy generation, the field of algal biofuels face a major obstacle of high productivity cost which is approximately sometimes 10–100 times

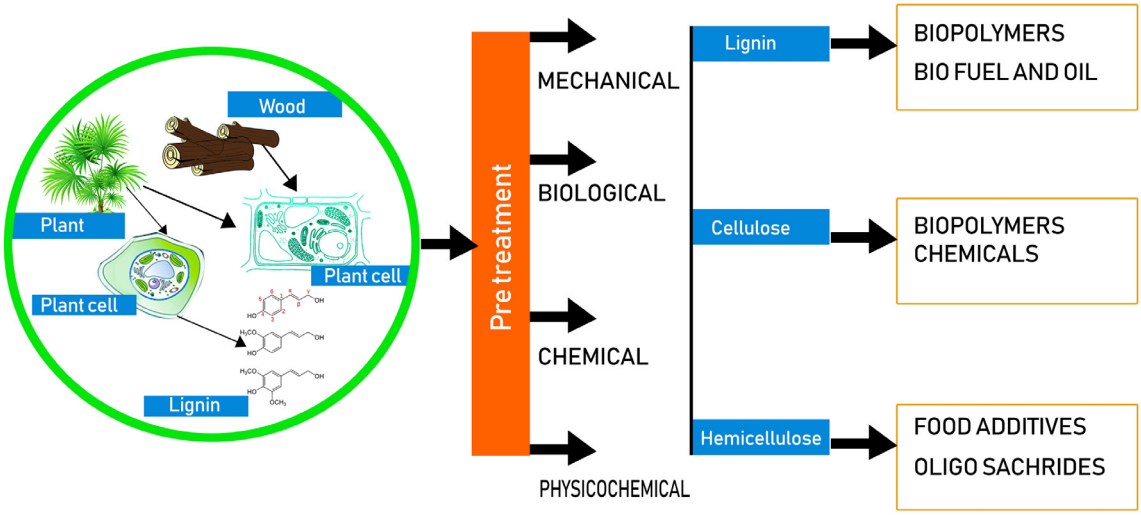


Fig. 1. Pretreatment of lignocelluloses to yield products.

Table 2
An outline of LCA inventories utilising lignocellulosic and algal biomass.

Sl No.	Biomass	Pretreatment/Process	Product yielded	Environmental impact	Ref
1	*Chlorella vulgaris* (microalgae)	Continuous recirculation of culture ponds through thickener; flocculation, dewatering.	biodiesel	Global warming potential	(Lardon et al., 2009)
2	Rice straw	Co firing	Power	Acidification, global warming potential (GWP), eutrophication	(Shafie et al., 2013)
3	Wood residue		Electricity	Global warming potential	(Hsu, 2012)
4	Energy crop and wheat straw		Electricity	Global warming potential	(Sebastián et al., 2011)
5	Willow		Electricity	Net energy ratio and net global warming potential	(Kauffman et al., 2011)
6	Corn stover	Fast pyrolysis	Bio gasoline	Global warming potential	(Han et al., 2013)
7	Forest residue		Gasoline, diesel,	Global warming potential and net energy value (NEV)	(Han et al., 2013)
8	Short rotation poplar			Cumulative energy demand, global warming, ozone, layer depletion, photochemical oxidant formation, land competition, acidification, eutrophication	(Kauffman et al., 2011)
9	Logging residue, Hybrid poplar, willow and waste wood		Electricity	Global warming potential	(Fan et al., 2011)
10	Woodchip	Ablative pyrolysis		Global warming, ozone depletion, photochemical ozone creation potential, acidification, eutrophication	(Faix et al., 2010)
11	Corn stover and switch grass	Slow pyrolysis	Bio-char	Global warming potential	(Iribarren et al., 2012)
12	Forest residue	Gasification	Heat and power	Global warming, ozone layer depletion, photochemical oxidization, acidification, eutrophication, toxicity, abiotic depletion	(Hsu, 2012)
13	Poplar energy crop		Electricity	Global warming, ozone layer depletion, smog, acidification, eutrophication, solid waste, energy consumption	(Rafaschieri et al., 1999)
14	Willow biomass		Heat and power Hydrogen	Global warming, smog, acidification, eutrophication, carcinogenesis, heavy metals, smog	(Kimming et al., 2011)
15	Rice husk and Wood waste	Combustion	Electricity	Global warming, acidification, eutrophication, ecotoxicity	(Faix et al., 2010; Shafie et al., 2012)
16	Forest residue, Maize residue, and Pine	Atmospheric pressure gasification + upgrading	Methanol	Industrial application, to reduce the production cost of chemicals	(Amigun et al., 2010; Sarkar et al., 2011; Shabangu et al., 2014)
17	Agricultural Biomass, Natural gas	Fast pyrolysis + gasification + upgrading	Ethanol	Biomass-to-Liquid Fuel Production	(Trippe et al., 2010)
18	Lignocellulosic biomass	Gasification syngas-DME-Ethylene	Dimethyl ether and Ethylene	Implications of negative CO2 emissions	(Haro et al., 2013)
19	Hydrogen Forest residue and straw	Battelle Columbus Laboratory (BCL)	Hydrogen	Upgrading of bitumen from oil sands	(Sarkar and Kumar, 2010)

greater than petroleum based techniques; depending upon the methodologies adopted for production (Hannon et al., 2010). Such production costs are to be reduced at different stages of algal growth, harvesting, dewatering, pretreatment and bioconversion to fuels.

5.1. LCA based solutions to algal growth

Algal biofuels synthesis commonly contains stages of algal growth, harvest and extraction which further delineates to dry, wet as well as acoustic methods based on different algal species, its biofuel yield and characteristic growth parameters. The choice of algae primarily influences the life cycle of biofuel yield based on the fact that each algal source varies in its fuel yield (Demirbas and Fatih Demirbas, 2011). Natural high lipid yielding strains of *Chlorella protothecoides* (Cheng et al., 2009), *Chlorella zofingiensis* (Liu et al., 2011), *Ochromonas danica* (Lin et al., 2019) etc and even potent lipid yielding strains developed with the aid of metabolic engineering or recombinant technology could be promising workforce for the synthesis of biofuels (Sharma et al., 2018).

LCA analysis of algal growth indicates that wastewater based units could serve as promising surfaces for cultivation, thereby reducing the expense required to set up photoreactors when needed. Macroalgae are proven to be ideal candidates in aquaculture farms to produce biofuels and various value added chemicals, thereby reducing the waste effluent mediated eutrophication and adjoining problems by integrating aquaculture farms with algal growing nets (St Peter and Pietrak, 2010). Yet other studies with macroalgae viz, Macrocystis pyrifera have been done at lab scale and found promising; however scaling to large scale is needed to visualise the commercialised production (Aitken et al., 2014).

5.2. LCA based solutions to pretreatment

The pretreatment strategy adopted for any bioprocess varies greatly from each biomass used as well as the product intended to be produced from the substrate. For instance macroalgae being a rich source of carbohydrate is utilised for fermentative production of biofules such as biogas, bioethanol etc is pretreated mostly with chemico-enzymatic methods, whereas microalgae a dominant lipid source is used for the converted mediated production of biodiesel are mechanically disrupted (Yoo et al., 2015). A wide array of methods such as physical, chemical, mechanical, enzymatic, hydrothermal methods are employed for the algal treatment (Fig. 2). Quite often the major portion of the cost of algal by product synthesis is spent in extended by such extraction techniques.

Conventionally thermal dewatering is adopted in most cases of algae bioprocess units; however the consistent efforts and intensive research has paved the way of better extraction methods such as wet extraction, hydrothermal liquefaction, electroporation, jet engine extraction etc. As per the studies with *Chlorella* it was noted that flocculation of algae by pH adjustments to alkaline range (using lime) as well as use of synthetic flocculants is preferred to energy consuming methods of centrifugation normally used for algal harvest. The analysis of dry method of lipid extraction from algae indicates that dry extraction can be done using a conveyor belt dryer followed by counter current extraction using hexane (Lardon et al., 2009). The wet extraction of *Chlorella* grown in nitrogen limiting conditions in the above study stood out as an effective strategy than the dry method producing an effective 105 MJ of energy after production; thereby resulting less energy- fertilizer demands and reduced negative impacts to the ecosystem.

The LCA analysis of algal biofuel production by conventional methods as per USLCI, indicated that thermal dewatering process alone accounted for significant amount of fossil energy of 3556 kJ/kg of water removed (Sander and Murthy, 2010). Thus alternatives to replace the this fossil fuel used for dewatering methods of centrifugation and pressing needs to be found out. In such a scenario, the use of solar dryer can be optimised and practised for algal dewatering utilising the solar based dewatering carried out in sludge (Solmaz and Okaygün, 2018). Though such solar based strategies are not practical worldwide, collaborative efforts utilising the resources in tropical countries could help to find effective solutions in this direction.

Assessment on the life cycle of algal based hydrothermal liquefaction and lipid extraction instigated the use of less algae was needed; but it resulted in higher levels of CO_2 emissions and lesser energy was produced even to meet requirements of algal growth and harvest (Frank et al., 2013). Novel methods such as confined impinging jet mixers permits turbulent mixing of lipid with solvents and thus they permit faster lipid recovery of algae even bypassing the steps of dewatering (Tseng et al., 2019). The prospect of inducing reversible and irreversible pores in algae to facilitate easy recovery of their byproducts has been explored in Chlamydomonas reinhardtii by giving short pulses of 5 μs (Bodenes et al., 2019).

A fifteen year study initiated by National Alliance for Advanced Biofuels and Bioproducts has identified novel strategies of algal fuel synthesis with 45% reduction of green gas emissions against base methods (Shi et al., 2019). This comprehensive study involving multiple LCA components identified that the use of acoustic harvesting and acoustic extraction of algal lipids had a great impact of LCA by reducing the GHG emissions. It is also noted that the conversion of microalgae to butanol rather than to biodiesel is found to pose less environmental effects on climate change and human health (Wu et al., 2019).

The LCA analysis of macroalgal biofuels involved analysis of the environmental impacts at different stages of growth, harvest, milling, pretreatment and acidogenic digestion needed for its conversion to organic acids. The study indicated that the major energy consumption was during the distillation phase due to the requirement of steam and additional electricity to run the concerned equipment (St Peter and Pietrak, 2010). Apart from all the above mentioned pretreatment strategies of lignocellulose and algal biomass, biological treatments using bacterial or fungal enzymes have been found effective in better energy yield and productivity, however, such methods are not always adopted in large scale options due to some technoeconomic problems demanding long pretreatment time and its sure extensive research in this direction would lead to reversal of such situations in near future (Zabed et al., 2019).

6. Conclusion

The life cycle analysis giving emphasis on pretreatment of biomass is comparatively less than other stages of biofuel production. Generalised comparison of different algal production strategies will not give conclusive, yet compiling worldwide efforts targeting a common aim of economised commercial biofuel synthesis augments novel technological advancements in this direction. Quite often the life cycle analysis of a multitude of production strategies generated after years of research may help to develop sustainably feasible routes of synthesis. Moreover, the routes of optimisation and LCA analysis have to be concentrated on a single type of feed stock to get practically validated results.

Authors contribution

All the authors have equally contributed to the work contributing different sections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

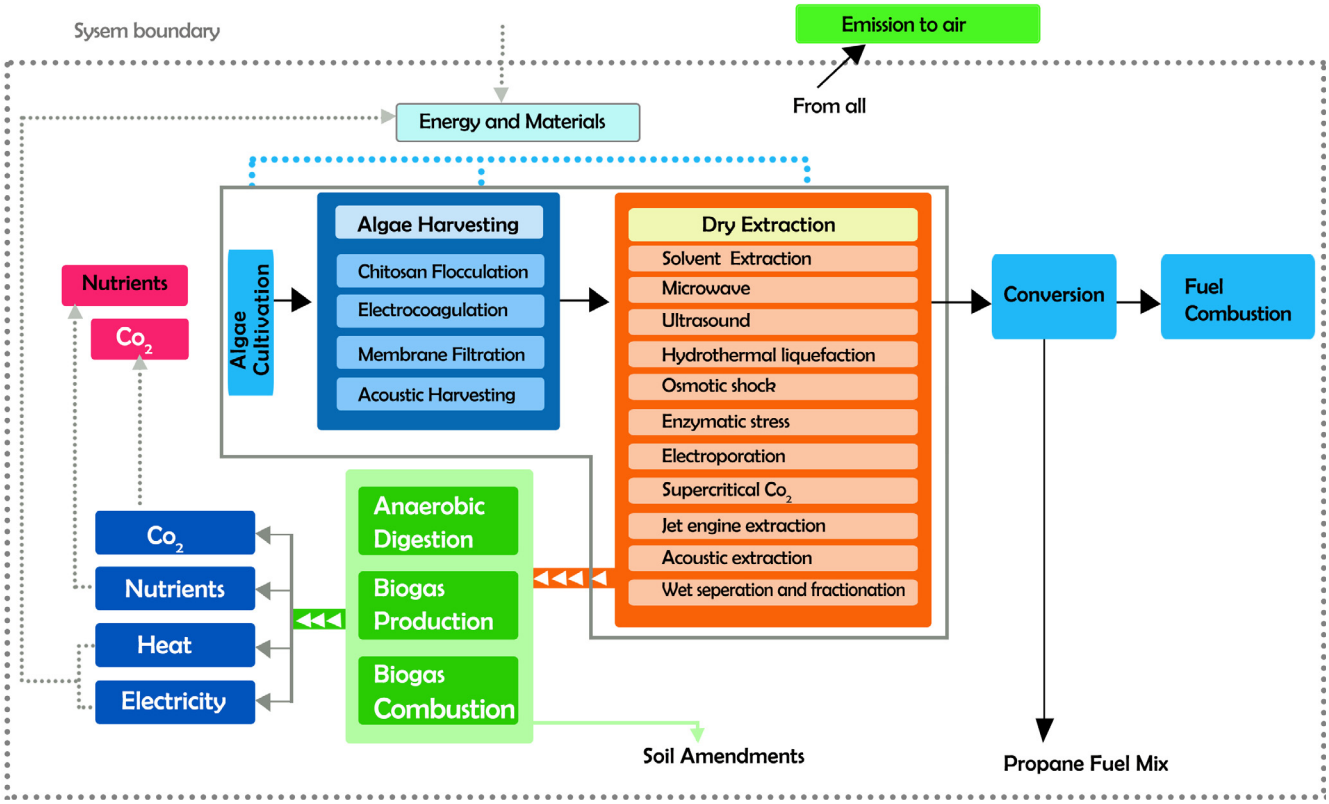


Fig. 2. Algal pretreatment to yield energy an overview.

Acknowledgements

The author Embalil Mathachan Aneesh thanks University Grants Commission, Government of India (UGC Research Award – F30-6/20-16(SA-II)) and DBT (BT/IN/Indo-US/Foldscope/39/2015) for funding. Raveendran Sindhu acknowledges Department of Science and Technology for sanctioning a project under DST WOS-B scheme.

References

Ahorsu, R., Medina, F., Constantí, M., 2018. Significance and challenges of biomass as a suitable feedstock for bioenergy and biochemical production: a review. Energies 11 (12), 3366.
Aitken, D., Bulboa, C., Godoy-Faundez, A., Turrion-Gomez, J.L., Antizar-Ladislao, B., 2014. Life cycle assessment of macroalgae cultivation and processing for biofuel production. J. Cleaner Prod. 75, 45–56.
Akhtar, N., Gupta, K., Goyal, D., Goyal, A., 2016. Recent advances in pretreatment technologies for efficient hydrolysis of lignocellulosic biomass. Environ. Prog. Sustainable Energy 35 (2), 489–511.
Amigun, B., Gorgens, J., Knoetze, H., 2010. Biomethanol production from gasification of non-woody plant in South Africa: optimum scale and economic performance. Energy Policy 38 (1), 312–322.
Arevalo-Gallegos, A., Ahmad, Z., Asgher, M., Parra-Saldivar, R., Iqbal, H.M., 2017. Lignocellulose: a sustainable material to produce value-added products with a zero waste approach—a review. Int. J. Biol. Macromol. 99, 308–318.
Asgher, M., Ahmad, Z., Iqbal, H.M., 2017. Bacterial cellulose-assisted de-lignified wheat straw-PVA based bio-composites with novel characteristics. Carbohydr. Polym. 161, 244–252.
Ayehunie, S., Belay, A., Baba, T.W., Ruprecht, R.M., 1998. Inhibition of HIV-1 replication by an aqueous extract of Spirulina platensis (Arthrospira platensis). J. Acquired Immune Deficiency Syndromes Human Retrovirol. 18 (1), 7–12.
Balan, V., 2014. Current challenges in commercially producing biofuels from lignocellulosic biomass. ISRN Biotechnol. 2014, 1–31 463074.
Balan, V., Chiaramonti, D., Kumar, S., 2013. Review of US and EU initiatives toward development, demonstration, and commercialization of lignocellulosic biofuels. Biofuels, Bioprod. Biorefin. 7 (6), 732–759.
Bankar, S.B., Survase, S.A., Ojamo, H., Granström, T., 2013. Biobutanol: the outlook of an academic and industrialist. RSC Adv. 3 (47), 24734–24757.
Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A.M., Passarelli, V., Vesentini, N., Gualtieri, P., 2008. Oddities and curiosities in the algal world, in: Algal Toxins: Nature, Occurrence, Effect and Detection, Springer, pp. 353–391.
Basso, M., Cerrella, E., Cukierman, A., 2002. Lignocellulosic materials as potential biosorbents of trace toxic metals from wastewater. Ind. Eng. Chem. Res. 41 (15), 3580–3585.
Behera, S., Arora, R., Nandhagopal, N., Kumar, S., 2014. Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew. Sustain. Energy Rev. 36, 91–106.
Bilal, M., Asgher, M., Iqbal, H.M., Hu, H., Zhang, X., 2017. Biotransformation of lignocellulosic materials into value-added products—a review. Int. J. Biol. Macromol. 98, 447–458.
Bodenes, P., Bensalem, S., Français, O., Pareau, D., Le Pioufle, B., Lopes, F., 2019. Inducing reversible or irreversible pores in Chlamydomonas reinhardtii with electroporation: Impact of treatment parameters. Algal Res. 37, 124–132.
Borowitzka, M.A., 2013. High-value products from microalgae—their development and commercialisation. J. Appl. Phycol. 25 (3), 743–756.
Borrion, A.L., McManus, M.C., Hammond, G.P., 2012. Environmental life cycle assessment of lignocellulosic conversion to ethanol: a review. Renew. Sustain. Energy Rev. 16 (7), 4638–4650.
Brennan, L., Owende, P., 2010. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14 (2), 557–577.
Candido, R.G., Mori, N.R., Gonçalves, A.R., 2019. Sugarcane straw as feedstock for 2G ethanol: evaluation of pretreatments and enzymatic hydrolysis. Ind. Crops Prod. 142, 111845.
Cara, C., Ruiz, E., Oliva, J.M., Sáez, F., Castro, E., 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresour. Technol. 99 (6), 1869–1876.
Cheng, Y., Zhou, W., Gao, C., Lan, K., Gao, Y., Wu, Q., 2009. Biodiesel production from Jerusalem artichoke (Helianthus Tuberosus L.) tuber by heterotrophic microalgae Chlorella protothecoides. J. Chem. Technol. Biotechnol. 84 (5), 777–781.
Chuetor, S., Champreda, V., Laosiripojana, N., 2019. Evaluation of combined semi-humid chemo-mechanical pretreatment of lignocellulosic biomass in energy efficiency and waste generation. Bioresour. Technol. 292, 121966.
da Silva Vaz, B., Moreira, J.B., de Morais, M.G., Costa, J.A.V., 2016. Microalgae as a new source of bioactive compounds in food supplements. Curr. Opin. Food Sci. 7, 73–77.
Demirbas, A., 2009. Biofuels securing the planet's future energy needs. Energy Convers. Manage. 50 (9), 2239–2249.
Demirbas, A., Fatih Demirbas, M., 2011. Importance of algae oil as a source of biodiesel. Energy Convers. Manage. 52 (1), 163–170.
Derwenskus, F., Metz, F., Gille, A., Schmid-Staiger, U., Briviba, K., Schließmann, U., Hirth, T., 2019. Pressurized extraction of unsaturated fatty acids and carotenoids from wet chlorella vulgaris and phaeodactylum tricornutum biomass using subcritical liquids. GCB Bioenergy 11 (1), 335–344.
Faix, A., Schweinle, J., Schöll, S., Becker, G., Meier, D., 2010. (GTI-tcbiomass) life-cycle assessment of the BTO®-process (biomass-to-oil) with combined heat and power generation. Environ. Prog. Sustainable Energy 29 (2), 193–202.
Fan, J., Kalnes, T.N., Alward, M., Klinger, J., Sadehvandi, A., Shonnard, D.R., 2011. Life

cycle assessment of electricity generation using fast pyrolysis bio-oil. Renewable Energy 36 (2), 632–641.
Finkbeiner, M., 2013. From the 40s to the 70s—the Future of LCA in the ISO 14000 Family. Springer.
Frank, E.D., Elgowainy, A., Han, J., Wang, Z., 2013. Life cycle comparison of hydrothermal liquefaction and lipid extraction pathways to renewable diesel from algae. Mitig. Adapt. Strat. Glob. Change 18 (1), 137–158.
Galbe, M., Zacchi, G., 2012. Pretreatment: the key to efficient utilization of lignocellulosic materials. Biomass Bioenergy 46, 70–78.
Gruber, P.R., Kamm, M., Kamm, B., 2006. Biorefineries: Industrial Processes and Products: Status Quo and Future Directions. Wiley-VCH.
Han, J., Elgowainy, A., Dunn, J.B., Wang, M.Q., 2013. Life cycle analysis of fuel production from fast pyrolysis of biomass. Bioresour. Technol. 133, 421–428.
Hannon, M., Gimpel, J., Tran, M., Rasala, B., Mayfield, S., 2010. Biofuels from algae: challenges and potential. Biofuels 1 (5), 763–784.
Haro, P., Trippe, F., Stahl, R., Henrich, E., 2013. Bio-syngas to gasoline and olefins via DME–a comprehensive techno-economic assessment. Appl. Energy 108, 54–65.
Hayashi, K., Hayashi, T., Kojima, I., 1996. A natural sulfated polysaccharide, calcium spirulan, isolated from Spirulina platensis: in vitro and ex vivo evaluation of anti-herpes simplex virus and anti-human immunodeficiency virus activities. AIDS Res. Hum. Retroviruses 12 (15), 1463–1471.
Hill, J., Nelson, E., Tilman, D., Polasky, S., Tiffany, D., 2006. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. 103 (30), 11206–11210.
Hsu, D.D., 2012. Life cycle assessment of gasoline and diesel produced via fast pyrolysis and hydroprocessing. Biomass Bioenergy 45, 41–47.
Huleihel, M., Ishanu, V., Tal, J., Arad, S.M., 2001. Antiviral effect of red microalgal polysaccharides on Herpes simplex and Varicella zoster viruses. J. Appl. Phycol. 13 (2), 127–134.
Iqbal, H.M.N., 2015. Development of Bio-composites with Novel Characteristics through Enzymatic Grafting. University of Westminster. http://www.westminster.ac.uk/westminsterresearch.
Iqbal, H.M.N., Asgher, M., Bhatti, H.N., 2011. Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of Trametes versicolor. BioResources 6 (2), 1273–1287.
Iribarren, D., Peters, J.F., Dufour, J., 2012. Life cycle assessment of transportation fuels from biomass pyrolysis. Fuel 97, 812–821.
Isikgor, F.H., Becer, C.R., 2015. Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym. Chem. 6 (25), 4497–4559.
Kauffman, N., Hayes, D., Brown, R., 2011. A life cycle assessment of advanced biofuel production from a hectare of corn. Fuel 90 (11), 3306–3314.
Kimming, M., Sundberg, C., Nordberg, Å., Baky, A., Bernesson, S., Norén, O., Hansson, P.-A., 2011. Biomass from agriculture in small-scale combined heat and power plants–A comparative life cycle assessment. Biomass Bioenergy 35 (4), 1572–1581.
Kristensen, J.B., Börjesson, J., Bruun, M.H., Tjerneld, F., Jørgensen, H., 2007. Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. Enzyme Microb. Technol. 40 (4), 888–895.
Kuila, A., Sharma, V., 2017. Lignocellulosic Biomass Production and Industrial Applications. John Wiley & Sons.
Kumar, D., Murthy, G.S., 2012. Life cycle assessment of energy and GHG emissions during ethanol production from grass straws using various pretreatment processes. Int. J. Life Cycle Assessment 17 (4), 388–401.
Kumar, S., Singh, S.P., Mishra, I.M., Adhikari, D.K., 2009. Recent advances in production of bioethanol from lignocellulosic biomass. Chem. Eng. Technol. 32 (4), 517–526.
Langlois, J., Sassi, J.-F., Jard, G., Steyer, J.-P., Delgenes, J.-P., Hélias, A., 2012. Life cycle assessment of biomethane from offshore-cultivated seaweed. Biofuels Bioprod. Biorefin. 6 (4), 387–404.
Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P., Bernard, O., 2009. Life-cycle assessment of biodiesel production from microalgae. Environ. Sci. Technol. 43 (17), 6475–6481.
Lask, J., Wagner, M., Trindade, L.M., Lewandowski, I., 2019. Life cycle assessment of ethanol production from miscanthus: a comparison of production pathways at two European sites. GCB Bioenergy 11 (1), 269–288.
Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog. Energy Combust. Sci. 38 (4), 449–467.
Lin, Z., Li, C., Ju, L.-K., 2019. Glycerol and acetate additions to maximize lipid content in high-density cultures of phagotrophic alga Ochromonas danica. J. Am. Oil. Chem. Soc. 96 (3), 231–238.
Liu, J., Huang, J., Sun, Z., Zhong, Y., Jiang, Y., Chen, F., 2011. Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic Chlorella zofingiensis: assessment of algal oils for biodiesel production. Bioresour. Technol. 102 (1), 106–110.
Loow, Y.-L., Wu, T.Y., Tan, K.A., Lim, Y.S., Siow, L.F., Md. Jahim, J., Mohammad, A.W., Teoh, W.H., 2015. Recent advances in the application of inorganic salt pretreatment for transforming lignocellulosic biomass into reducing sugars. J. Agric. Food. Chem. 63 (38), 8349–8363.
Manna, S., Saha, P., Chowdhury, S., Thomas, S., Sharma, V., 2017. Alkali treatment to improve physical, mechanical and chemical properties of lignocellulosic natural fibers for use in various applications. Lignocellulosic Biomass Prod. Indus. Appl. 47–63.
Marin, E., Briceño, M.I., Caballero-George, C., 2013. Critical evaluation of biodegradable polymers used in nanodrugs. Int. J. Nanomed. 8, 3071.
Medipally, S.R., Yusoff, F.M., Banerjee, S., Shariff, M., 2015. Microalgae as sustainable renewable energy feedstock for biofuel production. BioMed. Res. Int. 2015, 1–13. https://doi.org/10.1155/2015/519513.
Morales, M., Collet, P., Lardon, L., Hélias, A., Steyer, J.-P., Bernard, O., 2019. Life-cycle assessment of microalgal-based biofuel. Biofuels Algae 507–550.
Muduli, A., 2010. HR Issues in Upstream Oil and Gas Industry of India: Some Reflections. Available at SSRN 2804578.
Peng, W., Wu, Q., Tu, P., 2000. Effects of temperature and holding time on production of renewable fuels from pyrolysis of Chlorella protothecoides. J. Appl. Phycol. 12 (2), 147–152.
Peng, W., Wu, Q., Tu, P., Zhao, N., 2001. Pyrolytic characteristics of microalgae as renewable energy source determined by thermogravimetric analysis. Bioresour. Technol. 80 (1), 1–7.
Petersson, A., Thomsen, M.H., Hauggaard-Nielsen, H., Thomsen, A.-B., 2007. Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean. Biomass Bioenergy 31 (11–12), 812–819.
Prakash, A., Sharma, V., Kumar, D., Kuila, A., Sharma, A.K., 2017. Utilization of lignocellulosic biomass for biobutanol production. Lignocellulosic Biomass Prod. Ind. Appl. 247–263.
Prasad, A., Sotenko, M., Blenkinsopp, T., Coles, S.R., 2016. Life cycle assessment of lignocellulosic biomass pretreatment methods in biofuel production. Int. J. Life Cycle Assess. 21 (1), 44–50.
Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Appl. Microbiol. Biotechnol. 65 (6), 635–648.
Rafaschieri, A., Rapaccini, M., Manfrida, G., 1999. Life cycle assessment of electricity production from poplar energy crops compared with conventional fossil fuels. Energy Convers. Manage. 40 (14), 1477–1493.
Rebello, S., Anoopkumar, A., Puthur, S., Sindhu, R., Binod, P., Pandey, A., Aneesh, E.M., 2018. Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour. Technol. Rep. 3, 1–6.
Reddy, N., Yang, Y., 2005. Biofibers from agricultural byproducts for industrial applications. Trends Biotechnol. 23 (1), 22–27.
Roux, J.-M., Lamotte, H., Achard, J.-L., 2017. An overview of microalgae lipid extraction in a biorefinery framework. Energy Procedia 112, 680–688.
Saifuddin, N., Priatharsini, P., 2016. Developments in bio-hydrogen production from algae: a review. Res. J. Appl. Sci. Eng. Technol. 12 (9), 968–982.
Sander, K., Murthy, G.S., 2010. Life cycle analysis of algae biodiesel. Int. J. Life Cycle Assess. 15 (7), 704–714.
Sarkar, S., Kumar, A., 2010. Biohydrogen production from forest and agricultural residues for upgrading of bitumen from oil sands. Energy 35 (2), 582–591.
Sarkar, S., Kumar, A., Sultana, A., 2011. Biofuels and biochemicals production from forest biomass in Western Canada. Energy 36 (10), 6251–6262.
Sawayama, S., Minowa, T., Yokoyama, S.-Y., 1999. Possibility of renewable energy production and CO2 mitigation by thermochemical liquefaction of microalgae. Biomass Bioenergy 17 (1), 33–39.
Schau, E.M., Fet, A.M., 2008. LCA studies of food products as background for environmental product declarations. Int. J. Life Cycle Assess. 13 (3), 255–264.
Schwartz, R.E., Hirsch, C.F., Sesin, D.F., Flor, J.E., Chartrain, M., Fromtling, R.E., Harris, G.H., Salvatore, M.J., Liesch, J.M., Yudin, K., 1990. Pharmaceuticals from cultured algae. J. Ind. Microbiol. 5 (2–3), 113–123.
Sebastián, F., Royo, J., Gómez, M., 2011. Cofiring versus biomass-fired power plants: GHG (Greenhouse Gases) emissions savings comparison by means of LCA (Life Cycle Assessment) methodology. Energy 36 (4), 2029–2037.
Shabangu, S., Woolf, D., Fisher, E.M., Angenent, L.T., Lehmann, J., 2014. Techno-economic assessment of biomass slow pyrolysis into different biochar and methanol concepts. Fuel 117, 742–748.
Shafie, S., Mahlia, T., Masjuki, H., Rismanchi, B., 2012. Life cycle assessment (LCA) of electricity generation from rice husk in Malaysia. Energy Procedia 14, 499–504.
Shafie, S., Mahlia, T., Masjuki, H., 2013. Life cycle assessment of rice straw co-firing with coal power generation in Malaysia. Energy 57, 284–294.
Sharma, P.K., Saharia, M., Srivstava, R., Kumar, S., Sahoo, L., 2018. Tailoring Microalgae for Efficient Biofuel Production. Front. Mar. Sci. 5 (382).
Sharma, P., Sharma, N., 2017. Industrial and biotechnological applications of algae: a review. J. Adv. Plant Biol. 1 (1), 01.
Shi, R., Handler, R.M., Shonnard, D.R., 2019. Life cycle assessment of novel technologies for algae harvesting and oil extraction in the renewable diesel pathway. Algal Res. 37, 248–259.
Shirvani, T., Yan, X., Inderwildi, O.R., Edwards, P.P., King, D.A., 2011. Life cycle energy and greenhouse gas analysis for algae-derived biodiesel. Energy Environ. Sci. 4 (10), 3773–3778.
Singhvi, M., Gokhale, D., 2013. Biomass to biodegradable polymer (PLA). RSC Adv. 3 (33), 13558–13568.
Smullen, E., Finnan, J., Dowling, D., Mulcahy, P., 2019. The environmental performance of pretreatment technologies for the bioconversion of lignocellulosic biomass to ethanol. Renewable Energy 142, 527–534.
Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K., Tignor, M., Miller, H., 2007. Climate Change 2007: The Physical Science Basis Cambridge. Cambridge University Press, United Kingdom and New York, USA.
Solmaz, S., Okaygün, M., 2018. A novel technology for dewatering and pasteurization of digested water sewage sludge utilizing concentrated solar energy.
St Peter, A., Pietrak, M., 2010. Life cycle assessment of macro algae as a bio-fuel feedstock source. 2010 AIChE Spring Meeting, San Antonio, TX.
Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A., 2005. Principles and Applications of Soil Microbiology. Pearson Prentice Hall Upper Saddle River, NJ.
Trippe, F., Fröhling, M., Schultmann, F., Stahl, R., Henrich, E., 2010. Techno-economic analysis of fast pyrolysis as a process step within biomass-to-liquid fuel production. Waste Biomass Valorization 1 (4), 415–430.
Tseng, Y.-H., Mohanty, S.K., McLennan, J.D., Pease, L.F., 2019. Algal lipid extraction using confined impinging jet mixers. Chem. Eng. Sci.: X 1, 100002.
Ubando, A.T., Rivera, D.R.T., Chen, W.-H., Culaba, A.B., 2019. A comprehensive review of life cycle assessment (LCA) of microalgal and lignocellulosic bioenergy products

from thermochemical processes. Bioresour. Technol. 291, 121837.
Van Fan, Y., Klemes, J.J., Lee, C.T., 2018. Pre-and post-treatment assessment for the anaerobic digestion of lignocellulosic waste: P-graph. Chem. Eng. Trans. 63, 1–6.
Wu, W., Lei, Y.-C., Chang, J.-S., 2019. Life cycle assessment of upgraded microalgae-to-biofuel chains. Bioresour. Technol. 288, 121492.
Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y., 2011. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour. Technol. 102 (1), 159–165.
Yoo, G., Park, M.S., Yang, J.-W., 2015. Chapter 12 – chemical pretreatment of algal biomass. In: Pandey, A., Negi, S., Binod, P., Larroche, C. (Eds.), Pretreatment of Biomass. Elsevier, Amsterdam, pp. 227–258.
Zabed, H.M., Akter, S., Yun, J., Zhang, G., Awad, F.N., Qi, X., Sahu, J., 2019. Recent advances in biological pretreatment of microalgae and lignocellulosic biomass for biofuel production. Renew. Sustain. Energy Rev. 105, 105–128.
Zhang, X., Fevre, M., Jones, G.O., Waymouth, R.M., 2017. Catalysis as an enabling science for sustainable polymers. Chem. Rev. 118 (2), 839–885.
Zhang, C., 2019. Lignocellulosic ethanol: technology and economics, in: Alcohol Fuels-Current Technologies and Future Prospect, IntechOpen.

Energy, Environment, and Sustainability
Series Editors: Avinash Kumar Agarwal · Ashok Pandey

Binod Parameswaran
Sunita Varjani
Sindhu Raveendran *Editors*

Green Bio-processes

Enzymes in Industrial Food Processing

Springer

Chapter 15
Industrial Enzymes as Feed Supplements—Advantages to Nutrition and Global Environment

Sharrel Rebello, Divya Balakrishnan, A. N. Anoopkumar, Raveendran Sindhu, Parameswaran Binod, Ashok Pandey and Embalil Mathachan Aneesh

Abstract The relevance of enzyme fortification in animal feeds has been well established and exploited to generate a high income generating sector of industrial enzymes. Apart from aiding the better nutritional uptake and utility of the food constituents from the animal feed, the economic benefits gained by the production of better meat yield from livestock has prompted the acceptability of these enzymes in the feedstock. The current review outlines the various types of enzymes supplemented in animal feeds, their functional role and advantages of feed enzymes on animal growth and productivity. The effectiveness of feed enzymes in reducing the release of unused residual metabolites into the environment and the contributory role of these enzymes in diminishing the aftermaths of global warming are also discussed. The various strategies adopted in the individual and combinatorial generation of feed enzymes, their substrates and the characteristics of such feed enzymes are also presented in a concise manner. Finally, the advancements in the industrial production of feed enzymes and its supplementation are reviewed.

Keywords Feed enzymes · Applications · Advantages · Phytase

S. Rebello (✉) · A. N. Anoopkumar · E. M. Aneesh
Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Kerala, India
e-mail: sharrel.rebello@gmail.com

D. Balakrishnan
Cashew Exporters Promotion Council of India (CEPCI) Laboratory and Research Institute, Kollam 691001, India

R. Sindhu · P. Binod
Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum 695019, India

A. Pandey
CSIR-Indian Institute of Toxicology Research (CSIR-IITR), 31 MG Marg, Lucknow 226001, India

B. Parameswaran et al. (eds.), *Green Bio-processes*, Energy, Environment, and Sustainability, https://doi.org/10.1007/978-981-13-3263-0_15

15.1 Introduction

Enzyme supplementation in feed has gathered great momentum in the modern world due to the increased interest for improved nutritional quality of food. Research and relevance of feed enzymes primarily took its origin in the United States, emphasizing their nutritive benefits in barley supported food products (Jensen et al. 1957; Willingham et al. 1961). Early studies on phytases, for example, augmented the phosphorus availability on poultry diets which enhanced the growth performance and facilitated digestion (Singh 2008). Irrespective of the wide utility of enzymes in a wide array of products, research on feed enzymes is still at a state of infancy. The development of target specific enzymes is however, gaining momentum in the last 3 decades. More research carried out in fermentation and microbiological technologies resulted new opportunity to produce feed enzymes in more cost-effective and in commercial formulations (Ravindran 2013).

Enzymes otherwise nicknamed as the biological catalysts are proteins with a compact three-dimensional molecular structure and substrate specificity, capable of accelerating chemical reactions and released by biological cells (Aehle 2007). Apart from its common role of adding nutritive value to food; enzymes differ in their specific mechanisms of action, molecular characteristics, stability (demanding or disregarding protective layers), site of action and efficiency of catalytic role. Thus different recipes of enzyme preparations are utilized in different food products to meet the nutritive demand of the food consumer as specified by the nutritionist.

15.2 Types of Feed Enzymes

Poultry feed, as well as most of the food, occurs as a mixture of proteins, fats, carbohydrates and proteins; demanding the use of enzyme cocktails to enable the breakdown of its nutritive molecules and better absorption by consumers (Selle and Ravindran 2007). Thus generally feed enzymes can be basically classified as cellulase (ß-glucanases), phytases, proteases, lipases and galactosidases as enlisted in Table 15.1. Sometimes they are merely grouped as carbohydrases (lysing any carbohydrate), proteases (breaking proteins) and phytases (lysing phytate content) based on the target molecules they act on. Enzyme addition to feed may have a varied effect on different animals influencing the rates of nutrient utilization and digestive ability (Juanpere et al. 2005). The combinatorial use of multienzymes along with phytase augmented the nutrient utility and performance of broiler chickens compared to unsupplemented (Attia et al. 2012; Choct 2006; Rani et al. 2003). However, generalization of enzyme recipes for entire animals does not seem to be effective; as different organisms respond differently to enzyme supplementation. In rainbow trout, for instance, phytase supplemented with protease showed a reverse impact on the growth performance; whereas an enzyme mix of pectinase, phytase, cellulase or enzyme mix had an anti-nutritive effect on nutrient utilization (Yigit and Keser 2016; Yigit et al. 2018) (Table 15.2).

Table 15.1 List of feed enzymes and their targeted feedstuff

Enzyme	Target substrate	Target feedstuff
Phytases	Phytic acid	All plant-derived ingredients
β-Glucanases	β-Glucan	Barley, oats, and rye
Xylanases	Arabinoxylans	Wheat, rye, triticale, barley, fibrous plant materials
α-Galactosidases	Oligosaccharides	Soybean meal, grain legumes
Proteases	Proteins	All plant protein sources
Amylase	Starch	Cereal grains, grain legumes
Lipases	Lipids	Lipids in feed ingredients
Mannanases, cellulases, hemi cellulases pectinases	Cell wall matrix (fiber components)	Plant-derived ingredients, fibrous plant materials

Table 15.2 A list of some commercially available feed enzyme with their characteristics

Trade name of enzyme	Type of enzyme	Company	Characteristics
Ronozyme Hi Phos	Phytase	Ronozyme	Increases phosphorus availability
QUANTUM® Blue	Phytase	ROAL. AB Vista	release phytate bound nutrients
HiZyme P-5000	Phytase	Vetline India	Aid phosphorus use in poultry feeds
FINASE® EC	*E. coli* derived phytase	ROAL. AB Vista	Used in pig and poultry feeds
RONOZYME® ProAct	Protease	Ronozyme	Improve protein digestion
RONOZYME® RumiStar™	Amylase	Ronozyme	Improves corn utilization in dairy diets
ECONASE® GT	Beta-glucanase	ROAL. AB Vista	Used in barley- and oat-based diets for piglets and broilers
ECONASE® XT	Beta 1-4, endo-xylanase	ROAL. AB Vista	Maximise energy utilization in pig and poultry feeds
HiZyme (Multi-Enzyme)	Blend of enzymes	Vetline India	Aids better-feed utilization, increased growth rate and production in broilers
CBT XL	Combination of non-starch polysaccharide (NSP) Enzymes with Probiotics	V. Excel International	Combination of heat tolerant enzymes targeting feed with high oil and fat content, supplemented with probiotics
AveMix® enzyme	Phytases, protease and non non-starch polysaccharide (NSP) hydrolase	Avevebiochem	Aid in polysaccharide, protein and phytate digestion

Carbohydrases include NSP (Non-starch polysaccharides) degrading enzyme that degrade fiber or NSP found on the plant cell wall, constituents large feed enzyme segment. Certain enzymes included in the segment is xylanases, beta-glucanases, xyloglucanses, galactomannanases, pectinases and debranching enzymes such as arabinofuranosidases and ferulic acid esterases. Among these enzymes, most widely used and important enzyme classes are xylanases and arabinoxylans that constituents a major part of NSP as feed ingredients (Le et al. 2013). These enzymes reduce the anti-nutritional factors of NSP by degrading the soluble fiber to reduce gut viscosity and enhance the nutrient absorption. Moreover, the degradation of polysaccharides yields oligosaccharides that act as prebiotics which benefits the gut microflora. Another class of carbohydrases widely used in the poultry industry is amylase, used in fast-growing broilers to improve starch digestibility level. Moreover, amylase supplemented with cellulase results in the enhancement of milk yield and back fat thickness. Commercially available enzymes are derived from *Bacillus* of different species viz, *halmapalus*, *licheniformis* and *stearothermophilus* (Gessesse et al. 2011). One of the basic problems with starch is that is it harder to absorb in the gut in form of pelleted feed as opposed to crushed feed and starch digestion seems to be less effective in fast-growing modern boiler breeds than low growing breeds; necessitating amylase in their diet (Svihus and Hetland 2001).

The other important class of enzyme is protease, included in feed with the purpose of increasing protein hydrolysis and thus improving the nitrogen utilization (Oxenboll et al. 2011). Protein digestion enables the better amino acid availability and enhanced absorption of other valuable nutrients. Another advantage of protein digestion is the reduced release of undigested protein in manure thereby reducing the drastic environmental effects such as eutrophication and acidification.

Phytases are enzymes supplemented to break down the anti-nutrient factor phytate present commonly in animal feed; thereby enabling the better functional utility of biomolecules (Rebello et al. 2018). Such phytate breakdown will enable the mobilization of various nutrient minerals from the animal feed into its body for utilization (Rebello et al. 2017).

15.3 Mode of Action and Functional Role of Enzymes

The use of enzymes in animal feed is of great commercial importance. Consistent increase in the price of feed ingredients had been a major limitation in most of the developing countries. (http://www.enzymesinc.com/vegetarian-vs.-animal-derived-enzymes.html). The ultimate aim of adding enzymes is to improve growth and profitability through enhanced digestion of dietary components (protein, amino acids, starch, lipids, and energy) in ingredients. However, the acceptance of feed enzymes could also be due to the necessity of varied feed formulations (in the presence of different feedstuffs), development of homogenous nutritive content even in different batches of production, reduced manure output, lowering of water

content in excreta, better digestibility and absorption of nutrients, upliftment of gut immunity of animals and development of uniformly healthy animals (Bedford and Cowieson 2012; Collett 2012; Jaroni et al. 1999).

Different feed enzymes in the industry have different modes of action due to substrate specificity, yet their exact mechanism is still unclear. This would involve sometimes the degradation of anti-nutrient factors or undigestible bonds in feed or even the breaking of physical cell walls aiding nutrient release (Bedford and Partridge 2001). In such an environment, it normally results in an altered pattern of digestion and the necessity of endogenous proteins for digestion is greatly reduced (Cowieson et al. 2009). The subsequent reduction in intestinal weight, alterations in intestinal microflora has a direct effect on its constituent enzymes and it often enhances the digestion rates (Svihus 2010).

15.4 Benefits on Nutrition

Various animal experiments bring significant data indicating the limited digestibility of nutrient elements in the small intestine of poultry (Low and Longland 1990; Pettersson and Åman 1989). Therefore, sufficient enzyme production might be renowned as the major factor responsible for the aforesaid state. The feed enzymes used in poultry might change the nutritional profile of feed elements to increase the efficiency of egg and meat production (Bedford 2000). Hence, appropriate use of feed enzymes in poultry might enrich the nutrient content and dietary energy together with the economic benefits for the public (Pettersson and Åman 1989).

Recently, considerable interest has been shown in the use of phytase as a feed additive, as it not only increases the availability of phosphate in plants but also reduces environmental pollution. The type and amount of cereals included in the diet, the level of anti-nutritive factor in the cereal, the concentration of enzymes used, the type and age of the animal (younger animals respond better to enzymes than older animals), type of gut micro-flora in digestive track and finally the physiology of the bird (Khattak et al. 2006). Although the majority of research trials of feed enzyme were conducted on broilers, the responses of egg-laying hens to enzyme-supplemented feeds are also well-documented. The use of an enzyme complex containing carbohydrases and phytase improves the growth potential of broilers, egg-laying hens, ducks, and Japanese quail (Yang et al. 2010). Certain enzymes, such as cellulase, xylanase, phytase, etc., required for the digestion of NSPs and phytates are not produced by birds. Hence, supplementation of NSP degrading enzymes and phytase not only reduce the anti-nutritive effects of NSPs and phytates but releases some nutrients to be utilized by the birds (Attia 2012).

15.5 Economic Advantages

The extensive characteristic feature of the poultry sector has allowed the faster uptake of advanced technologies including the use of feed enzymes. The extended use of these feed enzymes in animal nutrition is becoming a most widely used practice to overcome the harmful impacts of anti-nutritional elements (Ravindran 2013). The global market of enzymes is rapidly raised up to 7% during 2015. Based on the regional perception, North America was documented as the prevalent consumer of enzyme products in the regional markets of Western Europe. Similarly, the demand for feed enzymes in Asia/Pacific region including Japan, China, and India have increased rapidly (Adrio and Demain 2014; Li et al. 2012). In addition to this, the feed enzyme consumption in China was promptly growing at an annual rate of 7.5% in 2013 (Li et al. 2012).

The current research on farming systems receives much more attention towards feed enzymes for animal nutrition (Choct 2006). The economic advantages of most of all the enzymes used for poultry nutrition are associated with the reduction of feeding cost. The supplementation of different kinds of enzymes such as proteases, carbohydrases, lipases, and phytases on diet might upsurge the production efficiency and quality of poultry (Bedford and Partridge 2001).

One of the major economic advantages of supplementing feed enzymes into poultry is the enhanced production performances (Chen et al. 2002). Previous studies have reported that 10% of the total production performance of broilers can be improved by using the aforesaid feed enzymes (Cowieson and Ravindran 2008). However, the performance of feed enzymes on animal nutrition absolutely depends on the quality and quantity of feedstuff used together with the environmental conditions (Acamovic 2001). Perić et al. in 2011 reported that the supplementation of multi-enzyme complex of feed enzymes in animal nutrition improved economic advantages of poultry including reduced production cost (Perić et al. 2011).

15.6 Environmental Benefits

The production of poultry over the world has been rapidly increased during the sixties; the livestock population is found to be nearly 4 billion animals, and 500 million tons of manure has been produced in every year (Oxenboll et al. 2011). The overproduction of poultry is considered as one of the major drivers for enhanced production of nitrogen fertilizers and the emission of the aforesaid nitrogen-containing compounds might generate adverse impacts over the environment by atmospheric and water pollution (Crutzen et al. 2016). In order to increase the utilization of nutrients and reduce environmental pollution caused by poultry waste, several factors in the animal feeding aspects must be documented.

Nitrogen-containing compounds from the poultry waste are considered as one of the major perspectives for air and water pollution (Kendall et al. 1999). A number

of environmental benefits have been offered when protease is used as an additive in animal nutrition. Oxenboll et al. in 2011 reported that the use of protease as an additive in animal feed could contribute predominantly to reduce the quantity of nitrous compounds enter into the aquatic environment together with the reduction of acidification and eutrophication (Carpenter et al. 1998; Erisman et al. 2007; Oxenboll et al. 2011). The addition of feed enzymes to broiler diet offered better protein digestibility together with the drastic reduction of Nitrogen output from the broiler waste (Charlton and Pugh 1995). Manipulation of specific feeding enzymes to animal nutrition has greatly affected excretion of Nitrogen in urine and feces (Parsons et al. 1992).

The emission of ammonia can be reduced by employing low protein diet and specific nutrition techniques in animal feeding (Backus et al. 1997). Abdel and Tahir in 2015 reported that the supplementation of phytase enzyme in broilers has significantly reduced the phosphorus pollution from poultry waste, (Abdel-Megeed and Tahir 2015). The administration of phytase enzyme in broiler chicken has made substantial effects on pH and phosphorous content of excreta. They also discussed that the feed enzyme supplementation might decrease feed cost for phosphorous supplementation and the importance of inorganic phosphorus as a non-renewable element for the sustainable farming (Abdel-Megeed and Tahir 2015) (Fig. 15.1).

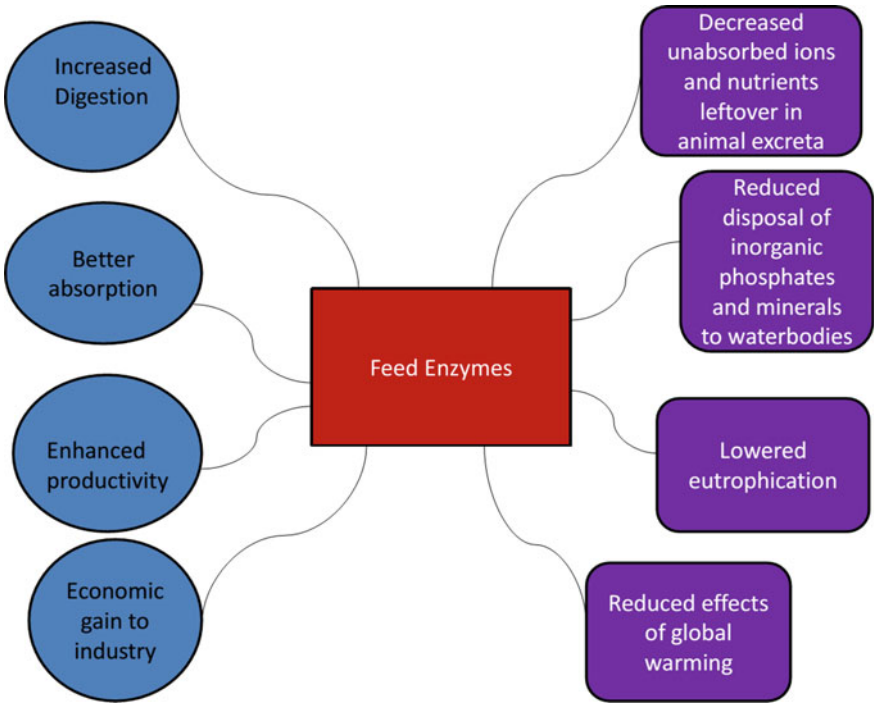


Fig. 15.1 Impacts of feed enzyme supplementation an overview

15.7 Industrial Production of Feed Enzymes

The industrial production of enzymes plays a significant role in modern biotechnology since the enzymes have been used in various biological systems including animal nutrition (Headon and Walsh 1994). The advanced developments in the biotechnology including genetic engineering have now enabled the industrial production of enzymes easy (Singhania et al. 2010). Hence the field of enzymology has been reflected as one of the most established branches of biotechnology. The discovery of novel enzymes together with the understanding of formerly reported enzymes and their functions offers several innovative applications for the industrial production and utilization of the aforesaid enzymes (Headon and Walsh 1994; Kirk et al. 2002; Volesky et al. 1984).

The amylases of plant and microbial (fungal and bacterial amylases) origin have played a major role in animal nutrition as feed enzymes since the industrial production of amylases offers low cost, consistency, and less time-consuming processes (Burhan et al. 2003). The solid-state fermentation (SSF) and submerged fermentation (SmF) is considered as the principal techniques for the rapid production of amylases at low cost (Hamliton et al. 1999; Haq et al. 1997; Pandey 2008). Several *Bacillus* bacterial species such as *Bacillus subtilis*, *B. licheniformis*, and *Bacillus stearothermophilus* are widely employed for the industrial production of α-amylases (Janeček 2002). The industrial sectors predominantly use *B. subtilis* as major producers for the large-scale production of proteases (Deviram et al. 2015). In addition to this, the microbial organisms from the genus *Aspergillus* have been also renowned as a good producer for the industrial production of α-amylases and proteases (Ward et al. 2009; Vihinen and Mantsiila 1989).

The Solid-state fermentation (SSF) technique has gained much more importance for the industrial production of α-Galactosidase due to its economic benefits such as improved enzyme yield, increased product stability, and lower cost (Hölker et al. 2004). Manipulation of several parameters such as pH, temperature, carbon sources, nitrogen sources, moisture, metal ions, and surfactants has been required for the large-scale production of feed enzymes (Ashraf et al. 2005; Burhan et al. 2003; Chandra et al. 1980; Ramesh and Lonsane 1990).

The fore-stomach tissue of lambs, pancreatic tissues of pigs, and plants can be used as an excellent source for the production of lipases. However, microbial lipases are presently receiving much more attention than others because of its economic benefits and technical advantages together with the drawbacks of using animal lipases (Vakhlu 2006). The bacteria, yeast, fungi, and actinomycetes are renowned as admirable sources lipases (Ertuğrul et al. 2007). Submerged culture, solid-state fermentation methods, and immobilized cell culture (used in rare cases) have been used for the large-scale production of lipases (Chen et al. 1999; Hemachander et al. 2001). Several previous studies have been conducted to understand the optimal nutritional requirements for the large-scale production of lipases using submerged culture since the production processes is strongly

influenced by several factors such as culture pH, dissolved oxygen concentration, temperature followed by the concentration of nitrogen and carbon sources (Elibol and Ozer 2000).

15.8 Conclusion

The practise of enzyme supplementation in feeds becomes very essential in the viewpoint of increased nutrition, better productivity, and yield of livestock. Apart from the nutritional benefits, the reduction in cost associated with waste management of poultry can be greatly reduced by enzyme addition in animal feeds. Moreover, the problems associated with increased environmental pollution and global warming can be addressed effectively by the practise of enzyme uptake by inhabitant animals grown in farms.

Acknowledgements Sharrel Rebello acknowledges SERB for National Post Doctoral Fellowship (File no. PDF/2015/000472). One of the authors Raveendran Sindhu acknowledges Department of Science and Technology for sanctioning a project under WOS-B scheme.

References

Abdel-Megeed A, Tahir A (2015) Reduction of phosphorus pollution from broilers waste through supplementation of wheat based broilers feed with phytase. J Chem (2015)

Acamovic T (2001) Commercial application of enzyme technology for poultry production. World's Poult Sci J 57:225–242

Adrio JL, Demain AL (2014) Microbial enzymes: tools for biotechnological processes. Biomolecules 4:117–139

Aehle W (2007) Enzymes in industry: production and applications. Wiley, New York

Ashraf H, Qadeer M, Iqbal J (2005) Pearl millet, a source of alpha amylase production by Bacillus licheniformis. Bioresour Technol 96:1201–1204

Attia YA, El-Tahawy WS, Abd El-Hamid AE-HE, Hassan SS, Nizza A, El-Kelaway MI (2012) Effect of phytase with or without multienzyme supplementation on performance and nutrient digestibility of young broiler chicks fed mash or crumble diets. Ital J Anim Sci 11:e56

Backus G et al (1997) Comparative study of four housing systems for non-lactating sows. In: Proceedings of 5th International Symposium Livestock Environment, Bloomington, Minnesota, pp 273–279

Bedford MR (2000) Exogenous enzymes in monogastric nutrition—their current value and future benefits. Anim Feed Sci Technol 86:1–13

Bedford M, Cowieson A (2012) Exogenous enzymes and their effects on intestinal microbiology. Anim Feed Sci Technol 173:76–85

Bedford MR, Partridge GG (2001) Enzymes in farm animal nutrition. CABI

Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. Process Biochem 38:1397–1403

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl 8:559–568

Chandra A, Medda S, Bhadra A (1980) Production of extracellular thermostable α-amylase by *Bacillus licheniformis*. J Ferment Technol 58:1–10
Charlton P, Pugh R (1995) Expanding enzyme applications: higher amino acid and energy values for vegetable proteins. Feed Compounder (United Kingdom)
Chen JY, Wen CM, Chen TL (1999) Effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*. Biotechnol Bioeng 62:311–316
Chen X, Zhang B, Fischer JA (2002) A specific protein substrate for a deubiquitinating enzyme: liquid facets is the substrate of Fat facets. Genes Dev 16:289–294
Choct M (2006) Enzymes for the feed industry: past, present and future. World's Poult Sci J 62:5–16
Collett SR (2012) Nutrition and wet litter problems in poultry. Anim Feed Sci Technol 173:65–75
Cowieson A, Ravindran V (2008) Effect of exogenous enzymes in maize-based diets varying in nutrient density for young broilers: growth performance and digestibility of energy, minerals and amino acids. Br Poult Sci 49:37–44
Cowieson A, Bedford M, Selle P, Ravindran V (2009) Phytate and microbial phytase: implications for endogenous nitrogen losses and nutrient availability. World's Poult Sci J 65:401–418
Crutzen PJ, Mosier AR, Smith KA, Winiwarter W (2016) N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. In: Crutzen PJ (ed) A pioneer on atmospheric chemistry and climate change in the anthropocene. Springer, Berlin, pp 227–238
Deviram G et al (2015) Production, optimization and partial purification of protease from Bacillus subtilis
Elibol M, Ozer D (2000) Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. Process Biochem 36:325–329
Erisman J, Bleeker A, Galloway J, Sutton M (2007) Reduced nitrogen in ecology and the environment. Environ Pollut 150:140–149
Ertuğrul S, Dönmez G, Takaç S (2007) Isolation of lipase producing Bacillus sp. from olive mill wastewater and improving its enzyme activity. J Hazard Mater 149:720–724
Gessesse A, Mulaa F, Lyantagaye S, Nyina-Wamwiza L, Mattiasson B, Pandey A (2011) Industrial enzymes for sustainable bio-economy: large scale production and application in industry, environment, and agriculture in Eastern Africa
Hamliton L, Fogarty W, Kelly C (1999) Puri fication and properties of the raw starch degrading α amylase of Bacillus sp. IMD 434. Biotechnol Lett 21:111–115
Haq I, Ashraf H, Ali S, Qadeer M (1997) Submerged fermentation of alpha amylase by Bacillus licheniformis GCB-36. Biologia (Bratislava) 43:39–45
Headon D, Walsh G (1994) The industrial production of enzymes. Biotechnol Adv 12:635–646
Hemachander C, Bose N, Puvanakrishnan R (2001) Whole cell immobilization of *Ralstonia pickettii* for lipase production. Process Biochem 36:629–633
Hölker U, Höfer M, Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl Microbiol Biotechnol 64:175–186
Janeček Š (2002) How many conserved sequence regions are there in the α-amylase family. Biologia 57:29–41
Jaroni D, Scheideler SE, Beck M, Wyatt C (1999) The effect of dietary wheat middlings and enzyme supplementation. 1. Late egg production efficiency, egg yields, and egg composition in two strains of Leghorn hens. Poult Sci 78:841–847
Jensen LS, Fry RE, Allred JB, McGinnis J (1957) Improvement in the nutritional value of barley for chicks by enzyme supplementation. Poult Sci 36:919–921
Juanpere J, Perez-Vendrell A, Angulo E, Brufau J (2005) Assessment of potential interactions between phytase and glycosidase enzyme supplementation on nutrient digestibility in broilers. Poult Sci 84:571–580

Kendall D et al (1999) Effects of fiber addition (10% soybean hulls) to a reduced crude protein diet supplemented with synthetic amino acids versus a standard commercial diet on pig performance, pit composition, odor and ammonia levels in swine buildings. J Anim Sci 77:176
Khattak F, Pasha T, Hayat Z, Mahmud A (2006) Enzymes in poultry nutrition. J Anim Plant Sci 16:1–7
Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351
Le DM, Fojan P, Azem E, Pettersson D, Pedersen NR (2013) Visualization of the anticaging effect of Ronozyme WX xylanase on wheat substrates. Cereal Chem 90:439–444
Li S, Yang X, Yang S, Zhu M, Wang X (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J 2:e201209017
Low A, Longland A (1990) Carbohydrate and dietary fibre digestion in the pig and the possible influence of feed enzymes. Feed Compd 10:37–42
Oxenboll K, Pontoppidan K, Fru-Nji F (2011) Use of a protease in poultry feed offers promising environmental benefits. Int J Poult Sci 10:842–848
Pandey B (2008) Biotechnology. APH Publishing
Parsons C, Hashimoto K, Wedekind K, Han Y, Baker D (1992) Effect of overprocessing on availability of amino acids and energy in soybean meal. Poult Sci 71:133–140
Perić L, Sartowska K, Milošević N, Đukić-Stojčić M, Bjedov S, Nikolova N (2011) The effect of enzymes on the economics of poultry meat and egg production. Maced J Anim Sci 1:113–117
Pettersson D, Åman P (1989) Enzyme supplementation of a poultry diet containing rye and wheat. Br J Nutr 62:139–149
Ramesh M, Lonsane B (1990) Critical importance of moisture content of the medium in alpha-amylase production by *Bacillus licheniformis* M27 in a solid-state fermentation system. Appl Microbiol Biotechnol 33:501–505
Rani BK, Desai J, Reddy D, Radhakrishna P (2003) Effect of supplementation of enzymes for non starch polysaccharides in corn-soya diet in broilers. Indian J Anim Nutr 20:63–69
Ravindran V (2013) Feed enzymes: the science, practice, and metabolic realities. J Appl Poult Res 22:628–636
Rebello S, Jose L, Sindhu R, Aneesh EM (2017) Molecular advancements in the development of thermostable phytases. Appl Microbiol Biotechnol 101:2677–2689
Rebello S, Anoopkumar A, Puthur S, Sindhu R, Binod P, Pandey A, Aneesh EM (2018) Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife. Bioresour Technol Rep
Selle PH, Ravindran V (2007). Microbial phytase in poultry nutrition. Anim Feed Sci Technol 2007 May 15;135(1-2):1-41
Singh P (2008) Significance of phytic acid and supplemental phytase in chicken nutrition: a review. World's Poult Sci J 64:553–580
Singhania RR, Patel AK, Pandey A (2010) The industrial production of enzymes. Industrial biotechnology: sustainable growth and economic success, pp 207–226
Svihus B (2010) Effect of digestive tract conditions, feed processing and ingredients on response to NSP enzymes. Enzym Farm Anim Nutr 129
Svihus B, Hetland H (2001) Ileal starch digestibility in growing broiler chickens fed on a wheat-based diet is improved by mash feeding, dilution with cellulose or whole wheat inclusion. Br Poult Sci 42:633–637
Vakhlu J (2006) Yeast lipases: enzyme purification, biochemical properties and gene cloning. Electron J Biotechnol 9
Vihinen M, Mantsiila P (1989) Microbial amylolytic enzyme. Crit Rev Biochem Mol Biol 24:329–418
Volesky B, Luong JH, Aunstrup K (1984) Microbial enzymes: production, purification, and isolation. Crit Rev Biotechnol 2:119–146

Ward OP, Rao MB, Kulkarni A (2009). Proteases, Production. Appl Microbiol Indust 495–511
Willingham HE, Fry RE, Leong KC, Jensen LS, McGinnis J (1961) Studies on stability of enzyme supplements to pelleting, long storage, and other treatments. Poult Sci 40:854–857
Yang J et al (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42:565
Yigit N, Keser E (2016) Effect of cellulase, phytase and pectinase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) fry fed diets containing canola meal. J Appl Ichthyol 32:938–942
Yigit NO, Bahadir Koca S, Didinen BI, Diler I (2018) Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (Oncorhynchus mykiss, Walbaum) fed soybean meal-based diets. J Appl Anim Res 46:29–32

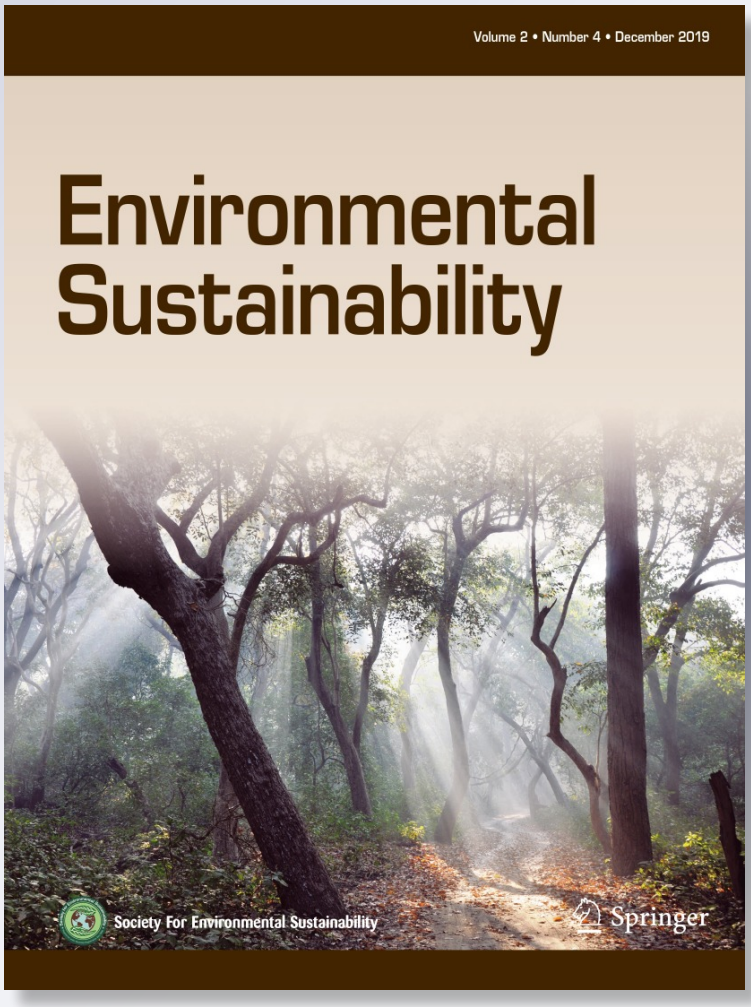
Synergistic control of storage pest rice weevil using Hypericum japonicum *and deltamethrin combinations: a key to combat pesticide resistance*

Sreedev Puthur, A. N. Anoopkumar, Sharrel Rebello & Embalil Mathachan Aneesh

Environmental Sustainability

e-ISSN 2523-8922
Volume 2
Number 4

Environmental Sustainability (2019)
2:411-417
DOI 10.1007/s42398-019-00086-w



Springer

Environmental Sustainability (2019) 2:411–417
https://doi.org/10.1007/s42398-019-00086-w

ORIGINAL ARTICLE

Synergistic control of storage pest rice weevil using *Hypericum japonicum* and deltamethrin combinations: a key to combat pesticide resistance

Sreedev Puthur[1] · A. N. Anoopkumar[1] · Sharrel Rebello[1] · Embalil Mathachan Aneesh[1]

Received: 31 October 2018 / Revised: 28 August 2019 / Accepted: 29 August 2019 / Published online: 5 September 2019

Abstract
The augmented rates of pest infestation in stored grains have forced humans to rely on pesticide application in stored food grains even in developed countries. The greater incidence and alarming rate of pesticide resistance and safety concerns about their use in the food industry also contribute to ill-being of grain consumers and environment, thereby alluring constructive attempts to reduce the levels of agrochemical use. The current research elucidated the synergistic role of *Hypericum japonicum* (a medicinal plant) and pyrethroid deltamethrin in controlling the most common storage pest rice weevil, *Sitophilus oryzae* even at low concentrations than recommended by FAO. The screening of pesticidal property of methanolic extract of *H. japonicum*, pyrethroid deltamethrin, and their combinatorial assay was evaluated by standard procedures under laboratory conditions and mortality was gauged after 24 h of exposure. The LC_{50} and LC_{90} concentrations of deltamethrin alone (0.725 mg/l and 3.577 mg/l respectively) and in combination with *Hypericum* methanolic extract (LC_{50} 0.119 mg/l and LC_{90} 1.27 mg/l respectively) were found to be potent. The study revealed that the supplementation of plant extract in the pest controlling formulation substantially reduced the effective individual LC_{90} concentration of the pesticide required for pest control. The plant extracts showed synergy towards deltamethrin with SF 6.09. This is the foremost report on the synergistic effect of *H. japonicum* with deltamethrin against rice weevil, which could serve as an effective and more safer storage pest control method against the indiscriminate pesticide use and abuse.

Keywords *Hypericum japonicum* · *Sitophilus oryzae* · Deltamethrin · Combinatorial bioassay

Introduction

The escalating population rates, as well as increased levels of plant raw material utility in industries, has always demanded the need for increased agricultural outputs worldwide (Ribeiro et al. 2003). Grain damage by pests has been a major test to the agricultural sector and the stored grain products being infested from the time of harvest to consumption. Majority of these pests belong to the coleopteran family and the most damaging candidates include *Sitophilus* Genus members (commonly called weevils) (Huignard et al. 2011; Tennyson et al. 2012). Rice weevil (*Sitophilus oryzae*) a serious pest of stored grains has a worldwide distribution especially in temperate areas, critically affecting the quality and quantity of the stored grains (Agarwal et al. 1979; Batta 2004). The damages caused by the pest alone accounts to 40% of the worldwide stored grain production (Mishra et al. 2013), as the adult weevils consume the endosperm of the grain and diminish its carbohydrate and protein content (Belloa et al. 2000).

In such a scenario, synthetic pesticides, especially contact insecticides are traditionally used to prevent and eliminate various insect pests (Cengiz et al. 2016). Deltamethrin is one such synthetic pyrethroid-based pesticide widely used in agriculture and veterinary applications for the management of rice weevils (Bekele et al. 2010; Vélez et al. 2017). Regardless of its benefits in pest control, the greatest resultant problem is that the continued application of synthetic pesticides leads to physiological resistance and undesirable ecological effects (Suresh et al. 2017). Moreover, the increasing pyrethroid resistance in grain weevils

✉ Embalil Mathachan Aneesh
aneeshembalil@gmail.com

[1] Communicable Disease Research Laboratory, Department of Zoology, St. Joseph's College, Irinjalakuda, Thrissur, India

also demands the use of higher pesticide concentrations to effectively control them (Salerno et al. 2002). Such chaos amidst the adverse effects of chemical control methods and need for quality pest free grains have envisaged the utility of plant-derived materials as alternative pest control agents, individually or in combination with pesticides at very low concentrations (de Andrade Dutra et al. 2016; Herrera et al. 2017). While some plant bioactives have a natural pesticidal role, others serve as repellants not harming the living organisms and most of them are found to be biodegradable (Mavundza et al. 2011; Sreedev 2016).

The present study has been undertaken to evaluate the synergistic property of methanolic extract of *H. japonicum* with pyrethroid deltamethrin for achieving the highest insecticidal activity with minimal pesticide usage. *H. japonicum* is a plant widely used as a traditional medicine in China and has great importance because it consists of many active compounds (Hosni et al. 2017; Li et al. 2007). The plant has been used for treatments of hepatitis, gastrointestinal disorders and tumors (Samaga and Rai 2013). Previous studies also proved that the methanolic extract of *H. japonicum* possesses significant larvicidal and anticancerous activity (Puthur et al. 2018), there by inviting more research interests in the plant. The current research paper primarily targets the effectiveness of deltamethrin and *H. japonicum* in the control of common rice pest *S. oryzae*. The study also tries to solve the burden of increased pesticide resistance incidence, by reducing the effective deltamethrin concentrations when used along with phytobased compounds.

Materials and methods

Details of sample

Samples of *H. japonicum* were collected from Pothumolla, Wayanad district with latitude 11°53′N and latitude 76°0′E, Kerala, India during June 2017 in the vegetative phase. Voucher specimens of the plant were maintained at CDRL Laboratory, Thrissur and authenticated by comparison with the corresponding herbarium documents. The adult rice weevil (*S. oryzae*) were collected from Chalakudy, Thrissur district Kerala. The pests were collected along with damaged rice grains and were properly stored in a container along with rice grains throughout the study period. Only the adult weevils were used for the subsequent experiment. The analytical grade Deltamethrin standard used for this study was procured from Sigma-Aldrich India.

Preparation of the plant extract

The collected plant material (*H. japonicum*) were washed, shade dried and powdered. The extraction of the plant was carried out using a Soxhlet apparatus by using methanol as an organic solvent. In reducing the pressure of 20–22 mmHg at 35 °C the solvent undergoes distillation by using a vacuum rotary evaporator. The extract was evaporated to complete aridness at normal room temperature and stored for further analysis (Anoopkumar et al. 2017).

Bioassay

Susceptibility tests were done against the pest using impregnated-paper assays on 80-mm diameter in glass petri dishes (Ali et al. 2012). Whatman No. 1 filter papers were cut into two halves and dipped into the five differently concentrated plant extract solutions for 5 s against distilled water as a control. After drying in a hood for 2 min, each paper was placed at the bottom of the Petri dish and adults of *S. oryzae* were introduced (into the Petri dish). Both the test and control beakers were placed under similar conditions of 25 ± 2 °C, 12 h light/dark regime, with no food for 24 h, and mortality was monitored. The pest was considered as dead if they were not responsive to gentle prodding with a fine needle. All the tests in the following experiments were done in triplicate using 20 individuals, and values were expressed as mean ± standard deviation. The results were expressed as percent mortality.

Similarly, deltamethrin stock solutions were diluted using distilled water and the susceptibility test was carried out with five varying concentrations using the above-mentioned protocol. Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated from a log dosage-probit mortality regression line using a computer software program (IBM SPPSS version. 17.0). Mortality was measured in percentage, and if the control mortality fluctuated, it was corrected using Abbott's formula (Abbott 1925).

Combinatorial bioassay

For combinatorial studies, varying concentrations of deltamethrin were prepared to range 1.125–2 mg/l. The LC_{50} value of plant extract obtained from the probit analysis was kept as a standard and each concentration of the pesticide formulation was mixed with the plant extract accordingly. Mortality obtained after 24 h were recorded. The resulting data from the observation were subjected to statistical analysis to calculate the LC_{50} and LC_{90} values. The synergistic factor (SF) (Kalyanasundaram and Das 1985) and the co-toxicity coefficient (CTC) (Sun and Johnson 1960) are also calculated.

Table 1 Efficacy of methanol extract of *H. japonicum* against *S. oryzae*

Extract	Concentration (mg/l)	Percent mortality	LC_{50} (LCL–UCL) (mg/l)	LC_{90} (LCL–UCL) (mg/l)	Chi square value	P value
Control	0	0	–	–	–	–
Plant extract	200	25				
	500	40	498.29 (603.82–725.48)	2507.79 (3567.24–6075.67)	3.680	0.15
	1000	60				
	2000	85				

LCL lower confidence limit, *UCL* upper confidence limit

*P < 0.15 significant difference at 1%

Table 2 Efficacy of deltamethrin against *S. oryzae*

Extract	Concentration (mg/l)	Percent mortality	LC_{50} (LCL–UCL) (mg/l)	LC_{90} (LCL–UCL) (mg/l)	Chi square value	P value
Control	0	0	–	–	–	–
Deltamethrin	0.125	10				
	0.25	20	0.725 (1.017–1.439)	3.577 (5.960–14.272)	5	0.50
	0.5	25				
	1	40				
	2	60				
	3	80				
	4	95				

LCL lower confidence limit, *UCL* upper confidence limit

*P < 0.50 significant difference at 5%

Results

Bioassay of individual components

The pesticide deltamethrin alone showed potent activity with LC_{50} and LC_{90} value of 0.725 mg/l and 3.577 mg/l causing the death of 50% and 90% of pest populations respectively in post-treatment. On the other hand, the plant extracts required a higher LC_{50} and LC_{90} value of 498.29 mg/l, 2507.79 mg/l respectively, to induce significant lethality of rice weevil. The bioassay results of methanolic extracts of *H. japonicum* and deltamethrin are summarized in Tables 1 and 2 respectively. The survival of the pest species varied significantly by doses and time of exposure. The LC_{50} value of plant extract was a bit on the higher side compared to deltamethrin. The survival values were found in good proportion to the concentration applied and ranged from 75 to 15% of low to a high dose of plant extract applied; whereas in deltamethrin, the values ranged from 90 and 10% for low to high doses. However, the survival rate in control was 100% throughout the experiment proving the pesticidal activity of the individual components considered in the above study. Figures 1

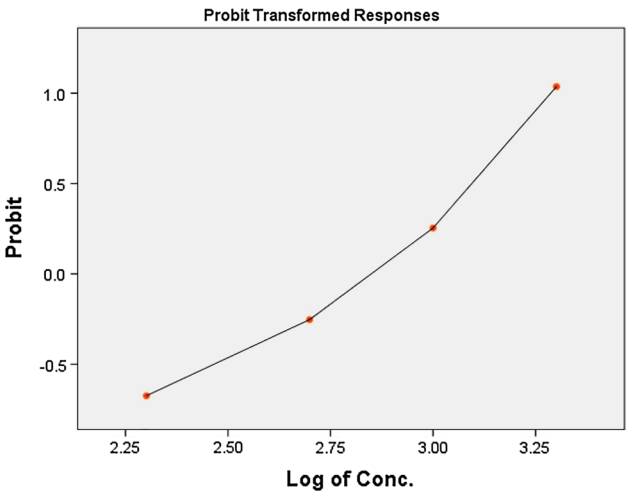


Fig. 1 Log concentration-percent mortality relationship of *S. oryzae* to *H. japonicum* methanolic extract

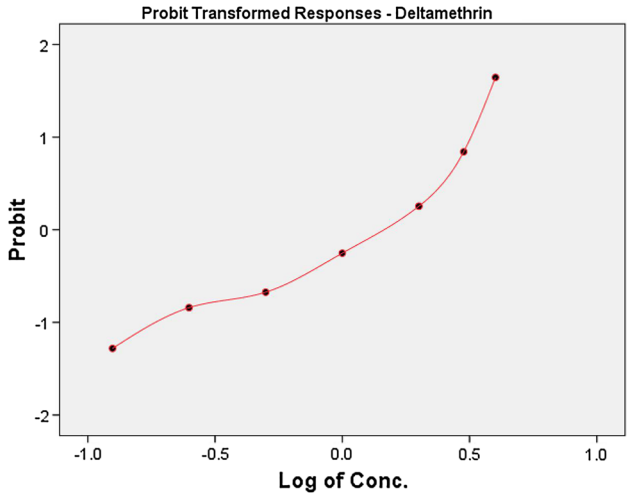


Fig. 2 Log concentration-percent mortality relationship of *S. oryzae* to deltamethrin

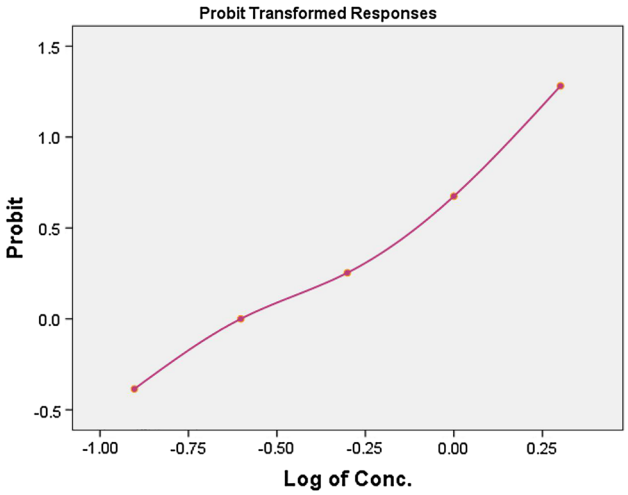


Fig. 3 Log concentration-percent mortality relationship *S. oryzae* to combinatorial bioassay

and 2 depict the log dose probit mortality responses of plant extract and deltamethrin against rice weevil.

Combinatorial bioassay

The combinatorial use of pesticide deltamethrin along with the methanolic plant extract yielded better pest control results compared to their individual effects against rice weevil as depicted in Table 3. Moreover, the study also succeeded in lowering the effective LC_{50} and LC_{90} values of deltamethrin to 0.119 mg/l and 1.27 mg/l respectively, when supplemented with *H. japonicum* phytoextract at a concentration of 498.29 mg/l (corresponding to its individual LC_{90} value) for a 24-h treatment. The combinatorial assay showed synergy with a synergistic factor of 6.09. The log dose probit mortality responses of combinatorial bioassay are shown in Fig. 3.

Discussion

S. oryzae (rice weevil) occurs as a cosmopolitan pest in rice, sorghum, maize, wheat and almost all grains with infestation caused by both adult and larvae (Srivastava and Subramanian 2016). The damage is characterized by the formation of an intact pericarp shell of grains when infested with rice weevils, leading to high levels of economic damage to stored crops worldwide (Hong et al. 2018). The individual use of deltamethrin was found effective in controlling rice weevils during this study, as high mortality rate was observed but the development of resistance is a major drawback that raises a caution. The growing pesticide resistance, especially against pyrethroids has been reported in a wide variety of pests attributed to knockdown resistance mutations in sodium channels and altered detoxification strategies (Haddi et al. 2018). Apart from pests, even vectors such as mosquitoes are reported to show pesticide resistance (Amelia-Yap et al.

Table 3 Synergistic effect of *H. japonicum* methanol extract and deltamethrin against *S. oryzae*

Extract	Concentration (mg/l)	Percent mortality	LC_{50} (LCL–UCL) (mg/l)	LC_{90} (LCL–UCL) (mg/l)	CTC	SF
Control	0	0	–	–	–	–
Plant extract + deltamethrin	498.2 + 0.125	35				
	498.29 + 0.25	50	0.119 (0.266–0.426)	1.271 (2.602–18.099)	87.5	6.09
	498.29 + 0.5	60				
	498.29 + 1	75				
	498.29 + 2	90				

LCL lower confidence limit, *UCL* upper confidence limit, *CTC* co-toxicity coefficient, *SF* synergistic factor

2018), thus demanding alternate strategies to control pests or reducing the unchecked usage of pesticides.

Literature survey revealed the exploration of various plant species for insecticidal properties. The lethal effect of *Datura alba* seed extract against onion thrips (Malik 2005), *Datura stramonium* leaf extract against *Tribolium castaneum* (Pascual-Villalobos and Robledo 1998) and *Datura fastuosa* extracts against rice weevil (Kamruzzaman et al. 2005) are some examples of herbal extracts against pests. *H. japonicum* is an annual herb widely distributed through Asia and used as traditional medicine for viral and bacterial disorders in China. This plant produces phloroglucinol flavonoid derivatives with potential antioxidant activity (Peng et al. 2006). References to the insecticidal activity of *Hypericum* species analyzed using essential oils was displayed in insecticidal action against *Brachiacantha dentipes* (Liu et al. 2007).

Significant mortality was shown by *H. japonicum* plant extract ($p < 0.15$) against *S. oryzae*, thereby raising its prospects as a herbal insecticide. The methanolic extract of *H. japonicum* comprises potent compounds which attribute its insecticidal activity (Puthur et al. 2018). There is a direct correlation between efficacy and concentration of phytoextract, the lethal effect was maximum at high concentration (Kuganathan et al. 2008). The present results agree with the studies conducted by Ali et al. (2012) in which *D. alba* leaf extracts active against *S. oryzae* and *Trogoderma granarium*.

Irrespective of its insecticidal properties, the individual use of botanicals face a major drawback of bio compound deterioration that promotes the prospects of synergistic use of plant extracts with insecticides (Nenaah 2011). Synergistic effects are defined as the action of multiple compounds on a pest that is greater than the action of individual compounds (Kumar and Parmar 1996; Scott et al. 2002). Muroi and Kubo (1993) reported the mechanism of synergy, such compounds will destroy the ability of the pest to metabolize toxins and leads to death. Plant extracts in combination with phenthoate and fenthion also showed significant synergism against malarial vector (Kalyanasundaram and Das 1985). Extracts of *Rhizophora apiculata*, *Caulerpa scalpelliformis* and *Dictyota dichotoma* showed synergism with synthetic insecticides against *Aedes aegypti* (Thangam and Kathiresan 1991).

According to the findings of the present study, methanolic extracts *H. japonicum* can be used as a natural weapon against stored grains pest *S. oryzae* and the extracts show synergy in combinational use with deltamethrin. Data verified by the above study thus suggests a combinatorial use of methanolic extracts of the plant at concentrations of 498.29 mg/l, and deltamethrin at 0.119 mg/l concentrations could serve as an effective decoction to reduce the pest population of stored grain by 50%. As per the regulations of FAO, pyrethroids are applied at a rate of 3 mg/l in stored grains to prevent infestation by pests (http://www.fao.org/docrep/t1838e/T1838E1g.htm). This would reduce the current pesticide use from 3 mg/l to lower concentrations gains relevance.

Our study revealed the pesticidal efficacy of *H. japonicum* plant extract, Deltamethrin and their combinatorial effect against *S. oryzae* with a synergy frequency of SF 6.09. This indicates that the insecticidal efficacy of the individual components is increased approximately seven times when used in combination. This method has the dual benefit in reducing the amount of synthetic insecticide and making the application more effective, the problems of insecticide resistance of pests can be overcome to an extent by using such control measures (Mohan et al. 2007; Morales-Rodriguez and Peck 2009). The exact mode of synergistic action is still not fully understood, however, previous studies conducted shows that plant secondary metabolites and synthetic pesticide will inhibit the acetylcholine esterase (AChE) activity of *S. oryzae* (Maazoun et al. 2017; Saad et al. 2018). This is the first report on synergistic and pest control role of *H. japonicum*. Thus synergistic combinations of *H. japonicum* methanolic extracts with substantially low concentrations of deltamethrin could be used as an effective solution for commercial grain storage. This investigation has the benefit of pesticide usage reduction maintaining environmental sustainability, and reducing the chance of developing toxicity to non-target organisms.

Acknowledgements We thank Principal, St. Joseph's College for the laboratory facilities provided.

Author contributions SP and EMA designed experiments and analyzed data. SP conducted experiments. ANA and SR did the statistical analysis. All authors contributed to writing the paper.

Funding We are greatly thankful to the University Grants Commission, New Delhi for providing funds under the UGC Research Award - F30-6/20-16(SA-II).

Compliance with ethical standards

Conflict of interest The Authors SP, ANA, SR, and EMA declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Abbott W (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267

Agarwal R, Singh K, Sirvastava P, Varma B (1979) Assessment of storage losses in wheat and different refractions in periodic arrivals in mandis of Haryana. Bull Grain Technol 17:202–204

Ali A, Ahmad F, Biondi A, Wang Y, Desneux N (2012) Potential for using *Datura alba* leaf extracts against two major stored grain pests, the khapra beetle *Trogoderma granarium* and the rice weevil *Sitophillus oryzae*. J Pest Sci 85:359–366

Amelia-Yap ZH, Chen CD, Sofian-Azirun M, Low VL (2018) Pyrethroid resistance in the dengue vector *Aedes aegypti* in Southeast Asia: present situation and prospects for management. Parasites Vectors 11:332. https://doi.org/10.1186/s13071-018-2899-0

Anoopkumar A, Puthur S, Rebello S, Aneesh EM (2017) Screening of a few traditionally used medicinal plants for their larvicidal efficacy against *Aedes aegypti* Linn (Diptera: Culicidae), a dengue fever vector. SOJ Microbiol Infect Dis 5(4):1–5

Batta Y (2004) Control of rice weevil (*Sitophilus oryzae* L., Coleoptera: Curculionidae) with various formulations of *Metarhizium anisopliae*. Crop Prot 23:103–108

Bekele J, Asmare K, Abebe G, Ayelet G, Gelaye E (2010) Evaluation of deltamethrin applications in the control of tsetse and trypanosomosis in the southern rift valley areas of Ethiopia. Vet Parasitol 168:177–184

Belloa G, Padina S, Lastrab C, Fabrizio M (2000) Laboratory evaluation of chemical biological control of rice weevil (*Sitophilus oryzae* L.) in store grain. J Stored Prod Res 37:77–84

Cengiz EI, Bayar AS, Kizmaz V (2016) The protective effect of vitamin E against changes in fatty acid composition of phospholipid subclasses in gill tissue of *Oreochromis niloticus* exposed to deltamethrin. Chemosphere 147:138–143

de Andrade Dutra K, de Oliveira JV, Navarro DMdAF, Santos JPO (2016) Control of *Callosobruchus maculatus* (FABR.) (Coleoptera: Chrysomelidae: Bruchinae) in *Vigna unguiculata* (L.) WALP. with essential oils from four *Citrus* spp. plants. J Stored Prod Res 68:25–32

Haddi K, Valbon WR, Viteri Jumbo LO, de Oliveira LO, Guedes RNC, Oliveira EE (2018) Diversity and convergence of mechanisms involved in pyrethroid resistance in the stored grain weevils, *Sitophilus* spp. Sci Rep 8:16361. https://doi.org/10.1038/s41598-018-34513-5

Herrera JM, Goñi ML, Gañan NA, Zygadlo JA (2017) An insecticide formulation of terpene ketones against *Sitophilus zeamais* and its incorporation into low density polyethylene films. Crop Prot 98:33–39

Hong K-J, Lee W, Park Y-J, Yang J-O (2018) First confirmation of the distribution of rice weevil, *Sitophilus oryzae*, in South Korea. J Asia Pac Biodivers 11:69–75. https://doi.org/10.1016/j.japb.2017.12.005

Hosni K, Msaaˆda K, Taârit MB, Marzouk B (2017) Fatty acid composition and tocopherol content in four Tunisian *Hypericum* species: *Hypericum perforatum*, *Hypericum tomentosum*, *Hypericum perfoliatum* and *Hypericum ericoides* Ssp. Roberti. Arab J Chem 10:S2736–S2741

Huignard J, Glitho IA, Monge J-P, Regnault-Roger C (2011) Insectes ravageurs des graines de légumineuses: Biologie des Bruchinae et lutte raisonnée en Afrique. Editions Quae, Versailles

Kalyanasundaram M, Das P (1985) Larvicidal and synergistic activity of plant extracts for mosquito control. Indian J Med Res 82:19–23

Kamruzzaman M, Shahjahan M, Mollah M (2005) Laboratory evaluation of plant extracts as antifeedant against the lesser mealworm, *Alphitobius diaperinus* and rice weevil, *Sitophilus oryzae*. Pak J Sci Ind Res 48:252

Kuganathan N, Saminathan S, Muttukrishna S (2008) Toxicity of *Datura alba* leaf extract to aphids and ants internet. J Toxicol 5:1559–3916

Kumar J, Parmar BS (1996) Physicochemical and chemical variation in neem oils and some bioactivity leads against *Spodoptera litura* F. J Agric Food Chem 44:2137–2143

Li J, Jiang B, Liu X, Zhang J, Chen X, Bi K (2007) Simultaneous determination of five bioactive flavonoids in *Hypericum japonicum* Thunb by high-performance liquid chromatography. Asian J Trad Med 2:75–81

Liu ZL, Goh SH, Ho SH (2007) Screening of Chinese medicinal herbs for bioactivity against *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst). J Stored Prod Res 43:290–296

Maazoun AM, Hlel TB, Hamdi SH, Belhadj F, Jemâa JMB, Marzouki MN (2017) Screening for insecticidal potential and acetylcholinesterase activity inhibition of *Urginea maritima* bulbs extract for the control of *Sitophilus oryzae* (L.). J Asia Pac Entomol 20:752–760

Malik MF (2005) Bionomic field studies and integrated pest management of thrips with species innovation through agro-ecosystem of onion (*Allium cepa*) in Balochistan, Pakistan. University of Balochistan, Quetta

Mavundza E, Maharaj R, Finnie J, Kabera G, Van Staden J (2011) An ethnobotanical survey of mosquito repellent plants in uMkhanyakude district, KwaZulu-Natal province, South Africa. J Ethnopharmacol 137:1516–1520

Mishra BB, Tripathi S, Tripathi C (2013) Bioactivity of two plant derived essential oils against the rice weevils *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae). Proc Natl Acad Sci India Sect B Biol Sci 83:171–175

Mohan L, Sharma P, Srivastava C (2007) Comparative efficacy of *Solanum xanthocarpum* extracts alone and in combination with a synthetic pyrethroid, cypermethrin, against malaria vector *Anopheles stephensi*. Southeast Asian J Trop Med Public Health 38:256

Morales-Rodriguez A, Peck DC (2009) Synergies between biological and neonicotinoid insecticides for the curative control of the white grubs *Amphimallon majale* and *Popillia japonica*. Biol Control 51:169–180

Muroi H, Kubo I (1993) Combination effects of antibacterial compounds in green tea flavor against *Streptococcus mutans*. J Agric Food Chem 41:1102–1105

Nenaah G (2011) Individual and synergistic toxicity of solanaceous glycoalkaloids against two coleopteran stored-product insects. J Pest Sci 84:77–86

Pascual-Villalobos M, Robledo A (1998) Screening for anti-insect activity in Mediterranean plants. Ind Crops Prod 8:183–194

Peng J, Fan G, Wu Y (2006) Preparative separation and isolation of three flavonoids and three phloroglucinol derivatives from *Hypericum japonicum* Thumb. using high-speed countercurrent chromatography by stepwise increasing the flow rate of the mobile phase. J Liq Chromatogr Relat Technol 29:1619–1632

Puthur S, Anoopkumar A, Rebello S, Aneesh EM (2018) *Hypericum japonicum*: a double-headed sword to combat vector control and cancer. Appl Biochem Biotechnol 186:1–11

Ribeiro B, Guedes R, Oliveira E, Santos J (2003) Insecticide resistance and synergism in Brazilian populations of *Sitophilus zeamais* (Coleoptera: Curculionidae). J Stored Prod Res 39:21–31

Saad MM, Abou-Taleb HK, Abdelgaleil SA (2018) Insecticidal activities of monoterpenes and phenylpropenes against Sitophilus oryzae and their inhibitory effects on acetylcholinesterase and adenosine triphosphatases. Appl Entomol Zool 53:173–181

Salerno G, Colazza S, Conti E (2002) Sub-lethal effects of deltamethrin on walking behaviour and response to host kairomone of the egg parasitoid *Trissolcus basalis*. Pest Manag Sci 58:663–668

Samaga PV, Rai VR (2013) Evaluation of pharmacological properties and phenolic profile of *Hypericum japonicum* Thunb. from Western Ghats of India. J Pharm Res 7:626–632

Scott I et al (2002) Insecticidal activity of *Piper tuberculatum* Jacq. extracts: synergistic interaction of piperamides. Agric For Entomol 4:137–144

Sreedev P (2016) A comparative analysis of larvicidal efficacy of *Piper longum* and *Ruta graveolens* against *Aedes aegypti* a dengue virus vector. Asia Pac J Res I(XXXVI):200–205

Srivastava C, Subramanian S (2016) Storage insect pests and their damage symptoms: an overview. Indian J Entomol 78:53–58

Sun Y-P, Johnson E (1960) Analysis of joint action of insecticides against house flies. J Econ Entomol 53:887–892

Suresh U et al (2017) Suaeda maritima-based herbal coils and green nanoparticles as potential biopesticides against the dengue vector *Aedes aegypti* and the tobacco cutworm *Spodoptera litura*. Physiol Mol Plant Pathol 101:225–235

Tennyson S, Ravindran KJ, Arivoli S (2012) Screening of twenty five plant extracts for larvicidal activity against *Culex quinquefasciatus* Say (Diptera: Culicidae). Asian Pac J Trop Biomed 2:S1130–S1134

Thangam TS, Kathiresan K (1991) Mosquito larvicidal activity of marine plant extracts with synthetic insecticides. Bot Mar 34:537–540

Vélez M, Barbosa W, Quintero J, Chediak M, Guedes R (2017) Deltamethrin-and spinosad-mediated survival, activity and avoidance of the grain weevils *Sitophilus granarius* and *S. zeamais*. J Stored Prod Res 74:56–65

Advanced Powder Technology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Advanced Powder Technology

journal homepage: www.elsevier.com/locate/apt

Original Research Paper

Acorus calamus mediated green synthesis of *ZnONPs*: A novel nano antioxidant to future perspective

Sreedev Puthur [a], K. Kavya Raj [a], A.N Anoopkumar [a], Sharrel Rebello [a], Embalil Mathachan Aneesh [b,*]

[a] Communicable Disease Research Laboratory (CDRL), St. Joseph's College, Irinjalakuda, Thrissur, Kerala, India
[b] Communicable Disease Research Laboratory (CDRL), Department of Zoology, St. Joseph's College, Irinjalakuda, Thrissur, Kerala, India

ARTICLE INFO

Article history:
Received 23 January 2019
Received in revised form 17 July 2019
Accepted 31 October 2020
Available online xxxx

Keywords:
Acorus Calamus
FTIR
ZnONPs
SEM
Antioxidant

ABSTRACT

Green synthesis of Nanoparticles gained much momentum because of its therapeutic uses and also has environmental benefits over traditional chemical methods. The present study describes the cost-effective eco-friendly synthesis of Zinc oxide nanoparticles from *Acorus Calamus* aqueous extract widely used as medicine in the traditional ayurvedic system of Kerala. The SEM analysis shows the synthesized *ZnONPs* are spherical and hexagonal wurtzite in structure and it was confirmed by the XRD data. The FTIR spectra revealed the role of Chemical moieties in the formation and stabilization of *ZnONPs*. The *Acorus Calamus ZnONPs* shows promising antioxidant activity and it shed light upon the role of phenolic compounds transferred from the plant during synthesis. The in vivo application of *ZnONPs* was very limited in the literature. The *Acorus calamus ZnONPs* may expect to serve as an alternative antioxidant supplement against disorders caused by oxidative stress and infections.

1. Introduction

The developments in the nanotechnology has opened up the novel and applied boundaries in therapeutic science. Nanoparticles owed much interest in industrial level because of their size, high surface ratio, and crystalline structure. Zinc oxide (ZnO), nanoparticles are metal oxides having the photocatalytic and photo-oxidizing capacity with biological species [1,2]-making it a prospective substance in medical areas. Moreover, ZnO NP has an implausible use in biological sensing, biological labeling, gene delivery, drug delivery and nanomedicine applications [3].

Zinc (Zn) was an essential mineral element in the animal kingdom for growth, immune function, metabolism, and oxygen free radical scavenging [4]. ZnO in low doses could be an alternative supplement form for zinc and it has a good biocompatibility to human cells over Zinc [5]. The ZnO nanocomposite has shown antibacterial and antifungal activity [6,7]. Various studies have proved that green synthesized nanoparticles have high biocidal efficacy against pathogens. ZnO nanoparticles have stronger chemical reactivity and undergo oxidation reactions with a variety of organic compounds and it can help to avoid adverse gastrointestinal reactions [8]. The use of chemical methods for nanoparticles synthesis was the common approach; however, it requires a high amount of chemicals, complex reactions and produces toxic byproducts. Recently, synthesis of NPs via eco-friendly routes has gained much momentum due to its low cost, non-toxicity and environmental compatibility.

Acorus calamus belonging to the family Acoraceae widely distributed in tropics and subtropics up to 1800 m altitude. It is one of the valuable plants in traditional Ayurveda medicine system, widely used in a number of infirmities like epilepsy, chronic diarrhea, bronchial catarrh, kidney and liver troubles, rheumatism, sinusitis and eczema [9,10]. The present study describes the green synthesis of ZnO NPs using *Acorus calamus*, characterization of the synthesized particles and its antioxidant properties.

2. Experimental details

2.1. Preparation of Acorus calamus plant extract

The fresh rhizome of *Acorus calamus* was collected from Wayanad District, Kerala, India. The rhizomes were washed with distilled water to remove dust particles and were shade dried at room temperature. 10gm of dried rhizome powder was boiled in 100 ml of distilled water for 20 min. The mixture was then cooled and filtered using Whatman No: 1 filter paper and stored at 4 °C for further use [11].

* Corresponding author.
E-mail address: aneeshembalil@stjosephs.edu.in (E.M. Aneesh).

https://doi.org/10.1016/j.apt.2020.10.016

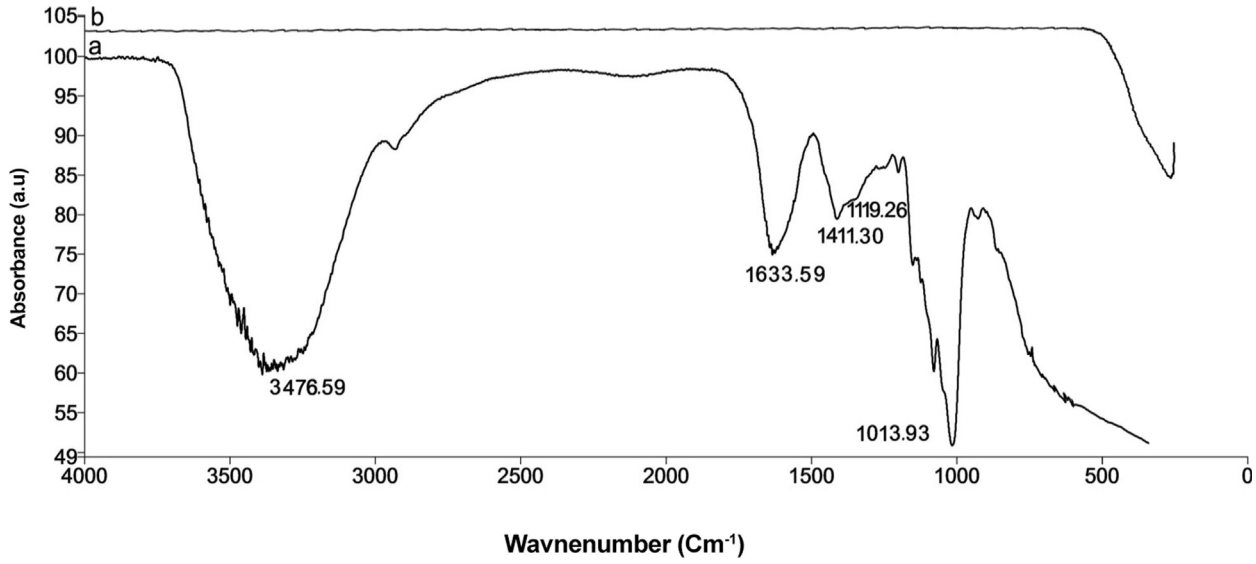


Fig. 1. FTIR spectra of (a) *Acorus calamus ZnONPs*, (b) pure ZnO NPs.

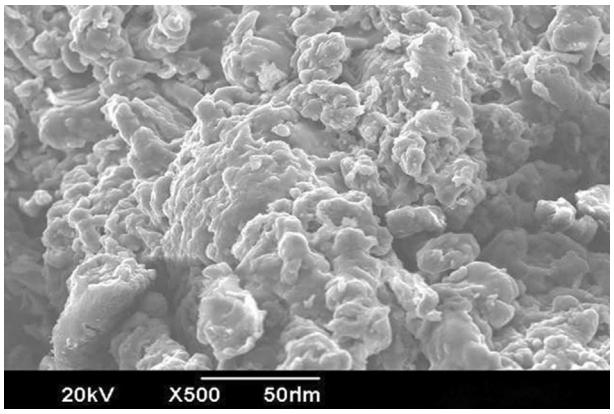


Fig. 2. FESEM image of *Acorus calamus ZnONPs.*

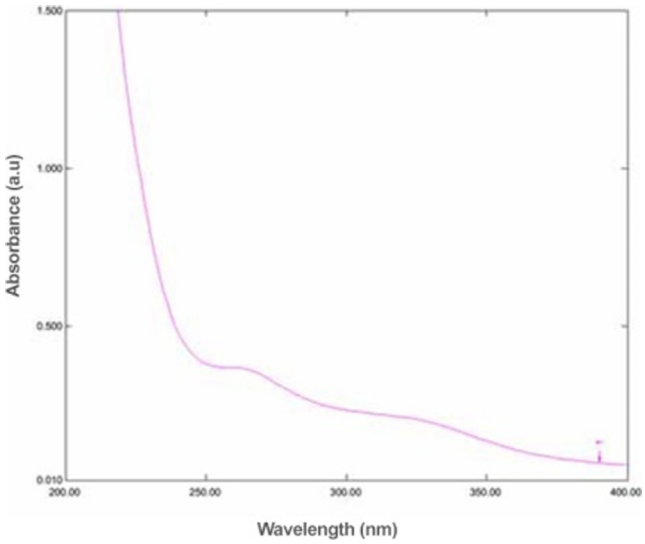


Fig. 3. UV–Vis absorption spectrum of the *Acorus calamus ZnONPs.*

2.2. Preparation of zinc nanoparticles

A 2 M solution of zinc acetate (10 ml) was mixed with 10 ml rhizome extract and boiled using a water bath. The temperature of the reaction mixture was maintained at 70 °C and the solution was agitated continuously for 30 min. An off-white precipitate formed, was then dried overnight at room temperature. The precipitate was washed with de-ionized water and annealing were carried out in a muffle furnace at 400 °C for 3 h [12].

2.3. Characterization of ZnONPs

The synthesized *ZnONPs* were characterized using UV–Vis spectrum, FTIR and SEM Analysis.

The UV–Vis spectrum of the synthesized ZnO nanoparticles was carried out on a UV– Vis Spectrophotometer (Shimadzu, India). The absorption maximum of the Nanoparticles was scanned between 100 nm and 400 nm. The chemical structure of *ZnONPs* was revealed using FTIR Spectrometer (PerkinElmer 1725x). The shape and size of synthesized ZnO nanoparticles were analyzed using a Scanning Electron Microscope (SEM) JSM-6390. The phase purity of the nanoparticle was determined using X-ray diffraction (XRD) analysis (Bruker AXS D8 Advance Twin Twin).

2.4. Test for antioxidant property

The total antioxidant capacity of *ZnONPs* was determined using the method described by Prieto[13]. *ZnONPs* were made up to different concentrations ranging from 62.5 to 1000 μg/l. Ascorbic acid was used as a standard; the ascorbic acid stock solution (1000 μg/L) was prepared in distilled water. In a series of test tubes 1 ml of different concentrations of *ZnONPs* were taken and mixed with 3 ml of the reaction mixture (0.6 M sulfuric acid, 4 M ammonium molybdate, and 28 M sodium phosphate) all test tubes were covered and incubated at 95 °C for 90 min. The mixture was then cooled and absorbance was recorded at 765 nm spectrophotometrically. The total antioxidant capacity of *ZnONPs* was expressed as ascorbic acid equivalent (AAE) per ml.

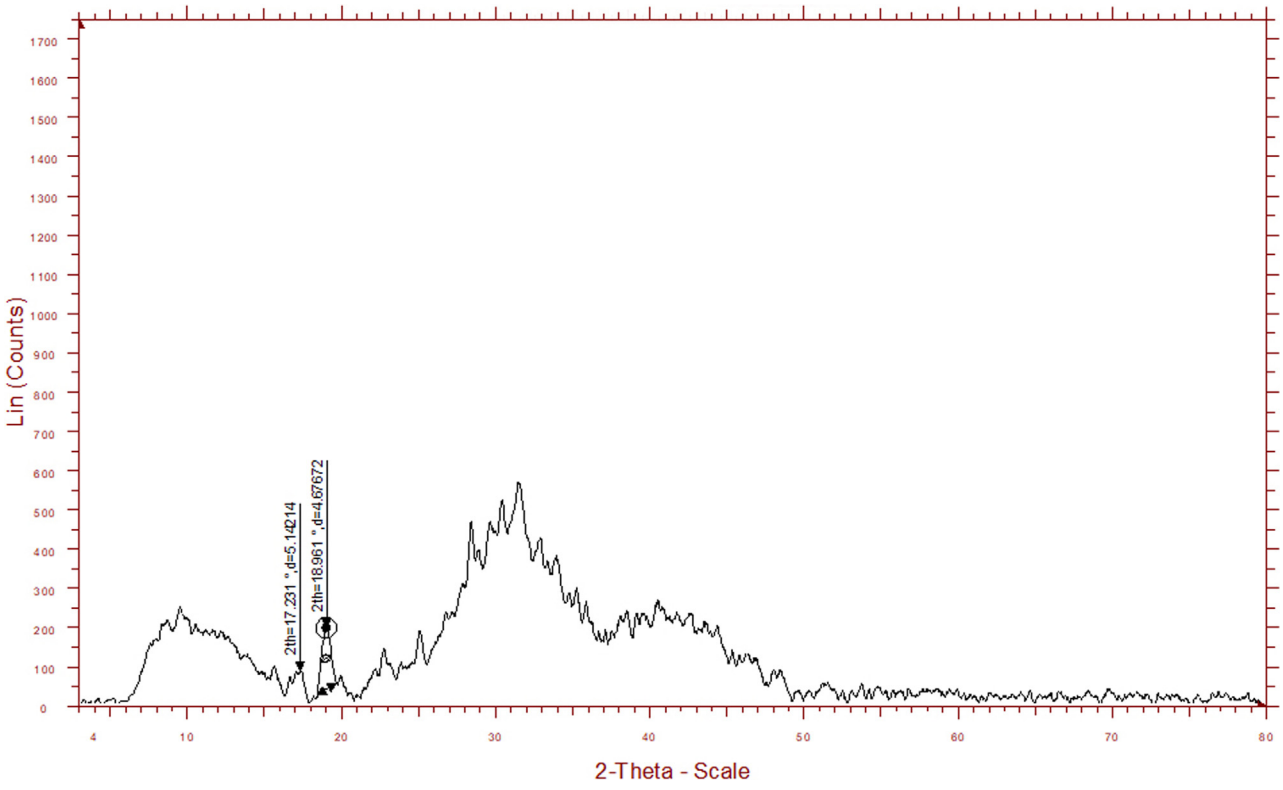


Fig. 4. XRD pattern of *Acorus calamus ZnONP.*

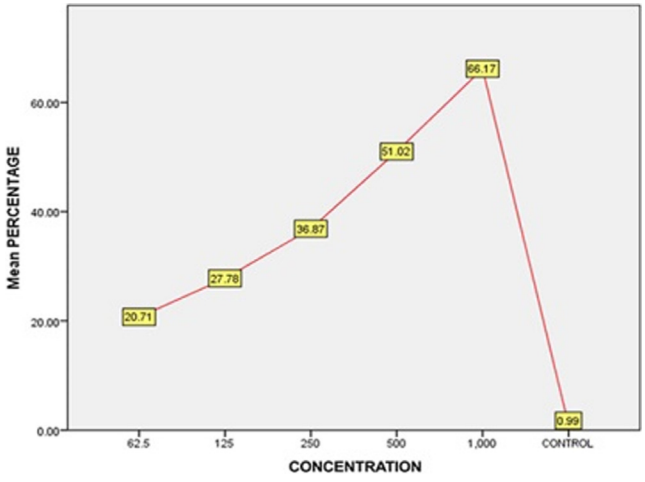


Fig. 5. Total Antioxidant activity of the *Acorus calamus ZnONPs.*

3. Results and discussion

The formation of *ZnONPs* was confirmed by way of visual examination. The colour of the reaction mixture was changed from dark brown to off-white in color during the end of the reaction, which indicates the formation of *ZnONPs.* The functional sulfate and cyclic peptide groups play a vital role in the formation and stabilization of *ZnONPs* [14].

FTIR confirmed the structure of *Acorus calamus* nanoparticles with band at 3476.59, 1633.59, 1411.30, 1199.26, 1013.93 cm^{-1} Fig. 1(a) displays the FTIR spectrum of *Acorus calamus ZnONPs,* in which the band at 1200–1300 cm^{-1} was not visible indicating the disappearance of stretching vibrations of sulfate groups and denotes the role of sulfate group in the formation of nanoparticles. The band intensity around 3476.59 to 1013.93 cm^{-1} indicates the C-O vibration. Another band at 1633.59 cm^{-1} is associated with the stretching vibrations of (NH) C═O group that are characteristics of proteins indicating the role of cyclic peptides involved in stabilizing the nanoparticles [15]. The signal at 448 cm^{-1} corresponds to the stretch band of zinc and oxygen. The FTIR spectrum of pure *ZnONPs* only shows a band at 430 $cm^{-1.}$

Fig. 2 represents the SEM image of synthesized nanoparticles; the pure *ZnONPs* formed were agglomerated with spherical and hexagonal wurtzite structures and the particle size ranging from 1 nm to 50 nm with some deviations. This agglomeration is probably due to polarization and electrostatic attraction of ZnO nanoparticles [16].

The UV–vis absorption spectrum of the pure ZnO nanoparticles was presented in Fig. 3 a high absorption peak in the 334 nm wavelength indicates the *ZnONPs* crystals basic band gap absorption due to the electron transitions from the valence band to the conduction band (O2p-Zn3d) [17].

Fig. 4 represents the XRD image of synthesized nanoparticles positions of the peaks was perfectly matching with cardnumber36-1451 of the Joint Committee on Powder Diffraction Standard (JCPDS). The peak intensity profile clearly indicates the wurtzite structure of synthesized *ZnONPs.* The mean particle sizes of the nanoparticles were estimated from FWHM using the Scherrer's equation [12]. The X-ray Wave length (1.5406A1), Bragg diffraction angle corresponding to the (18.96) plane, and full width at half maximum (FWHM) of the (18.96) plane, respectively. The mean particle size of the pure ZnO NPs was around 35 nm, which is well complemented with the measured Crystal diameter obtained from FESEM images.

Fig. 5 represents the graph exhibiting the total antioxidant activity evaluated using phosphomolybdate method. The *ZnONPs* has displayed high reducing capability of Mo (VI) to Mo (V) indicate its antioxidant properties [18]. The total antioxidant capacity

of ZnO-NPs was found to be 66% at 1000 μg/ml when compared with the standard ascorbic acid it was found to be 99%. This antioxidant activity may be due to the coating of the phenolic group present in plant extract on *ZnONPs* metal surface [19].

4. Conclusions

In the present investigation revealed an economical and an eco-friendly way to develop *ZnONPs* from aqueous extracts of *Acorus calamus*. FTIR analysis obtained was pointed out the role of sulfate groups in the formation of *ZnONPs*. The synthesized nanoparticles have spherical and hexagonal wurtzite structures and the particle size ranging from 1 nm to 50 nm with significant antioxidant activity. The *Acorus calamus ZnONPs* is expected to have prominent application as an antioxidant in pharmaceutical products and also in biomedical, cosmetic industries.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Principal, St. Joseph's College for the laboratory facilities provided.

References

[1] L. Vayssieres et al., Three-dimensional array of highly oriented crystalline ZnO microtubes, Chem. Mater. 13 (12) (2001) 4395–4398.
[2] R. Könenkamp et al., Nano-structures for solar cells with extremely thin absorbers, Physica E 14 (1–2) (2002) 219–223.
[3] J.R. Morones et al., The bactericidal effect of silver nanoparticles, Nanotechnology 16 (10) (2005) 2346.
[4] Y.-Z. Fang, S. Yang, G. Wu, Free radicals, antioxidants, and nutrition, Nutrition 18 (10) (2002) 872–879.
[5] N. Padmavathy, R. Vijayaraghavan, Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study, Sci. Technol. Adv. Mater. 9 (3) (2008) 035004.
[6] O. Yamamoto, Influence of particle size on the antibacterial activity of zinc oxide, Int. J. Inorg. Mater. 3 (7) (2001) 643–646.
[7] J. Sawai, T. Yoshikawa, Quantitative evaluation of antifungal activity of metallic oxide powders (MgO, CaO and ZnO) by an indirect conductimetric assay, J. Appl. Microbiol. 96 (4) (2004) 803–809.
[8] C.-Y. Zhao et al., Effects of dietary zinc oxide nanoparticles on growth performance and antioxidative status in broilers, Biol. Trace Elem. Res. 160 (3) (2014) 361–367.
[9] H. Imam et al., Sweet flag (Acorus calamus Linn.): An incredible medicinal herb, Int. J. Green Pharmacy (IJGP) 7 (4) (2013).
[10] P.K. Mukherjee et al., Plants of Indian origin in drug discovery, Expert Opin. Drug Discov. 2 (5) (2007) 633–657.
[11] M. Altikatoglu et al., Green Synthesis of Copper Oxide Nanoparticles Using Ocimum basilicum Extract and Their Antibacterial Activity, Fresenius Environ. Bull. 26 (12) (2017) 217–222.
[12] H.A. Salam, R. Sivaraj, R. Venckatesh, Green synthesis and characterization of zinc oxide nanoparticles from Ocimum basilicum L. var. purpurascens Benth.-Lamiaceae leaf extract, Mater. Lett. 131 (2014) 16–18.
[13] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. 269 (2) (1999) 337–341.
[14] S. Azizi et al., Green biosynthesis and characterization of zinc oxide nanoparticles using brown marine macroalga Sargassum muticum aqueous extract, Mater. Lett. 116 (2014) 275–277.
[15] G. Sangeetha, S. Rajeshwari, R. Venckatesh, Green synthesis of zinc oxide nanoparticles by aloe barbadensis miller leaf extract: Structure and optical properties, Mater. Res. Bull. 46 (12) (2011) 2560–2566.
[16] E.K. Goharshadi et al., Ultrasound-assisted green synthesis of nanocrystalline ZnO in the ionic liquid [hmim][NTf2], Ultrason. Sonochem. 16 (1) (2009) 120–123.
[17] A.K. Zak et al., Starch-stabilized synthesis of ZnO nanopowders at low temperature and optical properties study, Adv. Powder Technol. 24 (3) (2013) 618–624.
[18] A.T. Khalil et al., Sageretia thea (Osbeck.) mediated synthesis of zinc oxide nanoparticles and its biological applications, Nanomedicine 12 (15) (2017) 1767–1789.
[19] T. Santhoshkumar et al., Green synthesis of titanium dioxide nanoparticles using Psidium guajava extract and its antibacterial and antioxidant properties, Asian Pacific J. Trop. Med. 7 (12) (2014) 968–976.

Bioresource Technology Reports 3 (2018) 1–6

Contents lists available at ScienceDirect

Bioresource Technology Reports

journal homepage: www.journals.elsevier.com/bioresource-technology-reports

Zinc oxide phytase nanocomposites as contributory tools to improved thermostability and shelflife

Sharrel Rebello[a], A.N. Anoopkumar[b], Sreedev Puthur[a], Raveendran Sindhu[c], Parameswaran Binod[c], Ashok Pandey[d], Embalil Mathachan Aneesh[a,*]

[a] Communicable Disease Research Laboratory, St Joseph's College, Irinjalakuda, Kerala, India
[b] Department of Zoology, Christ College, Irinjalakuda, University of Calicut, Kerala, India
[c] Microbial Processes and Technology Division, CSIR-National Institute of Interdisciplinary Science and Technology (CSIR-NIIST), Trivandrum 695 019, India
[d] CSIR - Indian Institute of Toxicology Research (CSIR-IITR), 31 MG Marg, Lucknow 226 001, India

ARTICLE INFO

Keywords:
Antimicrobials
Lactobacillus
Penicillium
Enzymes
Genotyping

ABSTRACT

The current study suggests the utility of ZnO nanoparticles to increase the thermotolerance of phytase enzymes; thereby aiding their effective utilization to provide better phosphate uptake when applied in animal feeds. Microbial isolates with predominant phytase activity were isolated from industrial wastewater to obtain a promising thermotolerant isolate of *Penicillium decumbens*. The purified phytase showed activities at mash preparatory temperature (32.59 ± 0.045 $Uml^{-1} min^{-1}$ at 55 °C), animal feed pelletising temperature (37.83 ± 0.127 $Uml^{-1} min^{-1}$ at 80 °C) and steam sterilization temperature (18.56 ± 0.027 $Uml^{-1} min^{-1}$ at 100 °C) of animal feeds as per standard phytase assays. The supplementation of ZnO nanoparticles found to increase thermostability of phytase from 18.56 $Uml^{-1} min^{-1}$ to 30.5 $Uml^{-1} min^{-1}$ at 100 °C. The antibacterial role of the nanocomposites was checked against human pathogens to obtain satisfactory results against standard antibiotics. Thus the incorporation of ZnO nanoparticles in combination with phytase provided a dual benefit of increasing its thermostability and antimicrobial property thereby increasing its shelflife.

1. Introduction

Phytases have gained much attention as a prominent feed and fodder enzyme accounting to about 83.6% of the global market in 2015 (https://www.millioninsights.com/). The relevance of phytases in nutrition, increased mineral uptake, better energy generation and cleaving of antinutrient dietary elements has greatly increased the industrial demand for these enzymes (Rebello et al., 2017). Apart from increasing the phosphate uptake, nutrition and growth of phytase supplemented animals in a farm, phytase in animal diet can avoid the need of supplementing easily absorbable inorganic phosphates. The animals also nutritionally get benefited by better absorption of Ca, Zn, Fe, N_2 maintenance and effective utilization of proteins (Lei and Stahl, 2000). Almost 60% of the feed phosphate including phytate as well as myoinositol hexakisphosphate can be hydrolyzed to inorganic phosphate in a stepwise reaction, enabling effectual consumption of phosphates. Studies also reveal that a single unit of the livestock industry of US could yearly contribute almost 100,000 metric tonnes of nitrogen and almost one-fourth phosphorus (Mallin and Cahoon, 2003). As per reported animal farms often face the burden of phosphate-rich manure disposal, which sometimes account for almost 5% of the production costs (Jones, 2012). Thus phytase appears as a life and environment replenishing enzyme with good applicability in cattle feeds.

Traced from its discovery in 1907, phytases have advanced and appeared around the market as either natural or genetically engineered counterpart superior for its better catalytic efficiency and enzymatic potential (Lei et al., 2013). Irrespective of occurrence of various phytases in the market, the need of thermostable enzymes becomes essential as the process of sterilization and pelletisation often denatures the enzyme. Comparative analysis of various phytases indicates that almost 30% of its activity is lost post-exposure to temperatures above 80 °C (Nampoothiri et al., 2004; Sato et al., 2016). Though there are reports of increased thermotolerance in some of the recombinant phytases derived from commonly found *Aspergillus niger* (Ushasree et al., 2015) and a beetle fungus (Tan et al., 2016), commercialized production is still underway. Metabolic industrial approaches and mutational analysis of natural variants of phytases are also attempted to obtain better enzymes (Qvirist et al., 2017). Apart from this, studies also indicate that various physical techniques like enzyme addition after sterilization of animal feed, supplementation of extraneous agents such

* Corresponding author.
E-mail address: aneeshembalil@gmail.com (E.M. Aneesh).

https://doi.org/10.1016/j.biteb.2018.05.007
Received 6 March 2018; Received in revised form 11 May 2018; Accepted 22 May 2018
Available online 26 May 2018
2589-014X/

Table 1
Screening of different microbial isolates for phytase activity.

Sno	Site of water effluent sampling	Isolate ID	Phytase activity ($Uml^{-1} min^{-1}$)	Taxonomic identification
1	Appollo Tyres, Kalamaserry	A	4.8 ± 0.17	*Aspergillus niger*
2	IRE, Kochi	B	2.1 ± 0.50	*Fusarium oxysporum*
3	Synthite	C	4.6 ± 0.04	*Penicillium chrysogenum*
4	FACT	D	12.9 ± 0.24	*Penicillium decumbens*
5	Neta Gelatin	E	8.2 ± 0.31	*Saccharomyces cerevesiae*
6	Kerala Pharmaceuticals, Athani	F	1.3 ± 0.08	*Aspergilllus niger*
7	KMML, Kollam	G	2.5 ± 0.14	*Pichia stiptitis*

as magnetite chitosan (Onem et al., 2016) and immobilization techniques can be used to increase enzyme thermotolerance (Cho et al., 2011). The current article outlays the screening, identification of thermotolerant phytases from *Penicillium decumbens* an environmental isolate and the use of ZnO nanoparticles to improve thermotolerance of its phytase.

2. Materials and methods

2.1. Screening and molecular identification of potent phytase producer

Microbial isolates with predominant phytase activity were isolated from industrial wastewater from four different companies of Kerala, India as enlisted in Table 1. Wastewater was enriched by inoculating 1 ml of water in 50 ml of phytate minimal medium (glucose-15 gl^{-1}, NH_4NO_3-5 gl^{-1}, KCl-0.5 gl^{-1}, $MgSO_4{\cdot}7H_2O$-0.5 gl^{-1}, $FeSO_4{\cdot}7H_2O$-0.01 gl^{-1}, $MnSO_4{\cdot}7H_2O$-0.01 gl^{-1}) provided with 1% rice bran as phytate supplement and incubated at room temperature of 28 °C overnight for enrichment of phytase producing isolates. Overnight grown cultures were spread plated onto sodium phytate (0.05 gl^{-1}) supplemented mineral agar based medium (with sodium phytate replacing rice bran in the above media) to obtain well isolated colonies after incubation at 28 °C for 2–3 days.

Individual colonies of seven fungal isolates (designated A, B, C,…G) obtained from different sites were individually inoculated in sodium phytate based minimal media, incubated at 28 °C for 3 days and assessed for its phytase activity (Heinonen and Lahti, 1981). Briefly, 50 µl of the culture supernatants were treated with 10 mM sodium phytate in acetate buffer and incubated at 55 °C for 30 min. The enzyme reaction after incubation was stopped by using 5% Trichloroacetic acid (TCA) and treated with 4 ml of colouring reagent solution (containing 5% ammonium molybdate in 5 N H_2SO_4 mixed in equal proportions with twice the volume of acetone). The inorganic phosphates liberated by phytase action, formed complexes with ammonium molybdate of the colouring reagent and were estimated spectrophotometrically at 400 nm against standard concentrations of 50 mM KH_2PO_4 to express the enzyme activity as $Uml^{-1} min^{-1}$. The enzyme extract primarily treated with TCA and mixed with reaction mix served as a negative control. One unit of phytase activity was expressed as the amount of enzymes required to produce 1 µmol of inorganic phosphate per ml per unit time of the reaction.

The different isolates were screened for the extent of their phytase activity and the best isolate D showing highest phytase activity was selected for further study. The isolate was phenotypically characterized based on its macroscopic and microscopic appearance (Hoog et al., 2000). The genotypic identification of the isolate was done using 18S rRNA PCR using universal eubacterial primers targeting 18S rRNA region of fungi. The PCR product was purified, sequenced, analyzed using the gapped BLAST against the NCBI database and DNA sequence was deposited in its genebank database.

2.2. Phytase purification, thermostability assay and resistance to gastric digestion

The phytase enzymes of isolate D was isolated using its cell-free extract obtained after 5 day incubation in phytate based media, precipitated by ammonium sulphate precipitation at 4 °C overnight, pelleted by centrifugation at 2800*g* for 30 min at 4 °C and was redissolved in phosphate buffered saline (PBS) after desalting with a sephadex based PD-10 desalting column (GE Healthcare) against PBS. The desalted protein was further purified by column chromatography on a DEAE (Diethylaminoethyl)-Sepharose CL-6B column and eluted with various salt gradients in elution buffer (200 mM Tris–HCl, pH 8.0). Sephadex G-100 gel filtration column pre-equilibrated with 200 mM acetate buffer (pH 5.0), was used for desalting and separation of proteins based on size. Fractions showing high absorbance at 280 nm were assayed for phytase activity. Aliquots of the protein were used for SDS-PAGE electrophoresis and the thermostability assays. The thermostability of the enzyme extracts was assessed by incubating it at temperatures viz. 37 °C, 55 °C, 80 °C and 100 °C for respective time durations. A long incubation at of 30 min was used for 55 °C and 37 °C whereas an incubation of 10 min was used at 80 °C and 100 °C considering the chance of inactivation and the lesser time exposure of enzyme to a higher temperature during animal feed processing. The units of phytase activity were estimated as described earlier. The usual time of steam exposure of animal feed pellets at 100 °C is around 90 s (https://www.cpm.net/downloads/Animal%20Feed% 20Pelleting.pdf).

The suitability of phytase in physiological conditions and resistance to gastric digestion was estimated by their incubation in simulated gastric juice and assessment of phytase activity post incubation at 37 °C for 30 min. Simulated gastric juice was prepared using 125 mM sodium chloride, 7 mM potassium chloride, 45 mM sodium bicarbonate and 3 gl^{-1}pepsin adjusted to a final pH of 2.5 as explained in another study (Zárate et al., 2000).

2.3. ZnO nanoparticle biosynthesis

The biosynthesis of ZnO nanoparticles was done using curd derived *Lactobacillus* strains obtained on its selective media (De Man, Rogosa and Sharpe agar, abbreviated as MRS agar), which was further used in the synthesis of nanoparticles as per modified protocol depicted in Fig. 1 (Selvarajan and Mohanasrinivasan, 2013). Briefly, log phase culture of *Lactobacillus* was mixed with 0.4 M NaOH and 0.1 M $ZnSO_4$ in equal volumes each and heated in a microwave for 1–2 min to produce nanoparticles. The settled nanoparticles were further washed with deionized water several times, centrifuged to collect them, homogenized and dried overnight to obtain a fine powder. The formation of nanoparticles was further verified by UV–Vis spectroscopy (Systronics, India) and the structural analysis of the nanoparticles was done using Scanning Electron Microscopy (Carl Zeiss, Germany).

2.4. Phytase-nanocomposite thermostability assays

The nanoparticles of different concentrations were treated with

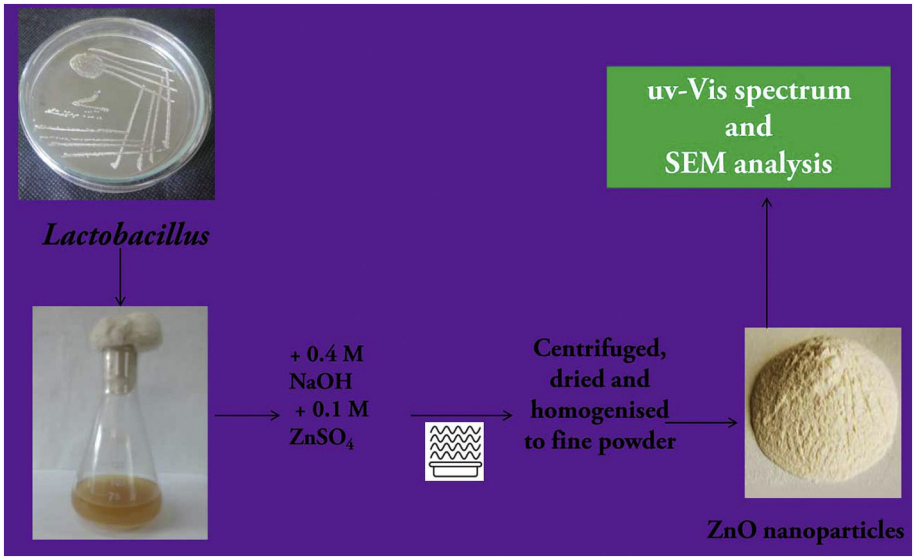


Fig. 1. Combinatorial use of *Lactobacillus* and microwaves in ZnO nanoparticle synthesis an outline.

phytase enzyme produced by isolate D (selected for study) and subjected to phytase assay as explained in Section 2.1. Variant concentrations of the nanoparticles viz. 1000 μg ml^{-1}, 500 μg ml^{-1}, 100 μg ml^{-1}, 40 μg ml^{-1}, 20 μg ml^{-1}, 10 μg ml^{-1}, 5 μg ml^{-1} and 2.5 μg ml^{-1} were treated with phytase enzyme. Briefly, 50 μl of the enzyme (with activity of 30 Uml^{-1} min^{-1}) were treated with 40 μl of nanoparticles of variant concentrations and 10 mM sodium phytate in acetate buffer and incubated at 55 °C for 30 min. The extent of phytase activity was analyzed at different temperatures (37 °C, 55 °C, 80 °C and 100 °C) and the level of inorganic phosphates liberated were assessed as per ammonium molybdate mediated phytase assay by spectrophotometric analysis at 400 nm.

2.5. Antimicrobial role of nanocomposites

Primary antimicrobial activity studies were done by agar well diffusion by inoculating test organisms (*Escherichia coli* MTCC 443, *Salmonella typhi* MTCC 733, and *Klebsiella pneumoniae* MTCC 109) with turbidity equal to 0.5 McFarland on Muller Hinton Agar (MHA) plates. The antimicrobial testing was done following the principles of the two fold dilution techniques at different range of ZnO nanoparticles (viz, 80 μg ml^{-1}, 40 μg ml^{-1}, 20 μg ml^{-1}, 10 μg ml^{-1}, 5 μg ml^{-1}, 2.5 μg ml^{-1}) to find the minimum inhibitory range of nanoparticles against the microbes. Fifty microlitre each of ZnO nanoparticles dissolved in DMSO at different concentrations (viz, 80 μg ml^{-1}, 40 μg ml^{-1}, 20 μg ml^{-1}, 10 μg ml^{-1}, 5 μg ml^{-1} and 2.5 μg ml^{-1}) was poured in each agar wells cut on microbe seeded MHA plates, pre-incubated at room temperature for half an hour till the nanoparticles diffused from wells and were further incubated at 37 °C for 18 h. The zone of inhibition of microbial growth around each agar well was noted after 18 h of incubation along with suitable positive control of ampicillin (40 mg ml^{-1}). The antibacterial activity was calculated by measuring zone of inhibition produced by nanoparticle against pathogenic bacteria.

The minimum inhibitory concentration (MIC) against the test microbes was further verified using microtitre plate method by using two fold dilution techniques initially taking concentrations of 20 μg ml^{-1}, 10 μg ml^{-1}, 5 μg ml^{-1}, 2.5 μg ml^{-1} of ZnO dissolved in DMSO in working volumes as per results obtained from agar well diffusion experiments. Briefly, 100 μl of each dilution of ZnO nanoparticles obtained after dilution was treated with 100 μl of test culture with absorbance equivalent to 0.5 Mc Farlands absorbance, incubated at 37 °C for 24 h and the absorbance at 620 nm was taken after incubation. The extent of inhibition was verified with respective positive and negative controls.

3. Results

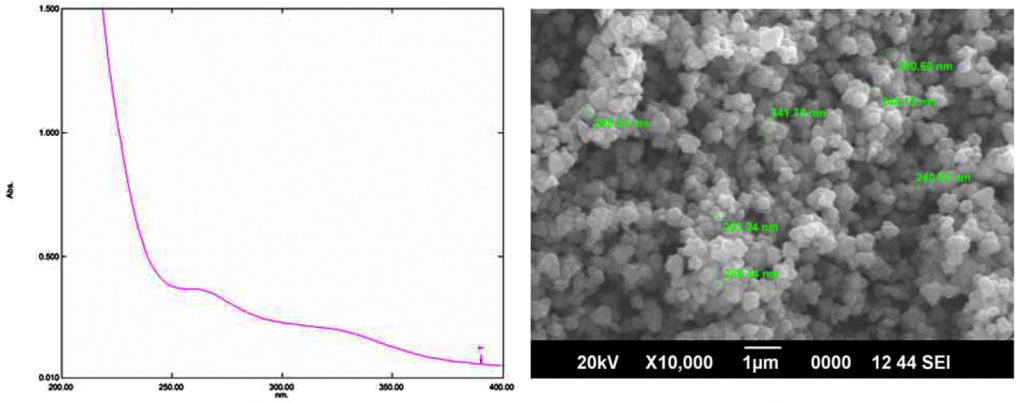
3.1. Identification of potent phytase producer

Seven phytase producing fungal isolates (A–G) were isolated from industrial effluents of seven companies located in Kerala after incubation on phytase based agar medium as noted in Table 1. The sites explored in the study reported to have different fungi (except for *Aspergillus niger* common at two sites) with different phytase production properties which formed the basis for further selection of isolates. The absence of phytase degrading bacteria was noted in all the sites indicating the predominant phytase production by fungi in these sites. Of the various phytase degrading isolates, the isolate D gave the maximum phytase activity after 3 days of incubation in phytate based broth media as per phytase assay. Thus the isolate D was selected, further analyzed for phytase production and thermostability studies.

The growth of the isolate D in liquid media initiated with formation of button likes globules of the fungus to fill the entire flask by seven days of incubation. Microscopic examination after staining of the isolate revealed the presence of spore characteristic of *Penicillium* sp. The sequenced 18S rRNA region of the isolate was deposited in the NCBI database with accession number MG386503 and analyzed by BLAST tool. The genotypic typing by 18S rRNA typing further confirmed the isolate D to be *Penicillium decumbens* as it showed 99% similarity to *Penicillium decumbens*.

3.2. Synthesis and characterization of ZnO nanoparticles

Biosynthesis of ZnO at nanoscale was done using *Lactobacillus* strains with the aim of providing an extra antimicrobial and probiotic role to enzyme ZnO nanocomposites. The formation of nanoparticles was screened using UV–Vis spectroscopy to obtain an absorption peak at around 260 nm. Scanning Electron Microscopy Analysis revealed the formation of globose nanoparticles in the range of 240 nm to 300 nm as shown in Fig. 2. The formation of nanoparticles was done with microwaves and *Lactobacillus* requiring limited processing time than other techniques needing longer duration of incubation of substrates with the microbes.



(A) (B)

Fig. 2. Characterization of nanoparticles by UV–Vis spectroscopy and SEM analysis.

3.3. Phytase-ZnO thermotolerance and resistance to gastric digestion

Phytase of *Penicillium decumbens* in the current study was thermostable till 80 °C with an enzyme activity of 37.83 ± 0.127 $Uml^{-1}\,min^{-1}$; but at a temperature of 100 °C its activity dropped. The presence of prominent activity at 80 °C indicated the thermophilic nature of the isolate; but inactivation at still higher temperatures demanded modes to improve its thermotolerance. The formation of ZnO-phytase nanoparticles at concentrations from 10 µg ml^{-1} till 40 µg ml^{-1} showed a beneficiary role on the activity of phytase aiding to improve its thermotolerance as noted in Table 2. However, higher ZnO nanoparticle concentrations were found to be inhibitory to phytase activity. As noted in Fig. 3, the formation of ZnO-phytase nanocomposites at merely 10 µg ml^{-1} concentrations aided to improve its thermotolerance at 100 °C.

The phytase enzyme was also found to be active at physiological conditions at 37 °C adding an extra-advantage to its utility in animal feeds; as most often enzymes derived from thermophiles seldom work at lower physiological temperatures. The utility of the phytase in physiological conditions of stomach with pH 2, presence of digestive enzymes such as pepsin was evaluated in simulated gastric juice. The purified enzyme was also found to exhibit an enzyme activity of 30.52 ± 0.08 $Uml^{-1}\,min^{-1}$ at 37 °C in simulated gastric juice; thus indicating its suitability and effectiveness for in vivo applications through animal feed incorporation. The activity of the enzyme was also found to be unaffected at pH of 2.5, indicating its suitability to function at physiological conditions.

3.4. Antimicrobial activity

The antimicrobial role of the ZnO nanoparticles were evaluated both by agar well diffusion and microtitre plate method to get similar results. ZnO nanoparticles were effective antibacterial agents against *Escherichia coli* MTCC 443, *Salmonella typhi* MTCC 733 and *Klebsiella pneumoniae* MTCC 109. The bacterial growth was totally inhibited on

Table 2
Effect of different concentrations of ZnO nanoparticles on phytase activity.

Nanoparticle concentration (µg ml^{-1})	Phytase activity at 37 °C ($Uml^{-1}\,min^{-1}$)
1000	10.43 ± 0.12
500	12.5 ± 0.20
100	20.89 ± 0.04
40	31.02 ± 0.15
20	32.20 ± 0.02
10	32.61 ± 0.05
5	32.12 ± 0.25
2.5	32.4 ± 0.01

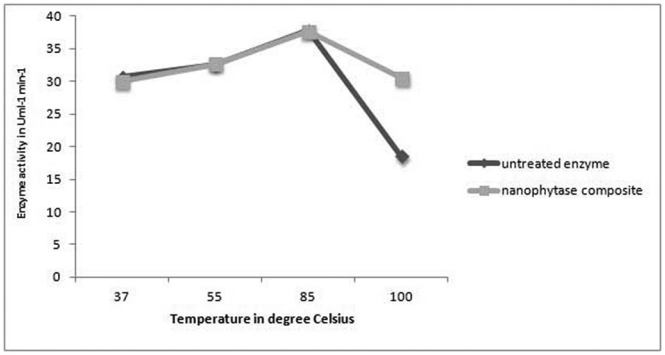


Fig. 3. Effect of ZnO treatment (10 µg ml^{-1}) on phytase activity at 100 °C.

Muller Hinton agars plates when treated with ZnO nanoparticles at high concentrations beyond 20 µg ml^{-1}. The agar well diffusion experiments indicated that concentrations of the nanocomposites at 40 µg ml^{-1} caused lysis of all three pathogens; leaving behind a clear agar plate after incubation. Thus the antimicrobial experiments on microtitre plates were conducted with ZnO nanoparticles at concentrations of 20 µg ml^{-1}, 10 µg ml^{-1}, 5 µg ml^{-1}, 2.5 µg ml^{-1} of ZnO; also taking into consideration the factor of enzyme inhibition at higher ZnO concentrations. The MIC_{50} refers to the minimum concentration of the compound that causes 50% inhibition to the test organism, whereas MIC_{90} refers to the minimum concentration of compound that causes 90% inhibition to the test organism as noted in previous studies (Cheng et al., 2017). The minimum inhibitory concentration (MIC_{50}) of the nanoparticles targeting the pathogens under this study could be commonly considered approximately as 10 µg ml^{-1}; whereas a minimum inhibitory concentration (MIC_{90}) of 20 µg ml^{-1} of ZnO is needed to inhibit almost the 90% population of the three cultures studied as observed in Fig. 4. The study also showed that similar antimicrobial activity was shown by the phytase-nanocomposites also indicating that interactions with phytase didn't hinder the antimicrobial activity of ZnO.

4. Discussion

The positive impacts of phytase supplementation in animal feeds such as reduced global warming, lesser environmental pollution, and better nutritional uptake; greatly relies on its residual phytase activity post sterilization of animal feeds. However, most of the phytases lose their phytase activity during pelleting processing; needing alternate techniques to add to their thermostability. Studies indicate the presence of different phytase producing microbes including bacterial isolates such as *Bacillus* (Yu and Chen, 2013), *Geobacillus* (Parhamfar et al.,

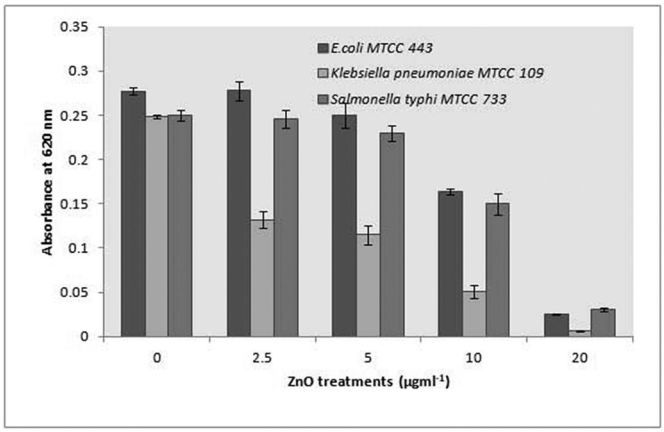


Fig. 4. Antimicrobial activity of ZnO nanoparticles against different microbes.

2015) as well as fungi such as *Penicillium oxalicum* (Lee et al., 2015), *Fusarium verticillioides* (Marlida et al., 2010), *Rhizopus* (Sato et al., 2016) etc.; but approximately 80% their activity is lost when processed at high temperatures. The isolate *Penicillium decumbens* of our study also showed a similar pattern of reduced enzyme activity at temperatures beyond 80 °C.

The current study analyzed the role of ZnO nanoparticles in the development of thermotolerance of phytase enzyme at 100 °C. The use of ZnO nanoparticles at concentrations from the range of 10 μg ml^{-1} to 40 μg ml^{-1} aided to improve the phytase activity even after treatment at high temperatures. The incorporation of ZnO nanoparticles at a concentration of 20 μg ml^{-1} caused the inhibition of pathogens; whereas 40 μg ml^{-1} concentrations resulted in the lysis of microbial cells indicating the antimicrobial role of the nanoparticles. The animal feed pellets itself undergo a standard sterilization protocol which kills pathogens in the feed; the additional incorporation of ZnO at low concentrations would thus be sufficient to overcome the chances of contamination post packing during its transport, storage and use. The use of *Lactobacillus* for ZnO synthesis also adds an additional probiotic benefit to the phytase nanocomposites in the form of antimicrobial activity; thereby biosynthesis of ZnO nanoparticles was used in the above study. *Lactobacillus* assisted ZnO nanoparticle synthesis is a safe and ecofriendly mode of nanoparticle production than chemical methods (Garmasheva et al., 2016). ZnO is considered to be non-toxic, biocompatible and stable metal oxide with unique optimal, electronic and photocatalytic properties which find various applications.

Phytases being a prospective additive in pelletised animal feeds, estimation on its activity at different temperatures of feed processing gains relevance. Animal feed processing involves steps of mash preparation (around 45–55 °C), pelletisation (around 80 °C) and finally steam sterilization (at 100 °C), thus the thermostability assays of our study were planned accordingly with enzyme exposure for different time durations at these temperatures (https://www.cpm.net/downloads/Animal%20Feed%20Pelleting.pdf). An increase in the thermotolerance of nanoparticle-treated enzyme at 100 °C was obtained at ZnO nanoparticles concentrations as low as 10 μg ml^{-1}, thereby suggesting nanoparticles at lower concentration was sufficient to improve enzyme thermotolerance.

The effectiveness of ZnO nanoparticles in enhancement of thermoactive properties and pH tolerance of enzymes such as cellulases is yet another instance (Srivastava et al., 2016). As per analyzed Zn based molecules as such are found to be thermostable; particularly ZnO can withstand high temperatures till 300 °C (Jones et al., 2013). Thus complexing enzymes with ZnO nanoparticles could aid to improve enzyme thermotolerance, provided the catalytic site and efficiency of the enzyme is not drastically affected. Apart from ZnO the use of immobilizing agents such as magnetite chitosan nanoparticles (Onem et al., 2016), alumina (Vinogradov and Avnir, 2015) and gold nanoparticles (Shankar et al., 2015) in increasing enzyme thermostability have been suggested. Immobilization of phytase onto gold nanoparticles by electrostatic forces found to reduce the activation energy of phytase and increased its thermostability (Shankar et al., 2015).

An evaluation on the influence of chemically derived ZnO nanoparticles on various biological enzymes viz. proteases and amylase (from *Aspergillus niger*), xylanase (from *F. oxysporum*) and phytases (from *Hypocrea lixii*) indicated that three of them except amylase was active even at high ZnO concentrations (Aroma et al., 2016). Biogenically synthesized ZnO nanoparticles in combination with phytase of *Penicillium decumbens* did not inhibit the enzyme activity at low concentrations till 20 μg ml^{-1} but higher concentrations inhibited the enzyme as shown in Table 2. This difference in observation of phytases could be due to the difference in the protein structure of phytases from *Penicillium* sp. and *Hypocrea lixii*.

Reports on the antimicrobial role of ZnO nanoparticles against methicillin-resistant *Staphylococcus aureus* (Dobrucka and Dlugaszewska, 2016; Joseph et al., 2016), *Klebsiella pneumonia* (Reddy et al., 2014), *Escherichia coli* (Baek et al., 2017), *Mycobacterium tuberculosis* (Patil and Taranath, 2016), *Campylobacter jejuni* (Xie et al., 2011) as well as various fungi (El-Feky et al., 2014; He et al., 2011) supports the antimicrobial role of ZnO nanoparticles against pathogenic microbe as observed in the current study. ZnO nanoparticles are proven to induce bacteriolysis of human pathogens such as *Salmonella typhimurium* and *Staphylococcus aureus* in approximately 8 h rather than merely inhibiting their growth (Tayel et al., 2011). This adds yet another advantage in using ZnO nanoparticles as bacteriostatic and bacteriolytic agents. Moreover, ZnO also provides an additional antibacterial effect by the generation of other reactive oxide radicals post-exposure to UV and visible light which causes oxidative stress, membrane lysis and cell apoptosis of exposed microbes (Fortunati et al., 2017; Padmavathy and Vijayaraghavan, 2008). ZnO is also considered as generally regarded as safe to be added in foods as per FDA and thus they can be added as active ingredients of food (Marcous et al., 2017).

5. Conclusion

The current study suggests the role ZnO nanoparticles at concentrations up to 10 μg ml^{-1} as effective doses to increase phytase thermotolerance and antimicrobial activity. Considerable loss of phytase activity applied in animal feeds can be addressed by simple methods such as ZnO nanoparticle immobilization to enhance its thermostability. This could also avoid the use of costly machinery needed to supplement phytase after the post pelletisation procedure. Combinatorial use of techniques of microwave aided *Lactobacillus* mediated ZnO nanoparticle synthesis along with phytases active at physiological conditions could greatly reduce the antinutrient role of phytate in food. The oxidative stress induced damage caused by ZnO nanoparticles against human pathogens also would aid to reduce the rate of spoilage of feed on storage and transport. Phytase constituting a major portion of industrial enzymes, the linking of simple nano-based techniques to increase its thermostability will surely benefit the phytase industry. This is the first attempt to link a probiotically synthesized ZnO nanoparticle with phytase to increase its thermostability.

Acknowledgement

The work was funded by Science and Engineering Research Board (F.NO.PDF/2015/000472), University Grants Commission, Government of India (F.NO.42-609/2013 SR) and Department of Science and Technology under DST WOS-B Scheme (F.No:SR/WOS-B/740/2016G).

Competing interest

The authors have no competing interest to declare.

References

Aroma, S.R., Namasivayam, S.K.R., Thyagarajan, R., 2016. An investigation of chemogenic zinc oxide nanoparticles mediated enzyme activity inhibition under in vitro condition. Pharm. Lett. 8, 425–431.

Baek, S., Joo, S.H., Kumar, N., Toborek, M., 2017. Antibacterial effect and toxicity pathways of industrial and sunscreen ZnO nanoparticles on *Escherichia coli*. J. Environ. Chem. Eng. 5 (3), 3024–3032.

Cheng, G., Xu, Y., Zhu, X., Xie, S., Wang, L., Huang, L., et al., 2017. The antibacterial activities of aditoprim and its efficacy in the treatment of swine streptococcosis. Sci. Report. 7, 41370. http://dx.doi.org/10.1038/srep41370.

Cho, E.A., Kim, E.J., Pan, J.G., 2011. Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. Enzym. Microb. Technol. 49, 66–71.

Dobrucka, R., Dlugaszewska, J., 2016. Biosynthesis and antibacterial activity of ZnO nanoparticles using *Trifolium pratense* flower extract. Saudi J. Biol. Sci. 23, 517–523.

El-Feky, O.M., Hassan, E.A., Fadel, S.M., Hassan, M.L., 2014. Use of ZnO nanoparticles for protecting oil paintings on paper support against dirt, fungal attack, and UV aging. J. Cult. Herit. 15, 165–172.

Fortunati, E., Puglia, D., Armentano, I., Valdes, A., Ramos, M., Juarez, N., Garrigos, M.C., Kenny, J.M., 2017. Multifunctional antimicrobial nanocomposites for food packaging applications. In: Food Preservation. Elsevier, pp. 265–303.

Garmasheva, I., Kovalenko, N., Voychuk, S., Ostapchuk, A., Livinska, O., Oleschenko, L., 2016. *Lactobacillus* species mediated synthesis of silver nanoparticles and their antibacterial activity against opportunistic pathogens in vitro. Bioimpacts 6, 19–223.

He, L., Liu, Y., Mustapha, A., Lin, M., 2011. Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. Microbiol. Res. 166, 207–215.

Heinonen, J.K., Lahti, R.J., 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal. Biochem. 113, 313–317.

Hoog, G.S.De., Guarro, J., Gene, J., Figueras, M.J., 2000. Atlas of Clinical Fungi. Central Bureau voor Schimmel Cultures/Universitat Rovira i virgili.

Jones, G., 2012. Phytase- for the environment and for profit. Pig Prog. 28, 11–13.

Jones, F., Tran, H., Lindberg, D., Zhao, L., Hupa, M., 2013. Thermal stability of zinc compounds. Energy Fuel 27, 5663–5669.

Joseph, B., Chacko, M., Rebello, S., Rishad, K.S., Ramesh Babu, N.G., Karthikeyan, S., 2016. Molecular taxonomic identification, biosynthesis and in vitro antibacterial activity of ZnO nanoparticles using *Boerhavia diffusa* against MRSA. Int. J. Toxicol. Pharma. Res. 8, 40–44.

Lee, S.H., Cho, J., Bok, J., Kang, S., Choi, Y., Lee, P.C.W., 2015. Characterization, gene cloning, and sequencing of a fungal phytase, PhyA, from *Penicillium oxalicum* PJ3. Prep. Biochem. Biotechnol. 45, 336–347. http://dx.doi.org/10.1080/10826068.2014.923446.

Lei, X.G., Stahl, C.H., 2000. Nutritional benefits of phytase and dietary determinants of its efficacy. J. Appl. Anim. Res. 17, 97–112.

Lei, X.G., Weaver, J.D., Mullaney, E., Ullah, A.H., Azain, M.J., 2013. Phytase, a new life for an “old” enzyme. Annu. Rev. Anim. Biosci. 1, 283–309.

Mallin, M.A., Cahoon, L.B., 2003. Industrialized animal production-a major source of nutrient and microbial pollution to aquatic ecosystems. Popul. Environ. 24, 369–385.

Marcous, A., Rasouli, S., Ardestani, F., 2017. Low-density polyethylene films loaded by titanium dioxide and zinc oxide nanoparticles as a new active packaging system against *Escherichia coli* O157: H7 in fresh calf minced meat. Packag. Technol. Sci. 30 (11), 693–701.

Marlida, Y., Delfita, R., Gusmanizar, N., Ciptaan, G., 2010. Identification characterization and production of phytase from endophytic fungi. World Acad. Sci. Eng. Technol. 65, 1043–1046. http://dx.doi.org/10.3923/pjn. 2010.471.474.

Nampoothiri, K.M., Tomes, G.J., Roopesh, K., Szakacs, G., Nagy, V., Soccol, C.R., Pandey, A., 2004. Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. Appl. Biochem. Biotechnol. 118, 205–214.

Onem, H., Cicek, S., Nadaroglu, H., 2016. Immobilization of a thermostable phytase from Pinar melkior (*Lactarius piperatus*) onto magnetite chitosan nanoparticles. CyTA J. Food 14, 74–83.

Padmavathy, N., Vijayaraghavan, R., 2008. Enhanced bioactivity of ZnO nanoparticles - an antimicrobial study. Sci. Technol. Adv. Mater. 9 (3), 1–7.

Parhamfar, M., Badoei-Dalfard, A., Khaleghi, M., Hassanshahian, M., 2015. Purification and characterization of an acidic, thermophilic phytase from a newly isolated *Geobacillus stearothermophilus* strain DM12. Prog. Biol. Sci. 5, 61–73.

Patil, B.N., Taranath, T.C., 2016. *Limonia acidissima* L. leaf mediated synthesis of zinc oxide nanoparticles: a potent tool against *Mycobacterium tuberculosis*. Int. J. Mycobacteriol. 5, 197–204.

Qvirist, L., Vorontsov, E., Veide, Vilg J., Andlid, T., 2017. Strain improvement of *Pichia kudriavzevii* TY13 for raised phytase production and reduced phosphate repression. Microb. Biotechnol. 10, 341–353.

Rebello, S., Jose, L., Sindhu, R., Aneesh, E.M., 2017. Molecular advancements in the development of thermostable phytases. Appl. Microbiol. Biotechnol. 101, 2677–2689.

Reddy, L.S., Nisha, M.M., Joice, M., Shilpa, P., 2014. Antimicrobial activity of zinc oxide (ZnO) nanoparticle against *Klebsiella pneumoniae*. Pharm. Biol. 52, 1388–1397.

Sato, V.S., Jorge, J.A., Guimaraes, L.H.S., 2016. Characterization of a thermotolerant phytase produced by *Rhizopus microsporus* var. *microsporus* biofilm on an inert support using sugarcane bagasse as carbon source. Appl. Biochem. Biotechnol. 179, 610–624. http://dx.doi.org/10.1007/s12010-016-2018-7.

Selvarajan, E., Mohanasrinivasan, V., 2013. Biosynthesis and characterization of ZnO nanoparticles using *Lactobacillus plantarum* VITES07. Mater. Lett. 112, 180–182.

Shankar, S., Soni, S.K., Daima, H.K., Selvakannan, P.R., Khire, J.M., Bhargava, S.K., Bansal, V., 2015. Charge-switchable gold nanoparticles for enhanced enzymatic thermostability. Phys. Chem. Chem. Phys. 17, 21517–21524.

Srivastava, N., Srivastava, M., Mishra, P.K., Ramteke, P.W., 2016. Application of ZnO nanoparticles for improving the thermal and pH stability of crude cellulase obtained from *Aspergillus fumigatus* AA001. Front. Microbiol. 7, 514.

Tan, H., Wu, X., Xie, L., Huang, Z., Peng, W., Gan, B., 2016. Identification and characterization of a mesophilic phytase highly resilient to high-temperatures from a fungus-garden associated metagenome. Appl. Microbiol. Biotechnol. 100, 2225–2241.

Tayel, A.A., El-Tras, W.F., Moussa, S., El-Baz, A.F., Mahrous, H., Salem, M.F., Brimer, L., 2011. Antibacterial action of zinc oxide nanoparticles against foodborne pathogens. J. Food Saf. 31, 211–218.

Ushasree, M.V., Vidya, J., Pandey, A., 2015. Replacement P212H altered the pH-temperature profile of phytase from *Aspergillus niger* NII 08121. Appl. Biochem. Biotechnol. 175, 3084–3092.

Vinogradov, V.V., Avnir, D., 2015. Exceptional thermal stability of industrially-important enzymes by entrapment within nano-boehmite derived alumina. RSC Adv. 5, 10862–10868.

Xie, Y., He, Y., Irwin, P.L., Jin, T., Shi, X., 2011. Antibacterial activity and mechanism of action of zinc oxide nanoparticles against *Campylobacter jejuni*. Appl. Environ. Microbiol. 77, 2325–2331.

Yu, P., Chen, Y., 2013. Purification and characterization of a novel neutral and heat-tolerant phytase from a newly isolated strain *Bacillus nealsonii* ZJ0702. BMC Biotechnol. 13, 78. http://dx.doi.org/10.1186/1472-6750-13-78.

Zárate, G., Chaia, A.P., González, S., Oliver, G., 2000. Viability and β-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. J. Food Prot. 63 (9), 1214–1221.

Hypericum japonicum: *a Double-Headed Sword to Combat Vector Control and Cancer*

Sreedev Puthur, A. N Anoopkumar, Sharrel Rebello & Embalil Mathachan Aneesh

Applied Biochemistry and Biotechnology
Part A: Enzyme Engineering and Biotechnology

ISSN 0273-2289

Appl Biochem Biotechnol
DOI 10.1007/s12010-018-2713-7

Springer

Appl Biochem Biotechnol
https://doi.org/10.1007/s12010-018-2713-7

Hypericum japonicum: a Double-Headed Sword to Combat Vector Control and Cancer

Sreedev Puthur[1] · **A. N Anoopkumar**[1] · **Sharrel Rebello**[1] · **Embalil Mathachan Aneesh**[2]

Received: 19 October 2017 / Accepted: 6 February 2018

Abstract Mosquito control with naturally derived herbal insecticides has gained much momentum, with the increased insecticide resistance of vectors and the multiple infectious diseases spread by them. Yet, recent studies also suggest that mosquitoes could probably transmit some cancerous cells or cancer-causing viruses from one individual to another between their blood meals. The current research thus focused on the screening and characterization of novel plants with both mosquitocidal and anticancerous properties. Accordingly, different solvent extracts of *Hypericum japonicum*, a key plant in Chinese medicine, were screened for its larvicidal efficacy using the fourth instar larvae of *Aedes aegypti* (major vector of Dengue and chikungunya). Methanolic extracts of the plant showed effective larvicidal property with LC_{50} 7.37 ppm and LC_{90}11.59 ppm values. The anticancerous property of the plant extract was also evaluated by in vitro cytotoxicity assay against Daltons Lymphoma Ascites (DLA) cells. The results indicated that *H. japonicum* plant extracts at very low concentrations of LC_{50}0.95 ppm and LC_{90}1.85 ppm were potent cytotoxic agents. To the best of our knowledge, this is the first and the foremost report of *Hypericum japonicum* as a potent mosquitocidal and anticancerous agent. Identification and characterization of such plant-derived bioactive plants thus could serve as a double-headed sword against the spread of infectious diseases and cancer.

Keywords *Aedes aegypti* · *Hypericum japonicum* · Larval bioassay · Cytotoxicity · DLA cells

Abbreviation

A. aegypti	*Aedes aegypti*
LCL	Lower confidence limit
UCL	Upper confidence limit

✉ Embalil Mathachan Aneesh
aneeshembalil@gmail.com

[1] Communicable Disease Research Laboratory, St. Joseph's College, Irinjalakuda, Thrissur, India

[2] Department of Zoology, St.Joseph's College, Irinjalakuda, Thrissur, Kerala 680121, India

Published online: 24 February 2018

LD	Lethal Dose
DLA	Daltons Lymphoma Ascites cells

Introduction

Mosquitoes are vectors of many devastating diseases which is one of the major reasons for depletion of the human population worldwide. Millions of people severely struggle by mosquito vectors and associated infections every year [3, 42]. Among them, dengue fever is widely distributed to tropics and subtropics and about 1.5 million people living in temperate regions are at a high risk of dengue virus transmission [14]. Mosquito-borne disease has a negative impact on economic development of a country by causing death, diminished productivity, and high medical costs in the tropical and subtropical countries [11, 37]. The huge outbreak of vector-borne diseases is due to the increased amount of vector population resulted by disturbed ecosystem, agricultural development, inadequate disposal facilities, and urbanization as well as water accumulation which favor mosquito production [12, 13].

Aedes aegypti, a significant vector of dengue fever in South-East Asia, Africa, and in America, is of prime concern because of its global distribution range and disease transmission capability [31]. Vector control continued to be the most efficient way to control mosquito population; it has advantages like mosquitoes are being killed in larval stages before they spread into different habitats and which also aids the habitat destruction of mosquitoes [19]. Due to the high-risk clinical conditions and economic imbalance caused by mosquito vectors, mosquito control is the prime goal of many new researchers over the past decade [28].

The usage of synthetic insecticides to control mosquito population is a common approach to control mosquitoes. However, in the environmental point of view, they are hazardous as majority portion of these insecticides applied are broken down into the air and deposited in plants and soils [29]. During the application of insecticides, only 0.1% reaches the target organism and the rest 99% influence the non-target species and causing toxicity [5]. Development of insecticidal resistance is one of the major hurdles of persistent use of synthetic insecticide, with the utilization of different classes of pesticides like pyrethroids, carbamates, and organophosphates [2]. This will contribute as one of the major drawbacks of synthetic insecticidal application.

Apart from the increasing pesticide resistance, studies indicate that some of the mosquito repellants as well as larvicidal agents on prolonged use lead to health hazards such as lung cancers [39]. Interestingly, the *Anopheles* and *Aedes* mosquitoes in the spread of malaria and cancer mortality have also been reported [23, 24]. Hypothesis on the role of mosquitoes in cancer spread also exist, for instance, hamster reticulum cell sarcoma cells could be transferred through *Aedes* mosquitoes as tumor cells remain alive in mosquitoes for about 8 h [6, 7]. Recent studies also justify the chances of spread of cancerous cells via mosquito bites [8].Thus, an urge for effective natural agents with both mosquitocidal and anticancerous role would be of great significance in the prevention of various infections. Thus, the current study evaluated the mosquito biocontrol efficiency and cytotoxic properties of *Hypericum japonicum* a plant widely used in Chinese traditional medicine system. The plant has been used for the treatments of bacterial, viral, and gastrointestinal disorders [40, 43].

Materials and Methods

Sample Collection

Samples of *Hypericum japonicum* were collected from Pothumolla, Wayanad district, Kerala, India. Voucher specimens of the plants were maintained at Communicable Disease Research Laboratory, Thrissur and authenticated by comparison with corresponding herbarium specimens.

Taxonomic Identification

Genomic DNA from the plant sample was extracted using NucleoSpin® Plant II Kit (Macherey-Nagel), and the molecular identification of the plant was done by rbcL typing using primers forward primer 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ and reverse primer 5′-TCGCATGTACCTGCAGTAGC-3′. PCR amplification reactions were carried out in a 20-μl reaction volume which contained 1X Phire PCR buffer (with 1.5 mM $MgCl_2$), 0.2 mM each dNTPs (dATP, dGTP, dCTP, and dTTP), 1 μl DNA, 0.2 μl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5 M Betaine, and 5 pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with initial denaturation at 98 °C for 30 s, followed by 40 cycles involving denaturation at 98 °C for 5 s, annealing at 58 °C for 10 s, extension at 72 °C for 15 s, followed by final extension at 72 °C for 60 s and cold holding at infinity. The PCR amplicon was purified by ExoSAP-IT Treatment (GE Healthcare) and sequenced using Big Dye Terminator v3.1. chemistry. The sequenced PCR amplicon was further BLAST for similarity in the NCBI database to genotypically identify the plant.

Extract Preparation

The whole plant of *Hypericum japonicum* was shade dried, powdered, and extracted by Soxhlet method using different organic solvents (acetone, hexane, petroleum ether, and methanol) based on polarity until exhaustion. Solvents were distilled in a vacuum rotary evaporator under reduced pressure of 20–22 mmHg at 35 °C, and the extract concentrates were further evaporated to complete dryness at room temperature for further analysis.

Mosquito Colony

Larvae of *Aedes aegypti* collected from their natural habitats were maintained and reared at ideal environmental parameters like temperature, humidity, and optimum nutritional requirements in the Communicable Disease Research Laboratory, Irinjalakuda, Kerala, of India for the study. The adults were reared in cages (30 × 30 × 30 cm) fitted with mosquito netting and fed first on freshly water-soaked raisin. Female mosquitoes were allowed to feed on albino rat placed in resting cage overnight on the third-day post-emergence. Small bowls filled with dechlorinated water were used to collect the egg rafts for hatching. The larvae were reared in large plastic trays (36 × 24 × 7 cm), containing dechlorinated water and fed with finely powdered dog biscuits and yeast mixture in the ratio of 2:1. The food was sprinkled on the surface of the water once daily, and the rearing water was changed daily until pupation. Pupae were collected daily, transferred to tiny bowls containing dechlorinated water, and placed in

cages for adult emergence. The test population was maintained in the laboratory under environmentally controlled conditions (26 ± 2 °C and 75 ± 5% relative humidity) with a photoperiod of 14 h light and 10 h dark.

Larval Bioassay

Larval susceptibility tests were done against the early fourth instar larvae of *Aedes aegypti* using different plant extracts at varying concentrations along with respective controls as per WHO standard procedures [44]. Briefly, the bioassay was performed in batches of 25 early fourth instar larvae against 1 ml of the extract at different concentrations; in glass beakers of 500 ml capacity to attain a final volume of 250 ml. The toxicity of each extract was determined with three various concentrations ranging from 2.5 to7.5 mg/l to provide a mortality range of 10 to 98%. Control beakers contained 25 test organisms and 249 ml of tap water along with 1.0 ml acetone (the diluents for various extracts). Both the test and control beakers were placed under similar conditions of 25 ± 2 °C, 12 h light/dark regime, with no food for 24 h, and mortality was monitored. The larvae were considered as dead or morbid if they were not responsive to gentle prodding with a fine needle. All the tests in the following experiments were done in triplicate, and values were as expressed as mean ± standard deviation. The results were expressed as percent mortality.

Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated from a log dosage-probit mortality regression line using computer software program (IBM SPPSS version.17.0). Larval mortality was measured in percentage, and if the control mortality was ranged between 5 and 20%, it was corrected using Abbott's formula [1]. The corrected mortality was determined by using the following formula.

$$\text{corrected} \quad \text{mortality} = \frac{\%\text{test} \quad \text{mortality} - \%\text{control} \quad \text{mortality}}{100 - \%\text{control} \quad \text{mortality}} \times 100$$

Characterization of Components with Mosquitocidal Properties

The methanolic phytoextract was completely dried over anhydrous calcium chloride and chromatographed over a thin layer of silica gel (GF254) with standard mobile phase of chloroform: ethanol in the ratio 7:2. The developed chromatogram was inspected under UV light, and major bands were detected. Ten grams of methanolic extract was grounded with 2 g of silica gel and loaded to the silica gel column and eluted stepwise using different gradients of chloroform: ethanol (0:100: 10:90, 90:10: 100:0). Of the four fractions obtained in TLC plate, the most active fraction was further purified by column chromatography, evaporated to complete dryness, and analyzed by gas chromatography mass spectrometry (GC-MS). Larval bioassay using the pure fraction was also conducted to find out its larvicidal efficacy against the fourth instar larvae of *Aedes aegypti.*

In Vitro Cytotoxicity Study

The in vitro cytotoxicity of the different plant extracts was done against Daltons Lymphoma Ascites (DLA) cells. DLA cells aspirated from the peritoneal cavity of tumor bearing mice were washed with phosphate-buffered saline (PBS) and assessed for its viability by trypan blue

method. Different extracts of the plant at varying concentrations were treated with viable DLA cells (with concentration of 1×10^6 cells per 100 μl), made up to 1000 μl using PBS and incubated for 3 h at 37 °C to assess its cytotoxicity potential in vitro. The extent of cytotoxicity of the plant extracts was measured after incubation by staining the cells with 100 μl of 1% trypan blue for 2–3 min and by counting the dead cells on a hemocytometer. The differentiation of dead and viable cells is based on the principle that while dead cells take up blue color, living cells remain colorless. The percentage of cell toxicity is expressed as the percentage of dead cells against the total number of cells (both dead and live).

Results and Discussion

Taxonomic Identification of Plant

Comparison of the various morphological characteristics of the test plant against known plant identification keys indicated that the plant belonged to *Hypericum japonicum* species. The morphological identification was further substantiated by rbcL PCR-based molecular barcoding of the plant. The rbcL targeted PCR resulted in a 1-kb amplicon which was sequenced, and the result showed 100% similarity to *Hypericum japonicum* on BLAST analysis against the NCBI database. *Hypericum japonicum* is widely found in the water logged areas of the grasslands and evergreen forests of the Western Ghats and Eastern Ghats (http://indiabiodiversity.org/ species/show/ 249777). The above plant is also reported in various countries of Asia including Bangladesh, Bhutan, China, Japan, Korea, Laos, Malaysia, Myanmar, Nepal, and the Philippines and is commonly used in traditional medicine [41].

Evaluation of Mosquitocidal Properties

It is important to test the susceptibility status of mosquito vectors in different areas to stop the resurgence of communicable diseases. The types of solvents used for the extraction affect the efficacy of volatile phytochemicals. Solvents from polar to non-polar range generally used for bioassay screening depending on the solubility of phytoconstituents [38]. The bioactivity of extracts depends on the chemistry of compound, nature of solvent, and procedures opting for extraction [26]. Table 1 presents the results of toxicity of the *Hypericum japonicum* whole plant extracts by different solvents tested against *Aedes aegypti* larvae. The methanolic extracts of *Hypericum japonicum* showed high larval mortality against the fourth instar larvae of *Aedes aegypti* with LC_{50}7.37 ppm and LC_{90}11.59 ppm. Significant lethality was also exhibited by petroleum ether and hexane extracts with LC_{50} 8.27 and 9.63 ppm, respectively, while least activity was shown by acetone extract with LC_{50} 13.15 ppm. The bioactive compound isolated from the same was found more effective than the parent fraction with LC_{50} 0.95 ppm and LC_{90} 1.85 ppm as presented in Table 2. The log dose probit mortality responses of extracts and fraction were shown in Figs. 1 and 2.

Significant larvicidal property was shown by both polar methanol ($p < 0.17$) and non-polar petroleum ether ($p < 0.17$) extracts. The bioactive fraction was found to be more effective ($p < 0.008$) than the rest of the plant extracts even with the parent methanolic extract. The present results are in consonance with the studies conducted by Madhu et al. 2011 [27] in which *Piper longum* was active against *Culex*

Table 1 Larvicidal efficacy of a few solvent extracts of *H. japonicum* against *A. aegypti*

Solvent extracts	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	Chi-square value	*P* value
Acetone	13.15 (12.72–13.59)	18.54 (18.30–20.46)	5.68	0.106
Hexane	9.63 (9.05–10.28)	19.53 (17.23–23.17)	3.03	0.055
Petroleum ether	8.27 (7.82–8.73)	15.20 (13.91–17.05)	6.01	0.066
Methanol	7.37 (6.23–8.55)	11.59 (10.32–18.60)	4.98	0.039

LCL lower confidence limit, *UCL* upper confidence limit

*$P < 0.05$ significant difference at 5%

quinquefasciatus. Literature survey revealed the exploration of various *Hypericum* species for insecticidal properties. *Hypericum perforatum* showed significant larvicidal activity against *Culex quinquefasciatus* with LD_{50} 496 ppm and LD_{90} 960 ppm, respectively [34]. Considerable work also is done with different plant species, for instance, *P. logum*, *P. ribesoides*, and *P. sarmentosum* showed parricidal activity against *Stegomyia aegypti* [10]. The ethanol extracts of *P. beetle* has effectively destroyed the larvae of potential mosquito vectors *A. aegypti*, *C. quinquefasciatus*, *A. dirus*, and *Monsonia uniformis* [21]. Apart from plants, recent techniques using microbial insecticide-derived hexamerin proteins [35] as well as RNA interference techniques [18] to control mosquitoes also have invited research interest.

GC-MS analysis of the active fraction of *Hypericum japonicum* was done and showed peaks corresponding to phytochemical constituents. Based on the comparison of the mass spectra of the constituents with the MAINLIB, REPLIB library, three major chemical compounds were identified as isopropyl hexadecanoate, phenol-2,4-bis(1,1-dimethylethyl), and benzenepropanoic acid. One of these compounds, viz., isopropyl hexadecanoate derived from various plants are found to have the ability to act as a natural pest controlling agent [9]. Various studies indicate that *Isopropyl* hexadecanoate or isopropyl palmitate is an effective insect repellant and effective insecticide which has resulted in various patents [17, 33]. Phenol 2, 4-bis(1,1-dimethylethyl) derivatives are reported in ayurvedic herbal extract Ashokarishtam and has both antibacterial and anti-inflammatory function [4]. As listed by USEPA, benzenepropanoic acid is used as inert components in pesticide formulations, and they could serve as free radical stabilizers in such pesticide formulations [36]. The study thus developed an active

Table 2 Mosquito larvicidal effects of bioactive fraction from methanol extract of *H. japonicum* against *A. aegypti*

Fraction	Concentration (ppm)	Percent mortality	LC_{50} (ppm) (LCL-UCL)	LC_{90} (ppm) (LCL-UCL)	Chi-square value	*P* value
Control	0	0	–	–	–	–
Bioactive fraction	0.4	16.1				
	0.8	27.0	0.95 (0.49–1.44)	1.85 (1.44–3.92)	11.66	0.000
	1.2	56.2				
	1.6	79.0				
	2	96.0				

LCL lower confidence limit, *UCL*, upper confidence limit

*$P < 0.00$ significant *P* value

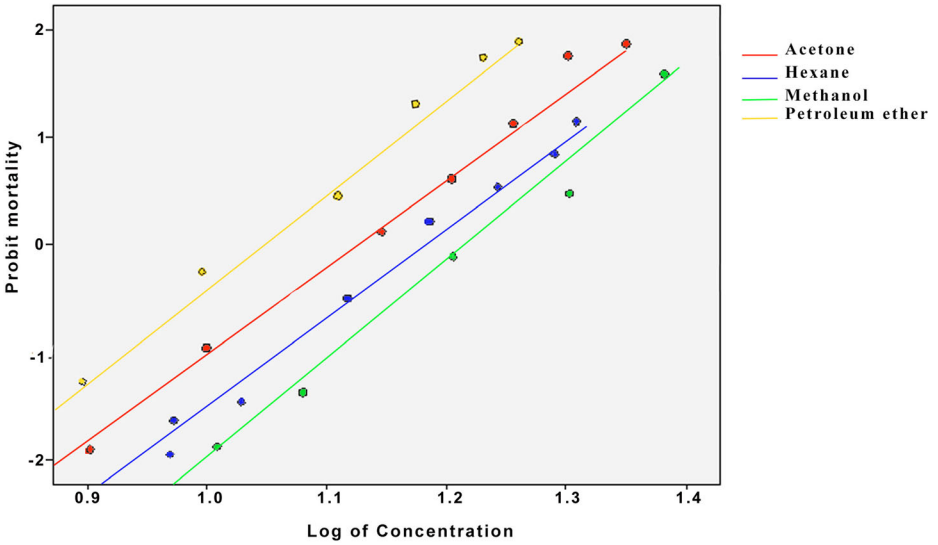


Fig. 1 Log concentration-percent mortality relationship of *A. aegypti* larvae to *H. japonicum* extracts

fraction from *Hypericum japonicum* which were very effective in biocontrol of mosquito larva even at LC_{50} 0.95 ppm which was lower than the parent extracts. Findings from the study strongly support observations made by Lee (2000) and Yang et al. 2002 [22, 45].

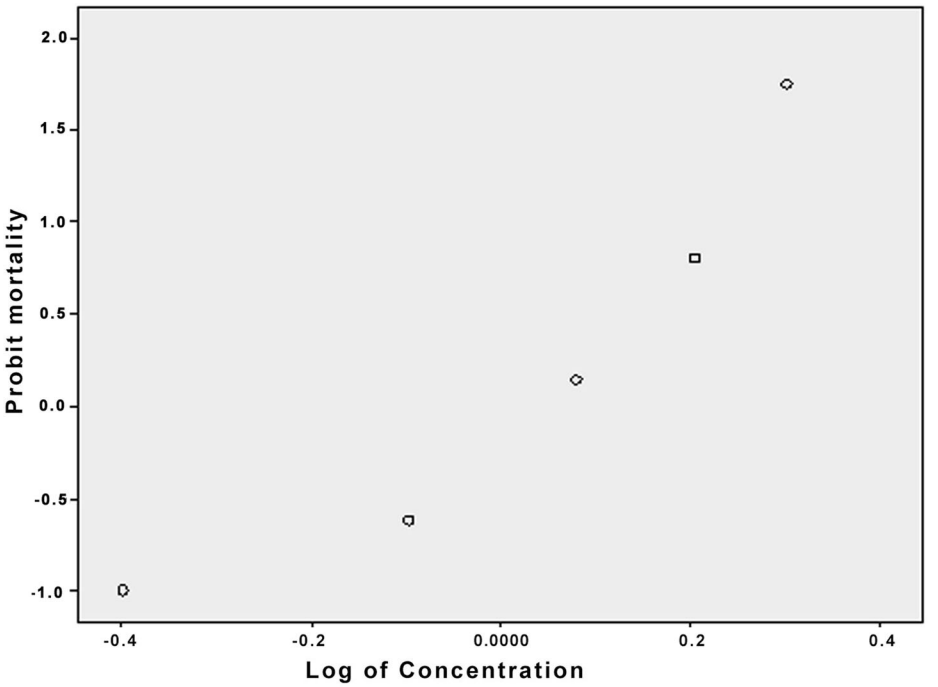


Fig. 2 Log concentration-percent mortality relationship of *A. aegypti* larvae to bioactive fraction, an active fraction obtained from *H. japoniccum*

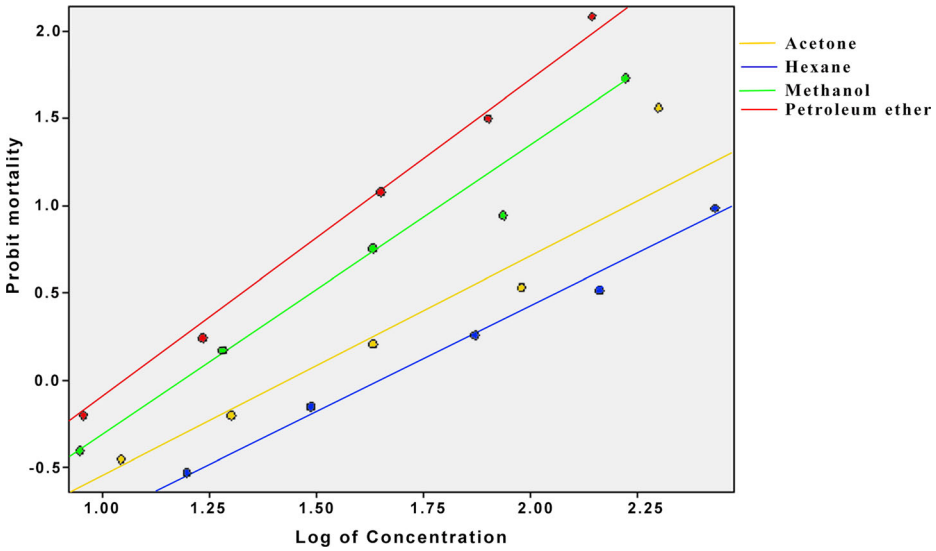


Fig. 3 Log concentration-percent mortality relationship of DLA cells to *H. japoniccum* extracts

In Vitro Cytotoxicity

Figure 3 represents the in vitro cytotoxicity of the *Hypericum japonicum* different solvent whole plant extracts tested against Dalton Lymphoma Cells DLA. The highest short-term in vitro cytotoxicity was shown by acetone extract and the least by methanolic extract. The dose required to kill 50% of DLA cells for acetone extract was 29.30 ppm, whereas the LD_{50} of petroleum ether and hexane extracts were 38.57 and 38.89 ppm, respectively. There is only slight dose variation as in the case of petroleum ether and hexane extracts. The least LD_{50} was shown by methanolic extract with a value of 60.20 ppm (Table 3).

The use of chemotherapeutic drugs in cancer treatment is nonselective and involves the risk of life-threatening host. The hunt, consequently, goes on to develop the drugs which selectively act on tumor cells. Naturally occurring active compounds exhibit cytotoxic activity against various cancer cell lines [20, 30]. The different extracts of *Hypericum japonicum* showed significant results with acetone extract ($p < 0.13$) followed by hexane ($p < 0.56$) and petroleum ether ($p < 0.59$) and the least activity by methanolic extract ($p < 0.64$). From the result, it is evident that there is no converse relationship between extract efficacy and solvent polarity in in vitro cytotoxicity screening; the highest efficacy was shown by acetone with

Table 3 Cytotoxic activity of a few solvent extracts of *H. japonicum* against DLA cells

Solvent extracts	LD_{50} (μg/ml)	LD_{90} (μg/ml)	Chi-square value	*P* value
Acetone	29.30	182.13	3.00	0.001
Hexane	38.89	294.21	5.60	0.132
Petroleum ether	38.57	245.10	5.91	0.116
Methanol	60.20	297.90	3.43	0.329

LD lethal dose

*$P < 0.01$ significant difference at 1%

polarity index (5.1) followed by hexane and petroleum ether of polarity index (0.1), and the least activity was shown by methanol with polarity index (5.1).

Plants of family *Hypericum* are reported to have antitumor activity from *H. perforatum*, *H. hookerianum*, *H. mysorence*, *H. patulum*, *H. polyanthemum*, and *H. drummondii* [32] with scanty reports on antitumor activity of *Hypericum japonicum* except for few [25]. Reports also support the antibacterial role of *Hypericum japonicum* thereby increasing its biopotential role [16]. The role of various terpenoid phloroglucinols of *Hypericum japonicum* are also reported to be effective against the proliferation of herpes simplex viruses [15].

Conclusion

An evaluation on the mosquitocidal and cytotoxic activity of *Hypericum japonicum* yielded highest activity using the methanolic and acetonic extracts, respectively. This indicates that it had the potential for both mosquitocidal and anticancerous property. Thus, effectiveness of the plant extract for simultaneous biocontrol and anticancerous role would be possible when a combination of both the extracts were used simultaneously. Data verified by the above study thus suggests a combinatorial use of methanolic extracts of the plant at concentrations of 7.37 ppm, and acetonic extracts at 29.30 ppm concentrations could serve as an effective decoction to reduce the mosquito population as well as cancer cell population by 50%. Since the use of herbal products is suggested for the control of mosquitoes, the problems of insecticide resistance of vectors can be overcome by using such control measures. To the best of our knowledge, this is the first report on mosquitocidal and anticancerous role of *Hypericum japonicum* simultaneously. This strategy of vector control thus could serve as an effective solution on the chance of cancerous cell transmission, as they are found to be viable in mosquitoes even when transmitted as indicated by previous research [8].

Acknowledgements The authors are thankful to the Principal of St. Joseph's College for the laboratory facilities provided. We acknowledge UGC, Govt. of India, and Kerala State Council for Science Technology and Environment (KSCSTE) for funds under Major Research Project (No. 27/SRSLS/2013/CSTE).

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interests.

References

1. Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology, 18*(2), 265–267.
2. Aneesh, E.M., & Vijayan, V. (2010). Laboratory selection of carbofuran tolerant line of Culex quinquefasciatus Say, the filarial vector at Mysore. *Journal of communicable Diseases, 42*, 201–207.
3. Anoopkumar, A., Puthur, S., Varghese, P., Rebello, S., & Aneesh, E. M. (2017). Life cycle, bio-ecology and DNA barcoding of mosquitoes Aedes aegypti (Linnaeus) and Aedes albopictus (Skuse). *The Journal of Communicable Diseases, 49*.
4. Aparna Ravi, M. G., Mudiganti Ram Krishna Rao, Kalaivani, V. S. Kalaiselvi, K. Prabhu, Shruthi Dinakar, G. V. Rao. (2015) GC MS analysis of an Ayurvedic medicine "Ashokarishtam" Der Pharmacia Lettre, 7, 45–52.

5. Arufe, M. I., Arellano, J. M., García, L., Albendín, G., & Sarasquete, C. (2007). Cholinesterase activity in gilthead seabream (Sparus aurata) larvae: characterization and sensitivity to the organophosphate azinphosmethyl. *Aquatic Toxicology, 84*(3), 328–336.
6. Banfield, W. G., Woke, P. A., & Mackay, C. M. (1966). Mosquito transmission of lymphomas. *Cancer, 19*(10), 1333–1336.
7. Banfield, W. G., Woke, P. A., MacKay, C. M., & Cooper, H. L. (1965). Mosquito transmission of a reticulum cell sarcoma of hamsters. *Science, 148*(3674), 1239–1240.
8. Benelli, G., Iacono, A. L., Canale, A., & Mehlhorn, H. (2016). Mosquito vectors and the spread of cancer: an overlooked connection? *Parasitology Research, 115*(6), 2131–2137.
9. Bessette, S. (2007) Pesticidal compositions containing isopropyl-containing compounds as pesticidally active ingredients. Google Patents.
10. Choochote, W., Chaithong, U., Kamsuk, K., Rattanachanpichai, E., Jitpakdi, A., Tippawangkosol, P., Chaiyasit, D., Champakaew, D., Tuetun, B., & Pitasawat, B. (2006). Adulticidal activity against Stegomyia aegypti (Diptera: Culicidae) of three Piper spp. *Revista do Instituto de Medicina Tropical de São Paulo, 48*(1), 33–37.
11. Fradin, M. S., & Day, J. F. (2002). Comparative efficacy of insect repellents against mosquito bites. *New England Journal of Medicine, 347*(1), 13–18.
12. Group, W. S. (2006). Malaria vector control and personal protection. *World Health Organization Technical Report Series, 936*, 1.
13. Gubler, D. J. (1998). Resurgent vector-borne diseases as a global health problem. *Emerging Infectious Diseases, 4*(3), 442–450.
14. Hales, S., De Wet, N., Maindonald, J., & Woodward, A. (2002). Potential effect of population and climate changes on global distribution of dengue fever: an empirical model. *The Lancet, 360*(9336), 830–834.
15. Hu, L., Xue, Y., Zhang, J., Zhu, H., Chen, C., Li, X.-N., Liu, J., Wang, Z., Zhang, Y., & Zhang, Y. (2016). (±)-Japonicols A-D, acylphloroglucinol-based meroterpenoid enantiomers with anti-KSHV activities from Hypericum japonicum. *Journal of Natural Products, 79*(5), 1322–1328.
16. Ishiguro, K., Yamaki, M., Kashihara, M., & Takagi, S. (1986). Sarothralen A and B, new antibiotic compounds from Hypericum japonicum. *Planta Medica, 52*(04), 288–290.
17. Jeanne, R. L. and Henderson, G. (1992) Non-insecticidal insect repellent. Google Patents.
18. Katoch, R., Sethi, A., Thakur, N., & Murdock, L. L. (2013). RNAi for insect control: current perspective and future challenges. *Applied Biochemistry and Biotechnology, 171*(4), 847–873.
19. Killeen, G. F., Fillinger, U., & Knols, B. G. (2002). Advantages of larval control for African malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. *Malaria Journal, 1*(1), 8.
20. Ko, W., Kang, T., Kim, N., Lee, S., Kim, Y., Ko, G., Ryu, S., & Lee, B. (2000). Lavandulylflavonoids: a new class of in vitro apoptogenic agents from Sophora flavescens. *Toxicology In Vitro, 14*(5), 429–433.
21. Komalamisra, N., Trongtokit, Y., Rongsriyam, Y., & Apiwathnasorn, C. (2005). Screening for larvicidal activity in some Thai plants against four mosquito vector species. *The Southeast Asian Journal of Tropical Medicine and Public Health, 36*(6), 1412–1422.
22. Lee, S. E. (2000) Mosquito larvicidal activity of pipernonaline, a piperidine alkaloid derived from long pepper, Piper longum. Journal of the American Mosquito Control Association- Mosquito News, 16, 245–247, 3.
23. Lehrer, S. (2010). Anopheles mosquito transmission of brain tumor. *Medical Hypotheses, 74*(1), 167–168.
24. Lehrer, S. (2010). Association between malaria incidence and all cancer mortality in fifty US States and the District of Columbia. *Anticancer Research, 30*(4), 1371–1373.
25. Liu, L.-S., Liu, M.-H., & He, J.-Y. (2014). Hypericum japonicum Thunb. ex Murray: phytochemistry, pharmacology, quality control and pharmacokinetics of an important herbal medicine. *Molecules, 19*(12), 10733–10754.
26. Madhu, S., & Vijayan, V. (2010). Evaluation of the larvicidal efficacy of extracts from three plants and their synergistic action with propoxur against larvae of the filarial vector Culex quinquefasciatus (Say). *Toxicological & Environmental Chemistry, 92*(1), 115–126.
27. Madhu, S., Vijayan, V., & Shaukath, A. (2011). Bioactivity guided isolation of mosquito larvicide from Piper longum. *Asian Pacific Journal of Tropical Medicine, 4*(2), 112–116.
28. Madhumathy, A., Aivazi, A.-A., & Vijayan, V. (2007). Larvicidal efficacy of Capsicum annum against Anopheles stephensi and Culex quinquefasciatus. *Journal of Vector Borne Diseases, 44*, 223.
29. Maurya, P., Mohan, L., Sharma, P., Batabyal, L., & Srivastava, C. (2007). Larvicidal efficacy of Aloe barbadensis and Cannabis sativa against the malaria vector Anopheles stephensi (Diptera: Culicidae). *Entomological Research, 37*(3), 153–156.
30. Miranda, C., Stevens, J., Helmrich, A., Henderson, M., Rodriguez, R., Yang, Y.-H., Deinzer, M., Barnes, D., & Buhler, D. (1999). Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines. *Food and Chemical Toxicology, 37*(4), 271–285.

31. Molyneux, D. H., Hotez, P. J., Fenwick, A., Newman, R. D., Greenwood, B., & Sachs, J. (2009). Neglected tropical diseases and the Global Fund. *The Lancet, 373*(9660), 296–297.
32. Narayanan Ravisankar, C. S., Sooriamuthu Seeni, Jerrine Joseph, Nanjian Raaman (2014) GC-MS analysis and anticancer activity of methanol extract of leaves of *Hypericum hookerianum* Wight & Arn International Journal of Pharmacy and Pharmaceutical Sciences 6**,** 515–519.
33. Newberry, G. D., Bain, O. G., Dyer, C. D. and Sterzi, D. (2017) Pesticide and a method of controlling a wide variety of pests. Google Patents.
34. Pavela, R. (2009). Larvicidal effects of some Euro-Asiatic plants against Culex quinquefasciatus Say larvae (Diptera: Culicidae). *Parasitology Research, 105*(3), 887–892.
35. Poopathi, S., Thirugnanasambantham, K., Mani, C., Mary, K. A., Mary, B. A., & Balagangadharan, K. (2014). Hexamerin a novel protein associated with Bacillus sphaericus resistance in Culex quinquefasciatus. *Applied Biochemistry and Biotechnology, 172*(5), 2299–2307.
36. Reid, B. L., Baker, R. B., Bao, N. N., Koufas, D. A., Kent, G. J. and Baur, P. (2016) Synergistic pesticide compositions. Google Patents.
37. Sachs, J., & Malaney, P. (2002). The economic and social burden of malaria. *Nature, 415*(6872), 680–685.
38. Shaalan, E. A.-S., Canyon, D., Younes, M. W. F., Abdel-Wahab, H., & Mansour, A.-H. (2005). A review of botanical phytochemicals with mosquitocidal potential. *Environment International, 31*(8), 1149–1166.
39. Shu-Chen, C., Ruey-Hong, W., Li-Jie, S., Ming-Chih, C., & Huei, L. (2008). Exposure to mosquito coil smoke may be a risk factor for lung cancer in Taiwan. *Journal of Epidemiology, 18*(1), 19–25.
40. Silva, B. A., Ferreres, F., Malva, J. O., & Dias, A. C. (2005). Phytochemical and antioxidant characterization of Hypericum perforatum alcoholic extracts. *Food Chemistry, 90*(1-2), 157–167.
41. Su, J., Fu, P., Shen, Y., Zhang, C., Liang, M., Liu, R., Li, H., & Zhang, W. (2008). Simultaneous analysis of flavonoids from Hypericum japonicum Thunb. ex Murray (Hypericaceae) by HPLC-DAD–ESI/MS. *Journal of Pharmaceutical and Biomedical Analysis, 46*(2), 342–348.
42. Taubes, G. (1997). A mosquito bites back. *New York Times Magazine, 24*, 40–46.
43. Unal, E. L., Mavi, A., Kara, A. A., Cakir, A., Şengül, M., & Yildirim, A. (2008). Antimicrobial and antioxidant activities of some plants used as remedies in Turkish traditional medicine. *Pharmaceutical Biology, 46*(3), 207–224.
44. WHO. (2005) World Health Organization: Guidelines for laboratory and field esting of mosquito larvicides. 2005, WHO/CDS/WHOPES/GCPP/2005.Geneva: WHO; 2005.
45. Yang, Y.-C., Lee, S.-G., Lee, H.-K., Kim, M.-K., Lee, S.-H., & Lee, H.-S. (2002). A piperidine amide extracted from Piper longum L. fruit shows activity against Aedes aegypti mosquito larvae. *Journal of Agricultural and Food Chemistry, 50*(13), 3765–3767.