STUDIES ON THE EFFECT OF *CLERODENDRUM INFORTUNATUM* L. AND *CHROMOLAENA ODORATA* (L.) KING & ROBINS. ON THE MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF SIXTH INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)

Thesis submitted to
the faculty of science, University of Calicut
For the Award of the Degree of

DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

JAGADEESH G. NAMBIAR

Under the Guidance of

Dr. K. R. RANJINI

PG & RESEARCH DEPARTMENT OF ZOOLOGY
MALABAR CHRISTIAN COLLEGE, CALICUT
UNIVERSITY OF CALICUT

OCTOBER 2017

PG & RESEARCH DEPARTMENT OF ZOOLOGY
MALABAR CHRISTIAN COLLEGE
CALICUT, KERALA

Dr. K.R. Ranjini
Associate Professor

Phone off: 0495 2765679
Res : 0495 2377380
Mob : 9447077381
e-mail: ranjini.k.r45@gmail.com

Date: 30. 10. 2017

CERTIFICATE

This is to certify that the work embodied in the accompanying thesis, entitled "**STUDIES ON THE EFFECT OF *CLERODENDRUM INFORTUNATUM* L. AND *CHROMOLAENA ODORATA* (L.) KING & ROBINS. ON THE MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF SIXTH INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)"** is a bonafide record for the work done by **Mr. JAGADEESH. G. NAMBIAR,** for the Ph.D course program of the University of Calicut from November 2012 to October 2017 under my direct supervision and guidance in fulfillment of requirements of degree of Doctor of Philosophy in Zoology under the faculty of Science of the University of Calicut. Further certified that no part of this thesis has not been submitted elsewhere for the award of any other degree or diploma.

Malabar Christian College **Dr. K. R. Ranjini**

DECLARATION

I, Jagadeesh G. Nambiar hereby declare that thesis entitled "**STUDIES ON THE EFFECT OF *CLERODENDRUM INFORTUNATUM* L. AND *CHROMOLAENA ODORATA* (L.) KING & ROBINS. ON THE MIDGUT TISSUE, FAT BODY AND HAEMOLYMPH OF SIXTH INSTAR LARVAE OF *ORTHAGA EXVINACEA* HAMPSON (LEPIDOPTERA: PYRALIDAE)"** is an authentic record of the work carried out under the supervision and guidance of Dr. K. R. Ranjini**,** Associate Professor, P.G. and Research Department of Zoology, Malabar Christian College, Calicut and that no part of this has been published previously or submitted for the award of any degree, diploma, title or recognition before.

Malabar Christian College **Jagadeesh G. Nambiar**

ACKNOWLEDGEMENT

First and foremost, I am greatly indebted for the almighty for His grace, mercy and guidance throughout my research work and for helping me to overcome all difficulties. Without His blessings, this achievement would not have been possible.

I express my immense debt of gratitude to my research supervisor ***Dr. K. R. Ranjini****, Associate Professor, P.G. and Research Department of Zoology, Malabar Christian College, Calicut for her invaluable guidance, punctilious attitude, constant encouragement, understanding and patience during the period of my research.*

I am extremely grateful to the Principal of Malabar Christian College, Calicut and also I would like to extent my gratitude to the Manager of Malabar Christian College, Calicut for providing facilities for my work.

With great pleasure, I express my sincere gratitude to the members of research advisory committee especially ***Prof. Dr. N. Ramani*** *and* ***Dr. Pushpalatha E****, Assistant Professor, Dept. of Zoology, University of Calicut, for timely and valuable suggestions to refine my research more.*

I gratefully acknowledge ***Dr. B. K. Chandrasekhar Sagar****, Additional Professor, Dept of Neuropathology, NIMHANS, Bangalore for providing ultra microscopic facilities and for his excellent guidance on electron microscopic studies.*

I am also indebted to ***Dr. Sulaiman C. T****, Senior Scientist, Dept of Phytochemistry, CMPR, Kottakal for guiding me in phytochemical studies and I would also extent my gratitude to* ***Mr. Deepak****, Research assistant, CMPR, Kottakal for assisting me in phytochemical studies.*

I express my profound gratitude to Mr. ***Dineep Devadasan****, Scientific Assistant, IUIC, Dept of environmental sciences, Mahatma Gandhi University, Kottayam for LC-Q-ToF and LC-MS/MS analysis and I am also grateful to* ***Dr. Ashish G. R****, Technical manager Care Keralam for helping me in HPTLC and GC-MS analysis.*

I sincerely thank ***Dr. Rekha Sivadasan****, Junior Scientist, CEPCI, CahsewBhavan, Kollam for assisting me in Electrophoretic analysis.*

I also extent my sincere thanks to ***Mr. Jabir****, Statistician for his help in data analysis.*

*I express my sincere thanks to **Mr. Raj Kumar**, Research scholar, Bharathiar University for his valuable suggestions.*

I gratefully acknowledge University Grant Commission for providing me the fellowship during the UGC major project period from 2012 to 2015.

*I would like to thank **Mr. Rajesh** and the staffs of Bina Photostat, Calicut University for their help during the final printing work of my thesis and also extend my gratitude to **Mr. Sajive Radhakrishnan** for supporting me in DTP works.*

*It is with immense pleasure I express my deep sense of gratitude to labmates **Mrs. Ranjini K. D** and **Mrs. Najiya Beegum T.P** for their co-operations during my research work.*

*I have no words to express my heartfelt gratitude to dearest friends, **Mr. Ranjith. R**. and **Mr. Unnikrishnan. T.** who have given encouragement during entire tenure of my research and their motivation had helped me to reach here.*

This journey would not have been possible without the great support of my family and I am speechless to express my deepest gratitude to them being biggest motivating force in my life, especially my brother who supported me in all aspects to complete this thesis.

Finally, I extent my sincere gratitude to those who helped me directly or indirectly during the period of my research work.

Jagadeesh G. Nambiar

Dedicated to

My Heavenly Mother

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CHAPTER I
GENERAL INTRODUCTION

1.1 Mango crops in India

Mango, 'The National Fruit of India' is the most popular and nutritionally rich fruit grown in the tropics. Due to its delicious taste, fragrance, unique flavour, nutritional richness and rich source of Vitamin A and C, mango is treated as the 'King of fruits'. Mango is the leading crop of India and it occupies 22% of the total under fruits comprising 1.2 million hectares with a total production of 11 million tonnes. India become first among world's mango producing countries accounting for about 50% of the world's mango production. Uttar Pradesh and Anthra Pradesh have largest mango cultivation areas, which comprises about 25% of total area in both states, followed by Maharashtra, Karnataka, Bihar, Tamil Nadu and Kerala. India's major destinations for exporting mango and mango pulps are UAE, Bangladesh, UK, Saudi Arabia, Nepal, Kuwait and USA. India has about 15 percent shares in world mango market. So the Mango production plays an important role in Indian agro-economy.

1.2 The insect pests that attack Mango crops

The pests and diseases are responsible for the reduction of mango crop yield. In India, due to insect pest attacks and diseases about 20% of the crop yield is lost per year, which approximately comes to Rs. 1500 crores (Singh *et al*., 2001). Mango crop is facing an enormous loss due to ravages of pests, a serious threat to mango industry and the crop is attacked by about 492 species of insects, 17 species of mites and 26 species of nematodes in the world level. Of these, 188 species have been reported from India (Tandon and Varghese, 1985; Srivatsava, 1998). Among these about 45% are found to cause serious

damage to the crop, which includes hoppers, mealy bug, inflorescence midge, fruit fly, leaf webber, shoot borer, bark eating caterpillar, stem borer, shoot gall psylla, scale insect and stone weevil and therefore they are considered as major pests of Mango crop. Among these, the leaf webbers have played their own role in reduction of crop yield.

1.2.1 The mango leaf webber *Orthaga exvinacea*.

Pyralidae, the third largest family of Lepidoptera comprises the two major leaf webbers *Orthaga exvinacea* and *Orthaga eudrausalis* which are the major pests responsible for the low productivity of Mango crop. *Orthaga exvinacea* Hampson commonly called as the mango leaf-webber, is one of the major pests of Mango crop. In larval stage, they defoliate the leaves and thereby reduce the crop yield but in adult stage they do not cause any damage to the crop. The infestation by this caterpillar directly affects flowering of the plant and thereby reduce the fruit formation.

In initial stage, the caterpillars feed by scrapping the leaf surface gregariously and later they web the leaves and terminal shoots into clusters and feed within. A webbed cluster of leaves may harbour several caterpillars in initial stage and their severe attack give burnt appearance to leaves. In late instar, they are very active and feed individually on the whole leaf lamina leaving only the midrib. The webbing of leaf by this caterpillar is in the form of a small tunnel or tent like structure, hence they are also known as “Tent caterpillars”. The active season of this pest in the mango orchards is reported from June to February, beyond that their activeness gets decreased up to May especially in summer season. According to Chowdhury (2015), infestations by this caterpillar may start as early as seedling stage and are active even during flowering and fruiting.

1.3 Pest status and management

The infestations of *O. exvinacea* were reported in different agro-climatic zones of India, especially in Andhra Pradesh, Uttar Pradesh, Uttaranchal and Kerala (Singh *et al*., 2006). Earlier, it was considered as minor pest of mango but since last few years, due to its severe infestation and extent of damage caused, it has been reported as a major pest of mango in Kerala (Cherian and Ananthanarayanan, 1943). The tremendous losses of crop yield due to the severe infestation of this pest were reported in different states of India. Kavita *et al.* (2005) observed that the mango leaf webber is a major pest in intensively cultivated areas of Andhra Pradesh and heavy infestation by this pest adversely affects the flowering as well as the growth of new flush. Due to the intensive effects of this pest attack, ninety percent of the completely defoliated / skeletonised shoot was reported to be dried and it did not fruit in coming season (Gajendra Singh, 1988). Within a short period of time, it gained a major pest status (Srivastava and Verghese, 1983; Srivastava, 1997).

In Kerala, heavy infestations of *O. exvinacea* were reported in Malabar region. The severely infested trees have a burnt appearance and heavy infestation by this pest caused complete failure of flowering (Verghese, 1998). It was also reported that the larvae cause serious injury to young grafts (Singh, 1968). The field survey reports of Shaw *et al.* (1996) in Madhya Pradesh showed that the incidence of *O. exvinacea* was greater in old trees than in young trees.

Farmers adopted different ways to control this pest. In general, the synthetic insecticides are considered to be the quick method for the control of insect pests. But the use of conventional insecticides in pest management programs around the world has resulted in environmental pollution, lethal effects on non-target organisms, pest resistance to insecticides and pest

resurgence. Eradication of crop pest by applying synthetic insecticides in field may double the crop yield rapidly, but at the same time it may involve serious health hazards for mammals and other animals. As a result of heavy rain these harmful residues may reach in different water bodies and thereby create a dangerous situation for the consumers and the environment. Synthetic insecticides can leave potentially toxic residues in food products and it can be deleterious to non-target organisms in the environment (Isman, 2006).

The above facts has created an awareness for an investigation on an alternate approach to control this pest which should be safer and ecologically acceptable and this has led to the use of botanicals in management of *O. exvinacea*.

1.4 Role of botanicals in pest management

The history of botanicals used in pest management was started about 4000 years back. Many archaeological studies revealed that the ancient farmers have used plant extracts for managing the pest. The earliest documented examples of plants being used as pesticides occur in different part of world especially in China, Egypt, Asia and Europe. In Rigveda (BC 2000) the insecticidal properties of different plant extracts have been mentioned and in BC 600, Charaka 'The father of medicine' has described the medicinal and insecticidal efficiency of Neem flowers, roots, fruits, leaves and bark.

Over the years more than 6000 species of plants have been screened and from this, nearly 2400 plants belonging to 235 different families were noticed to possess significant biological activity against insect pests (Grainge and Ahmed, 1988). In 19^{th} and 20^{th} centuries, a large scale plant screening programmes were conducted particularly in USA and China. The major families of plant showing pesticidal activity are *Meliaceae, Myrtaceae,*

Asteraceae, Euphorbiaceae, Leguminosae, Fabaceae. Among these, the plants under *Meliaceae* family have higher potentiality with broad spectrum of insecticidal activity and lesser adverse effects on beneficial insects. Among the screened group of plants, neem possess a wide range of insecticidal properties. In India alone, toxicity assessment of Neem have been successfully tried against 105 species of insects, 12 species of nematodes and 9 species of fungi (Singh and Kataria, 1991). According to Schmutterer and Singh (1995), there are a total of 417 species of insects susceptible to neem in the world over. This reported list includes almost all key pests of agriculture.

Botanical insecticides have been identified as attractive alternatives to synthetic chemical insecticides for pest management. Diversity of natural chemicals that occurs in different plants can be exploited to the development of environmentally safe methods for insect pest management (Sadek, 2003; Caramori *et al*., 2004). This diversity of phytochemicals have a wide range of properties including insecticidal activity, repellence to pests, antifeedant effects, insect growth regulation, toxicity to nematodes, mites and other agricultural pests and also antifungal, antiviral and antibacterial properties against pathogens (Prakash and Rao, 1986; 1997).

The potency of insecticidal activity of different plants are dependent on the active principle present in it. The first discovered plant based active components are nicotine, pyrethrum and rotenone. Nicotine present in tobacco is a well known alkaloid having insecticidal activity. There are about 12 alkaloids present in tobacco of which nicotine contribute about 97%. The nicotine having neurotoxic effect can enter in to the insect pest through cuticle, spiracle and ingested food. Pyrethroids are the insecticidal component present in the extract of the *Chrysanthemum coccineum*. Presence of esters is responsible for the toxicity of pyrethrum and its action as contact poison to kill the insect. Rotenone is the compound present in the roots of the plants

Derris and *Lonchocarpus*. It was first extracted in 1948, and used against leaf-eating caterpillars. The mode of action of rotenone pertains to a decline in oxygen consumption, leading to paralysis and death.

At present, many researchers have focussed their works on the insecticidal capabilities of different botanicals and their insect specificity for applying. The adverse effects of plant derived phytochemicals on insects are in several ways, lethal toxicity, growth retardation, oviposition deterrence, feeding inhibition, suppression of calling behaviour and reduction of fecundity and fertility (Mordue and Blackwell, 1993; Breuer and Schmidt, 1995; Hiremath *et al*., 1997; Zhao *et al.,* 1998; Muthukrishnan and Pushpalatha, 2001; Wheeler and Isman, 2001) and such a wide variety of effects of botanicals provide potential alternatives to the use of synthetic chemical insecticides (Sadek, 2003).

1.4.1 Significance of secondary metabolites

In the past, the secondary metabolites have only an unsatisfactory role in plant metabolism and growth because they were considered as the waste products resulting from the primary metabolism. But today, many researchers proved the predominant role of secondary metabolites in plant and insect interaction system. During the last several years, many scientists discovered a number of active compounds and proved their ecological and chemical defensive roles in plant protection and thereby they started a new area of scientific endeavour, often called ecological biochemistry (Harborne, 1988, 1989).

The plants are known as ‘nature’s chemical factory’ due to its diversity of chemical resources and their significant role in the plant protection system. Mainly they include alkaloids, terpenoids, phenolics and sulfur containing compounds. Many of these phytochemicals have been successfully used by

human to prove their insecticidal efficiency in insect pest management system.

Alkaloids are present in many vascular plants and include caffeine, cocaine, morphine and nicotine. The alkaloids are unevenly distributed in plant families except *Papaveracea*, in which all genera contained at least one alkaloid. A large number of useful pharmacological and insecticidal properties of alkaloids were exploited by man and they exhibit important deterrent factors against herbivorous pest. Petterson *et al*. (1991) reported that a group of quinolizidine alkaloids were effective feeding deterrents against a number of herbivores including insects, molluscs and mammals.

Terpenoids are the largest class of secondary metabolites and the synergetic effects of their derivatives against insect pest were recorded by many scientists. According to Harborne (1988), terpenes and related plant secondary metabolites (sesquiterpinoids and sterols) have been shown their insecticidal performance in management of several insect pests and this insecticidal activity is mainly due to their action as deterrents, toxins or as modifiers of insect growth.

A broad spectrum of insecticidal properties of phenolics has been identified by many researchers. According to Wuyts *et al*. (2006) phenolics are vital components having foliage defense structure against insects and also inhibit peristalsis of some nematodes. The major compounds under phenolics group are ligin, tannins, coumarin, furanocoumarines and flavonoids.

In recent alternative pest management, secondary metabolites of plant origin could be used in insect pest management and thereby aiming at protection of different agricultural crops by using their own mechanism through allelochemicals (Regnault and Roger, 2012). Today, many researchers are focusing on possible utilization of plant products such as

newer chemotherapeutics for plant protection because they are non-phytotoxic, ecofriendly and easily biodegradable (Isman, 2006). Hence the large spectra of plant derived compounds can be successfully exploited by human for preparing the biopesticides to develop an ecologically acceptable trend instead of synthetic insecticide.

1.5 Relevance of the study

Mango, the leading fruit crop of India faces enormous losses due to ravages of insect pests. Among these, the leaf webber *O. exvinacea* create a challenging situation to the crop yield production. Fast and effective pest eradication can be successfully done with synthetic chemical insecticides but its resultant pollution and environmental problems has forced to think about alternative methods which should safe and eco friendly. On the light of number of similar research works, it was widely accepted that the botanicals used pest management system is environmentally acceptable. In the present study an attempt was made to evaluate the toxic effect of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on sixth instar larvae of *O. exvinacea* and to prove the potency of these botanicals in management of this pest.

Clerodendrum infortunatum and *Chromolaena odorata* are locally available weeds which had proved their insecticidal property by many researchers. It was reported that *Clerodendron* spp. has insecticidal activity against a number of various pests (Roy Choudhary, 1994; Panday *et al*., 2005). Furthermore, the impact of *C. infortunatum* against *Oryctes rhinoceros* (Chandrika and Nair, 2000; Sreelatha and Geetha, 2010), and *Helopeltis theivora* (Roy *et al.,* 2009) were also reported. Pesticidal effect of *C. infortunatum* on the fat body of *Oryctes rhinoceros* causes drastic changes like reduction in the lobes of fat body and their derangement together with the disintegration of cell membrane, shrunken and scattered nucleus (Sreelatha

and Geetha, 2011). Many workers have proved the insecticidal activity of *C. odorata* in pest management programme. *C. odorata* leaves mixed with soil in sweet potato beds before planting reduces weevil infestation (Rajamma, 1982). Methanolic extracts of *C. odorata* leaves caused disruption of oocyte development and vitellogenesis in *Oryctes rhinoceros* (Sreelatha and Geetha, 2010). These effects reveal the potency of both botanicals to be used as natural biopesticides for the control of the pest, *O. exvinacea.*

The present investigation was an attempt to study the toxic effect of *Clerodendrum`infortunatum* and *Chromolaena odorata* on the histomorphology of the midgut tissue and the efficacy of both botanicals on different biochemical parameters of midgut, fat body and haemolymph of sixth instar larvae of *O. exvinacea*. The toxicity study of major fractions separated from both leaf extracts were carried out and a group of active toxic compounds were identified. By proving the insecticidal properties of active principles present in both leaf extracts, it is suggested that it can be tried in the management of not only this pest but also other various lepidopteran pests which are causing serious damages on vegetables and fruit crops.

1.6 Objectives

- To separate the major fractions from both leaf extracts by using column chromatography and to evaluate the toxicity of separated major fractions by estimating the mortality rate.
- To identify the active toxic components present in separated fraction by GCMS and LC-MS/MS.
- To study the effect of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on the histomorphology of the midgut epithelium of the sixth instar larvae of *Orthaga exvinacea*.

- To evaluate the effects of above leaf extracts on the protein concentration, amino acid and carbohydrate concentration in midgut tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea.*
- To evaluate the effects of leaf extracts on the protein profile of the midgut tissue, fat body and haemolymph of last instar larvae.
- To study the enzymatic changes that occur in both digestive enzymes (Protease and Amylase) and stress relared enzymes (ACP, ALP, SOD) under stress conditions.

CHAPETR II
REVIEW OF LITERATURE

2.1 Introduction

The successful survival of insect pest by its highly adaptive nature becomes a challenging factor for the researchers to develop different controlling measures. This is in fact has increased the number of research activities to overcome this situation through the invention of different controlling measures. Few years back, scientists have developed different types of synthetic insecticides and they were less bothered about its negative impact such as animal health hazards and environmental pollution. At present, due to the uncontrolled usage of synthetic insecticides serious threat has occurred to non-target organisms and environment. It has been estimated by Pimentel and Levitan (1986) that hardly 0.1% of the agrochemicals used in crop protection reach the target pest leaving the remaining 99.9% to enter the environment to cause hazards to non-target organisms including humans.

There are more than 500 species of insects and mites that are resistant to some form of pesticides and this has lead the countries to apply more products, combine pesticides, increase applications, or substitute with more toxic replacements. It was reported by USEPA (2011) that more than 2.5 million tons of pesticides are used in agricultural crops protection for every year and the global damage caused by synthetic insecticides reached more than $100 billion annually.

As result of the negative impacts of synthetic insecticides, it was forced to change our attitude to develop any kind of alternate approach in pest management system. Presently, many scientists have focused their research

work on developing different ecofriendly pest management approaches for crop protection.

Many literature studies show that the botanical used pest management programmes are more effective and environmentally acceptable. And also the role of secondary metabolites or active principles of plants has the efficiency to interfere the physiology of an insect pest and thereby controlling it.

This chapter deals with literature on the mango leaf webber, *Orthaga exvinacea* and its status in mango cultivation, negative impacts of synthetic insecticides, role of botanical insecticides in agriculture, significance of secondary metabolites of plants, botanicals assessment for the control of *Orthaga exvinacea* and evaluation of their toxicity, toxic effect of botanicals on histopathology, biochemical parameters and also on enzyme activity.

2.2 *Orthaga exvinacea* Hampson

The order Lepidoptera comprises one of the most destructive and challenging insects, the caterpillars of most of them are serious pest to the economically important plants like cereals, millets, pulses, oilseeds, fibre crops, sugarcane, fruit trees and vegetables. The caterpillars are mostly borers of stem, shoot or are feeders on leaves, shoots and flowers on plants. Some information on these pests is provided by Ayyar (1940, 1963), Lefroy (1971), Nair (1986), and Nair *et al.,* (1986). The leaf Webbers *Orthaga exvinacea* and *Orthaga euadrusalis* are considered as major pests responsible for low productivity in mango and cashew cultivation (Verghese, 1998). Among the serious pests affecting mango trees in South India, the mango leaf webber, *Orthaga exvinacea* Hampson was originally reported as a minor pest by Fletcher (1914), Ayyar (1940), Cheriyan (1942) and Ayyanna *et al.,* (1977) but since last few years, it has been reported as a major pest of mango in Kerala (Cherian and Ananthanarayanan, 1943).

According to Singh *et al*. (2006) *O. exvinacea* is widely distributed in different agro-climatic zones of India and has gained the status of serious pest. Kavita *et al*. (2005) reported that the mango leaf webber is a major pest in intensively cultivated areas of Andhra Pradesh and heavy infestation by this pest adversely affects the flowering as well as the growth of new flush.

Mishra (2001) observed that the mode of infection by *O. exvinacea* through damaging shoots and inflorescence occurred from April to December. The infested trees presented a burnt look and severe infestation resulted in complete failure of flowering (Verghese, 1998). A heavily infested tree shows many clusters of webbed and dried leaves, presenting it a conspicuous burnt up appearance (Rafeeq and Ranjini, 2011). Earlier, Cherian and Ananthanarayanan, (1943) noticed that infestation of *O. exvinacea* was more in young trees than in old trees, whereas Srivastava *et al*. (1982) reported severe pest attack even in very old trees.

Many researchers have studied the different aspects of mango leaf webber from time to time that includes biology and incidence of the pest (Haseeb *et al*., 2000; Singh, 2002; Beria *et al*., 2008; Reddy, 2013), screening of the germplasm to identify the resistance source (Reddy *et al*., 2001), exploration of entomopathogenic fungi (Asari *et al*., 1977) and efficacy of neem formulation and insecticides (Singh, 1999; Bhatia and Gupta, 2002). Many studies reported potent natural enemies against the larvae of *Orthaga* spp. (Srivastava and Tandon, 1980; Tandon and Srivastava, 1985). The large size of mango tree is the limiting factor for management of leaf webbers and also the mango orchard provides a safe micro-ecosystem for breeding of the pest in active period and for a safe living in the same orchard during off-season (Shukla *et al*., 2001).

2.3 Negative impact of synthetic insecticides

The undesirable detrimental effects of synthetic organic insecticides on living things and its surroundings were started from late 19th century. By the discovery of DDT in 1939, a revolutionary change could be made in agricultural pest management system but later its widespread usage has created serious environmental issues and this was brought to public attention through the book 'Silent spring' written by Rachel Carson in USA.

The major classes of synthetic insecticides are Organochlorines, Organophosphates, Carbamates and Pyrethroids. Organochlorines directly affect the reproductive, nervous, endocrine, and immune system of the pest and include DDT, toxaphene, dieldrin and aldrin. Organophosphates include diazinon, glyphosate, malathion etc. Carbamates like carbofuran, aldicarb, carbaryl affect the central nervous system of the pest. Fenpropanthrin, deltamethrin, cypermethrin like pyrethroids were found to be highly toxic but their mode of action is poorly described. The use of these synthetic insecticides with their different mode of action helped to increase the crop yield but at the same time their higher toxicity interfere the normal living conditions of environment.

Many observations were recorded relating to the harmful effects of synthetic insecticides which include acute, delayed and allergic effects. Acute effects are injuries or illness that appears immediately after exposure and slowly it disappear with proper medical care. Delayed effects are the most dangerous because the illness or injuries do not appear immediately, so it is very difficult to identify it initially and later it appears extremely. Allergic effects are harmful and they occur in response to certain substances but not all people develop it.

In India, the production of pesticides was started in 1952 with the establishment of a plant for the production of BHC, and India is now the second largest manufacturer of pesticides in Asia after China and ranks twelfth globally (Mathur, 1999). According to Varma and Dubey (1999), the discovery and use of DDT and BHC contribute a subsequent development in the field of crop protection through the marked chlorinated cyclo- dienes compounds but have also raised a number of ecological and medical problems. It was estimated that per every year 250,000 to 370,000 deaths were occurred due to pesticide poisoning worldwide (Gunnell *et al*., 2007).

Synthetic insecticides have a wide spectrum of activity against diverse groups of insects, so complete pest eradication can be successfully done from the field. There is no provision to control the action of these toxic compounds, hence the residual parts of insecticides applied in different ways penetrates into the surroundings of farmland ecosystems and act destructively, not only on the invertebrates, but also on the vertebrates (Chowanski *et al.,* 2014). Many researchers noticed various pesticidal issues such as hazards to human health and non-target organisms (Sighamony *et. al*., 1986), environmental pollution (Wright *et al*., 1993) and development of resistant strains (Yusof and Ho, 1992; White, 1995).

Miller (2004) noticed that over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, because they are sprayed or spread across entire agricultural fields. It was also reported by Carriger *et al*. (2006) that generally an estimation of only 0.1% of the pesticides applied reaches the target organisms and the remaining bulk quantity contaminates the surrounding environment. The repeated and enormous use of non-biodegradable pesticides lead to pollution of water, air and soil ecosystems and have also entered into the food chain and bioaccumulated in higher trophic levels causing many acute and chronic

illnesses in human beings (Mostafalou and Abdollahi, 2012).The pollution of water in different reservoirs with pesticides occur generally through run off or drainage induced by rain or irrigation (Larson *et al*., 2010) and also air may get polluted through different ways like spray drift, volatilization from the applied surface and aerial application of pesticides.

Synthetic insecticides have a long span of action, their retention in the environment for long periods of time, often several times exceeding the life period of different species and even generations of animals. Thus the intensive use of pesticides has led to the development of resistance in many targeted pest species (Tabashnik *et al*., 2009). The increased amount and higher frequency of pesticide applications have created a challenging situation to targeted pests causing them to either disperse to new environment and/or adapt to the novel conditions (Meyers and Bull, 2002; Cothran *et al*., 2013). The first well-documented case of insect resistance to insecticides was in 1946. It was resistance to dichlorodiphenyltrichloroethane (DDT) discovered in the housefly *Musca domestica* (Hammerstrom, 1958). Dhaliwal *et al*. (2006) reported the resistance developed in many insect species towards different insecticide groups as 291 species against cyclodiene, 263 species for DDT, 260 species for organophosphates, 85 species for carbamates, 48 species for pyrethroids, 12 species for fumigants and 40 species for other classes.

Many scientists proved the development of insecticide resistance in insect pest through their adaptive nature. Intensive use of insecticides is the main cause for the rapid development of insecticide resistance in many species of insects (Paris and Despres, 2012) and also it may lead to pest resurgence (Dhaliwal *et al*., 2006) and this pest resurgence was observed in bed bug, *Cimex lectularius* by Davies *et al*. (2012) and by Mironidis *et al*. (2013) in cotton bollworm, *Helicoverpa armigera*. After a limited time, the

frequency of insecticidal compound rises above critical value, which causes a given pesticide to become useless since a significant part of the pest population does not show sensitivity to it (Hammerstrom, 1958; Lenormand *et al*., 1999; Silva *et al*., 2012).

The insecticidal chemicals are equally harmful to human and other beneficial organisms and also it cause severe ecological disturbances. The major reported drawbacks in use of the pesticides include development of resistance and increasing costs of pesticides, excess use of pesticides led to resurgence of minor pest, ecological imbalance, pesticide residue in food and environmental pollution. These recorded negative impacts of synthetic insecticides prompted to change our pest management system with appropriate ecofriendly approach.

2.4 Use of botanicals in pest management

The botanical used pest management practices were started by ancient people and they were more bothered about environmental balance and health of other living organisms than the yield of crop production. But after those centuries, a revolutionary change was made by scientists through the development of synthetic inorganic insecticide in agriculture, it was only aiming at to increase the production of crop yield and they were less bothered about its environmental disturbances and harmful effects to the living organisms. But in recent years, an ecofriendly trend was developed in pest management system by using botanicals and other plant products. Many researchers noticed and screened a number of different plant families on the basis of their pesticidal effects and has developed some botanical based pesticides in pest management field.

Botanical pesticides are the relevant alternatives to reduce or replace the use of synthetic pesticides as they possess a wide range of properties

including toxicity to the pest, antifeedance, repellency, insect growth regulatory activities against agriculturally important pests (Prakash and Rao, 1986; Prakash *et al*., 1987 a, 1989, 1990) and they are being natural compounds, do not create any problems with persistence in the environment (Gebbinck *et al.,* 2002). Senthil-Nathan and Kalaivani (2005) also reported that botanical pesticides are highly effective, safe and ecologically acceptable. Fortunately, India has a prosperous condition of plants with potency of medicinal and insecticidal properties to develop biopesticides for helping farmers in pest management through an ecofriendly and economic way. Major advantages of botanical insecticides over synthetic insecticides are followed.

- Botanical pesticides do not cause any health hazards for non-target organisms and do not possess any environmental pollution because of their immediate degradation under normal environmental condition.
- There is no risk of developing pest resistance to these products and due to its high specificity in mode of action, it is less harmful to beneficial insects.
- Do not possess any adverse effect on plant's growth, yield quality and seed viability.
- Botanical pesticides are less expensive and easily available because of their natural occurrence.

2.4.1 Insecticidal activities of botanicals

Many workers reported that plants act as potent sterilents causing reproductive abnormalities including ovarian regression, abnormal arrested oocyte development and vitellogenesis (Singh, 2003; Sreelatha and Geetha, 2008; 2010). Limnoids from *Khaya senegallensis* have insecticidal properties against the cotton leaf worm *Spodoptera littoralis* (Aswad *et al*., 2003). Anjali

(2008) observed significant reduction in fecundity, hatchability and survival of eggs in *Epilachna dodecastigma* exposed to sublethal concentration of neem leaf cake and oil.

Ganesh Kumar and Sevarkodiyone (2009) reported that the seed extracts of *Annona squamosa* and *Lepidium sativum* showed a negative growth rate on the pupal development of *Spodoptera litura*. Application of *Clausena dentate* resulted in abnormalities in reproductive potential, inhibition of egg production, abnormal oocyte maturation and disturbance of protein synthesis in *Helicoverpa armigera* (Malarvanan *et al*., 2009). Anuradha *et al.* (2010) observed that the leaf extract of *Adathoda vasica* showed antifeedent activity on *Spodoptera litura* larvae.

Deepthy *et al.* (2010) noticed the growth inhibitory and insecticidal activity of *Vitex negundo* against *S. litura*. Martin and Gopalakrishnan (2010) recorded that three fractions obtained from methanolic extract of *Lantana wightiana* have insecticidal activity against fourth instar larvae of *S. litura*. Pratibha *et al*. (2010) reported that cold ethyl alcohol extract of *Annona squamosa* seeds are highly effective in controlling *S. litura* by causing a significant percent mortality at the larval, larval-pupal intermediate and pupal stages. Sahayaraj and Kombiah (2010) observed the insecticidal activities of neem gold on *Cosmopolites sordidus.* Studies on antifeedent and larvicidal activity of *Cassia fistula* on *S. litura* was carried out by Duraipandiyan (2011). Antifeedent activity of fistulin isolated from leaves of *Cassia fistula* was recorded by Arulpandi and Sangeetha (2012).

2.5 Role of secondary metabolites in pest management

Plants produce a wide range of organic compounds which have no direct role in plant growth and development but their insecticidal activities and importance in plant protection were enormously reported by many

scientists. Schafer and Wink (2009) reported that plants producing secondary metabolites have most prominent function in plant protection against pest and pathogens and they have the capacity to kill, retard or accelerate development or interfere with the life cycle of pest. The interruption of secondary metabolites on pest through different aspects include interfering in the development of pest, deformities, lengthening of developmental stages or may serve as repellents (Nenaah, 2011; Dinesh *et al*., 2014).

The mode of action of secondary metabolites to protect the plant itself was interestingly reported by many scientists. According to Miresmailli and Isman, (2014) the increased production of secondary metabolites from different parts of a plant is to protect from the high risk of pest attacks and hence these compounds also known as chemical weapons of plants. Sometimes these compounds can make the plant unfit for feeding by pest (Hirayama *et al*., 2007) or otherwise lure the pests towards poison and kill them (Tangtrakulwanich and Reddy, 2014). Rattan (2010) also noticed the functions of secondary metabolites present in plants as defensive, which inhibits reproduction and other biological processes.

The secondary metabolites involving alkaloids, terpenoids, phenolics, essential oils and other organic chemicals contribute a large spectrum of biological activities, such as attractants, antifeedants, deterrents, phagostimulants, or modify oviposition (Venkatachalam and Jebanesan, 2001). Plants produce different secondary metabolites like monoterpenes, sesquiterpene lactones and triterpenes which are believed to be a corner stone in plant natural defence mechanism and also they may have commercial applications (Heywood *et al*., 1977; Barney *et al*., 2005).

The plant essential oils also have a wide range of pesticidal activities which have long been used as fragrances and food xavorings and also demonstrated fumigant and contact insecticidal activities (Regnault-

Roger,1997).Terpenoids have great ecological value since they have been causing insecticidal activities like larval mortality, significant growth inhibition, repellent and fumigant activity. These plant terpenoids along with phenols, flavonoids, steroids play a prominent role in plant defences against phytophagous insects (Hartmann, 1996; Bruneton, 1999; Cox, 2004; Kubo, 2006; Aniszewski, 2007). The use of plant and plant-derived products to control pests in the developing world is well known and prior to the discovery of synthetic pesticides, these are the only pest-managing agents available to farmers around the world (Owen, 2004).

2.6 *Clerodendrum infortunatum* L.

The extraction is the initial and crucial step in the phytochemical analysis of plant, because it is necessary to separate the active components from the plant for categorizing them. Appropriate solvent selection is the major step in phytochemical extraction. According to Ghosh *et al*. (2012) the phytochemical extraction of active components from the plants depends upon the polarity of the solvents. The solubility of phytochemicals was different in different solvents (Cowan, 1999). The polarity of solvents range from the most polar solvents (water, acetic acid, ethylene glycol etc.) to the most non polar (pentane, hexane, petroleum ether etc.) and this wide range help to select the appropriate solvent for the extraction purpose. Generally polar solvents will always extract polar molecules and non-polar solvents extract non-polar molecules. Many researchers noticed the maximum efficiency of methanol for phytochemical extraction (Poojary *et al.*, 2015; Dhawan and Gupta, 2017).

There is a lot of scientific literature available on the medicinal, agricultural as well as the chemical aspects of plant species *Clerodendrum*. The various types of extracts from root, stem and leaves of *Clerodendrum viscosum* were reported to be used as therapeutic or prophylactic treatment of

tumours and certain skin diseases, pyreticosis, asthma, cataract, malaria, stomach disorders and diseases related with blood by tribal and village folks (Shrivastava and Patel, 2007; Das *et al*., 2009). Waliullah *et al*. (2014) conducted studies on the antibacterial and antifungal properties of *C. infortunatum* against various pathogens. Saidutty (1999) reported that the essential oils extracted from the leaves and root bark of *C. infortunatum* contained a number of different compounds which are partially used as drugs, laxative and remedy against some skin diseases or tumours.

Number of phytochemical extraction and compound isolation studies were reported in *Clerodendrum* species and most of them have the potency to control the insect pest. Several diterpenoid like insecticidal active compounds were isolated from *C. inerme* by Raha *et al*. (1991) and Rao *et al.* (1993). Akihisa *et al.* (1990) identified phenol-steroid compounds such as 22, 24-dimethyl 25-dehydrolophenol and 4 K-methyl steroid from *C. inerme* and Tian *et al.* (1993) isolated two novel abietene diterpene from *C. cyrtophyllum* and all these compounds have the insecticidal properties like growth inhibition and antifeedant activity on insects.

Many literatures presented the important antifeedant activity of this species on different insect pests. Kumari *et al*. (2003) reported the insecticidal and antifeedant activities of Clerodane diterpenoids isolated from *C. infortunatum* against the Cotton Bollworm, *Helicoverpa armigera* and a number of diterpene compounds were isolated from *C. tricotomum* possessing varying degrees of deterrent activity against *Spodoptera litura* (Kato *et al.,* 1972). Koul (1982) showed that 100% antifeedant activity at 50 ppm concentration of active compounds among diterpenes such as dihydroclerodin, and clerodinhemiacetal isolated from *C. divaricate*. Roy Choudhary (1994) observed the antifeedant and insecticidal activities of

Clerodendrum species against rice weevil and the same was reported by Roy *et al.* (2009) on *Helopeltis theivora.*

The insecticidal potency of phytochemical compounds present in *Clerodendrum* species were significantly proved by many scientists. Jirovetz *et al*. (1999) have studied the phytochemicals in essential oils extracted from the leaves and root bark of *C. infortunatum* by using different separation techniques like chromatographic-mass spectrometric and olfactory methods and among these phytochemicals, linalool showed insecticidal and molluscicidal properties.

It was observed that enormous reduction in the populations of the two pests in the field may be due to collective insecticidal, acaricidal, ovicidal, growth regulatory and antifeedant effects of *C. viscosum* (Gurusubramanian *et al*., 2008; Roy *et al.,* 2009 b). Sejbac *et al*. (1996) observed that the *C. viscosum* contains clerodin, lupane, clerodone, uncinatone and pectolinarigenin which possess insecticidal potency to the crop pests and also the contact response of this extract has led to suppression in their egg hatching, feeding and population size. (Roy *et al*., 2010 b).

Many workers have studied the toxic impact of *C. infortunatum* in insect pest control. Ahmed *et al.* (1981) reported insecticidal potential and biological activity of some selected Indian indigenous plants against *Musca domestica*. Pesticidal effect of *C. infortunatum* on the fat body of *Oryctes rhinoceros* caused drastic changes like reduction in the lobes of fat body and their impairment with the disintegration of cell membrane, shrunken and scattered nucleus (Sreelatha and Geetha, 2011). Furthermore, the impact of *Clerodendrum infortunatum* against *Oryctes rhinoceros* (Chandrika and Nair, 2000; Sreelatha and Geetha, 2010) and *Helopeltis theivora* (Roy *et al.,* 2009) were also recorded.

2.7 *Chromolaena odorata* (L.) King & Robins.

Chromolaena odorata formerly known as *Eupatorium odoratum*, is a weed categorized under sunflower family *Asteraceae* and originally located from southern Mexico south to Argentina and the Caribbean (Morton, 1981), but has been introduced into the Old World tropics where it has created an invasive role (Vaisakh and Pandey, 2012). Literature studies shows a large spectrum of applications of this plant in medicinal and agriculture field.

Many researchers evaluated the diverse properties of *C. odorata* in different aspects. Studies have revealed that the leaf extract of *E. odoratum* has a number of medicinal values such as antioxidant activity (Amatya and Tuladhar, 2011), anti-inflammatory activity (Owoyele *et al*., 2005; Hanh *et al*., 2011), allelopathic effects (Hu and Zhang, 2013), cyto-protective role, cytotoxicity and hepato-protective efficiency (Alisi *et al.,* 2011), analgesic (Vaisakh and Pandey, 2012), antimicrobial effects (Ngane *et al.,* 2006), insecticidal potency (Rajmohan and Logankumar, 2011), anti-diabetic and anti-cataract (Onkaramurthy *et al*., 2013), anti-schistosomiasis properties (Benson, 2012) and platelet-activating factor (PAF) receptor binding antagonist activity (Ling , 2007).

The potency of insecticidal properties of *C. odorata* has proved by many scientists. The ethanolic crude extract (Akob and Ewete, 2009) and essential oils (Bouda *et al*., 2001) of *C. odorata* was shown to possess insecticidal activity against *S. zeamais*, while its essential oil also showed toxic property to other insect pests (Felicien *et al*., 2012; Pamo *et al*., 2004). Zetan (2004) reported the significant antifeedant and toxic effect of the crude extract from *Eupatorium odoratum* against the *Helicoverpa armigera.* Essential oil extracts from their leaves have shown the efficacy on the mortality of the maize grain weevil, *Sitophilus zeamais* (Bouda *et al*., 2001).

According to Sreelatha and Geetha (2010) the methanolic leaf extract of *E. odoratum* can interrupt or disrupt the oocyte development and vitellogenesis in *Oryctes rhinoceros* and thereby control this pest. Rajamma (1982) proved the pesticidal efficiency of *E. odorata* by mixing the leaves with soil in sweet potato beds before planting which reduce weevil infestation. The essential oil from *C. odorata* has exhibited insecticidal (Bouda *et al.,* 2001), insect repellent (Cui *et al.,* 2009), and antibacterial (Inya-Agha *et al*., 1987; Bamba *et al.,* 1993) activities. Ahiati (2013) found that *C. odorata* oil to be effective against both the larvae and adults stages of mosquitoe. It was observed by Kelm and Nair (1998) that the presence of flavonoid, tannin and saponin occurred in leaf extracts of *C. odorata*. It was reported that saponins and triterpenoid group of secondary plant metabolites have different biological properties, such as antifeedant (Barbosa *et al.,* 1990) and growth inhibitory activities (Geyter *et al.,* 2011).

2.8 Histopathological effects of botanicals on insect pests.

Highly adaptive nature of insects facilitates a complex digestive mechanism for their survival in different conditions. Midgut is the main compartment for digestion in insect where digestion of food materials into useable small molecules and absorption of these nutrients into the cells, tissues and haemolymph takes place. So the complex cells present in midgut epithelium produce secretion into lumen for the digestion of food materials.

According to Rocha *et al.* (2010) the midgut of insects is the main part of the digestive tract in which digestion, absorption and transport of nutrients and inorganic ions occur and its wall comprises a single layer of epithelium and two muscle layers. Billingsly and Lehane (1996) observed that the midgut epithelium of lepidopteran larvae is a complex and dynamic tissue which is composed of 4 cell types: columnar, goblet, regenerative and endocrine.

Sutherland *et al*. (2002 b) conducted studies on insect midgut and revealed that midgut is the major site for digestion and absorption of nutrients and widely considered as a tissue where the synthesis and secretion of digestive enzymes occurs. This same was reported by many scientists that the digestive and absorptive epithelial cells are the most predominant cells in insect midgut and are responsible for processing of the diet and are actively involved in enzyme production and secretion, as well as absorption of nutrients (Martoja and Ballan Dufrancais, 1984; Dow, 1986; Baldwin *et al*., 1996; Billingsley and Lehane, 1996; Serra˜o and Da Cruz-Landim, 1996; Van Zyl and Van Der Linde, 2000).

Several scientific literatures show that the intake of toxic plant components into the midgut may create structural and functional interference in histomorphology and it leads to physiological disorders and thereby control the pest. Emara and Assar (2001) reported that when *Spodoptera littoralis* was treated with the extract of both *Clerodendrum inerma* and *Conyzadio scorids* there occurred slight and severe disintegration of the epithelium, fading of the boundaries of epithelial cells and detachment of epithelial cells and it led to several physiological disorders. Similar effects were noticed by Schmidt *et al.* (1997) and Salam and Ahmed (1997) on *S. littoralis* treated with the extract of *Melia azedarach.*

Several utrastructural studies were carried on the midgut of lepidopteran insects such as *Manduca sexta, Spodoptera frugiperda, Anticarsia gemmatalis, Alabama argillacea* (Hübner), *Diatraea saccharalis* and these studies suggested that the distribution and morphology of the epithelial cells and their variations along the different regions of midgut and these changes were studied only through the ultrastructural observations (Santos *et al*., 1984; Billingsley and Lehane, 1996; Levy *et al*., 2004; Pinheiro *et al*., 2008).

Many scientists focused on the ultrastructure and functions of different cellular components present in midgut epithelium. The digestive cell contains a prominent, often lobed nucleus in the central part of the cell and well-developed rough endoplasmic reticulum (rER) (Billingsley and Lehane, 1996). The midgut of insect is regarded as the best transporting epithelia, employed in intensive secretion of K+ and base equivalents (Chamberlin, 1990; Dow and O'Donnell, 1990), along with endosmotic fluid secretion (Zerahn, 1985), with the result the water content of gut components has been increased (Reynolds and Bellward, 1989). The peritrophic membrane in midgut separates the food from midgut tissue and plays an important role in the protection of the gut from food abrasion and various microbial challenges (Peters, 1992; Lehane, 1997).

According to Hung *et al.* (2000) the apical region of columnar cells with abundant mitochondria were involved in the absorption and metabolism of nutrients and the basal portion of columnar cells were involved in the nutrient and ion transport from the cell to the haemolymph. Terra *et al.* (1996) reported that the cellular components that fill the apical region of the columnar cells were involved in the elaboration of the digestive enzymes. Histological studies carried by Gerard (2002) on the midgut tissue of *Hofmannophila pseudospretella* reported the differences in the size and activity of middle midgut columnar and goblet cells depending on food quality.

Significant difference was observed by Peric-Maturaga *et al*. (2006) in size of columnar and goblet cells in gypsy moth larvae fed with suitable *Quercuscerris* leaves compared with larvae fed on *Robinia pseudoacacia* leaves which is associated with higher digestive activity. The histopathological effect of *Artemisia monosperma* on *S. littoralis* include the cytotoxic changes like extreme vacuolization and completely destroyed

epithelial membrane; elongated columnar cells with destructed cell boundaries (Adel *et al*., 2010). Jbiloua and Sayah (2008) noticed the cytoplasmic vacuoles and occurrence of large intercellular space due to the effects of methanolic extract of *Peganum harmala* seeds on *Tribolium castaneum* midgut cells through diet incorporation.

Sayed *et al.* (2011) reported that the larval midgut of *S. littoralis* treated with *Azadiracta indica* and *Citrullus colocynthis* extracts showed vacuolization and necrosis of the epithelial cells and destruction of epithelial cells and their boundaries. Abnormalities in ovarian follicular epithelial cells were found in brown plant hopper when induced by azadirachtin (Senthil-Nathan *et al*., 2008).

Histopathological studies carried out by Packiam *et al*. (2013) observed the changes such as cellular shrinkage, disintegration of peritrophic membrane and epithelium, necrosis, irregular nuclear arrangement etc. in the midgut of *Helicoverpa armigera* when treated with neem and pongam formulation. Barbara *et al.* (2007) suggested that the effect of plant cyclotides on the larval midgut of *H. armigera* showed that the cyclotides disrupt the plasma membrane of the epithelial cells forming holes or pores that lead to cell swelling and lysis.

According to Cottee (1984), the histological changes such as vacuolization of cytoplasm, reduction in the size of the nuclei, regeneration and necrosis of cells that occurred in *Schistocerca gregaria* and *Locusta migratoria* was mainly due to the active compounds present in botanicals. Nasiruddin and Mordue (1994) noticed the histopathological changes that occurred in the midgut of *S. gregaria* and *L. migratoria* after treatment with azadirachtin which consisted of swelling of cell organelles and rounding up of the cells, necrosis of midgut cells, enlargement of connective tissue with many invading cells and shrunken and swollen muscle layers.

Sayah *et al*. (1996) reported the potency of azadirachtin treatment on ovaries and fat bodies of earwig, *Labidura riparia* and observed the changes like separation of follicle cells from oocytes, degenerative changes in follicles, fragmentation of rough endoplasmic reticulum, multi vesicular bodies in adipocytes and lack of pinocytotic vesicles. Rembold and Annadurai (1993) noticed the azadirachtin induced changes like inhibition of cell proliferation and monolayer formation in *S. frugiperda* ovarian cell line (sf 9). Changes like elongation of cells and vacuolization, disruption of plasma membrane were also reported by Cohen *et al*. (1996) in *S. frugiperda* treated with a neem limonoid, nimbolide.

Gerard (2002) studied the changes occurred in the midgut epithelium of *Hofmannophila pseudospretella* associated with the introduction of keratin contained food diet and noticed the important differences in size and activity of columnar and goblet cells. Rocaglamide was an isolated compound from *Aglaiaelae agnoidea* and it possessed cytotoxicity at non-specific cellular levels on *H. armigera* (Koul *et al*., 2004).

The active principles present in plants possess the cytotoxicty and this can be exploited by many scientists to control several insect pests. The histopathological studies by Jing *et al*. (2005) on the toxic impact of ingested Jatropherol-I on the ultrastructure of midgut cells in silkworm *Bombyx mori* revealed that the changes occurred in the midgut epithelial cells due to the induction of phorbol-type plant isolated compound Jatropherol-I and it caused disintegration of the epithelial cells and it lead to severe turbulence in insect metabolism, especially in protein metabolism with alterations in activities of various midgut enzymes.

According to Halliwell and Gutteridge (1985), the plant flavonoids are group of allelochemicals with widely noted prooxidant effects and these effects include protein and lipid damage and DNA strand scission due to

excessive production of reactive oxygen and free radical species. It was reported that squamocin from *Annona squamosa* on *Aedes aegypti* larvae prevented the production of ATP by the electrons in the mitochondrial complex I and caused the death of the insect by affecting cellular respiration (Lummen, 1998; Takada *et al*., 2000). The effect of tannins on different species of Acridoidea showed the histopathological changes on midgut epithelia such as lesions and necrotic nuclei in *Locusta migratoria* (Bernays *et al*., 1980).

2.9 Biochemical effects of botanicals on insect pests.

The cytotoxic effects of plant extracts lead to serious digestive disorders and physiological problems in the insect pest. In addition to this, many scientists evidently proved that the active ingredients present in plant extracts can interfere the insect metabolism by creating qualitative and quantitative changes in biochemical parameters like protein, amino acid and carbohydrate.

Among the three regions of the gut, most of the studies were carried out particularly in midgut region because any kind of alterations that occurs in midgut will directly affect the growth and development of insects a as result of physiological changes and it mainly depend on meal intake, absorption and transformation (Mordue and Blackwell, 1993; Nisbet, 2000; De Sousa *et al*., 2009). Medhini *et al.* (2012) reported the antimicrobial activity of bioactive factors present in *Calendula officinalis* against the gut micro flora of the larvae of *S. litura* that contribute for the reduced digestive capacity. Similar observations were noticed by Adel *et al.* (2000) in *S. littoralis* against the activity of a number of saponins and sapogenins. The toxic components of *C. inerme* disrupt the process of digestion and absorption in *A. aegypti* larvae (Patil, 2008).

Proteins are the major biological factor that plays an important role in insect growth, development and various other physiological processes (Sahayaraj and Kombiah, 2010). According to Etebari *et al*. (2006) estimation of total protein is the major parameter in physiological studies, because insecticides reduce the feeding efficiency which in turn minimizes important vital components like proteins in the body.

Ulrichs *et al*. (2008) studied the efficacy of *Porterasia coarctata* leaf extracts on the midgut tissues of *S. litura* and noticed the reduction of the total proteins in larvae. According to Ranjini *et al.* (2015), the toxic effect of methanolic leaf extracts of *Hyptis suaveolens* and *Vitex negundo* reduced the amount of midgut protein in *O. exvinacea* larvae and thereby affect the growth, development and various other physiological processes. The volatile oils from *Cymbopogon citratus* and *Rosmarinus officinalis* decreased protein content in *Musca stabulan* and also induced biochemical disturbances (Khalaf, 1998). When *S. littoralis* larvae treated with *Eucalyptus* oil and its combination with gamma radiation exhibited reduction in the total protein content of midgut tissue (El-Naggar and Abdel-Fattah, 1999).

Vijayaraghavan *et al.* (2010) studied the toxic effect of plant extracts on biochemical components of cabbage leaf webber, *Crocidolomia binotalis* and reported the protein content in an insect is dependent upon its synthesis, breakdown, water movement between tissues and haemolymph. The reduction in protein content in larvae might be due to the reduction in synthesis of protein or increase in breakdown to detoxify the active principles present in the plant extracts. Similar studies were carried out by Kiran Morya *et al.* (2010) and observed that the leaf extracts of *Lantana camara* reduced the protein content of *Corcyra cephalonica*.

Rao and Subramanyam (1986) observed the changes in the hormones that regulate protein synthesis in *Schistocerca gregaria* treated with

azadirachtin. Sharma *et al*. (2009) revealed that the body wall made of chitin protein and other larval proteinaceous tissues were damaged with the total depletion in concentration of larval protein by the treatment of *Azadirachta indica* in *Culex* larvae. Senthilkumar *et al*. (2009) evaluated the interference of some phyto extracts with normal protein synthesis mechanism of *A. stephensi* and noticed the reduced protein levels in larvae.

El-Bermawy and Abdel-Fattah (2000) reported the quantitative changes occurred in total proteins in the larvae of *Tribolium confusum* with the treatment of vetiver oil and ethanol extracts of *Calotropis procera*. According to Khosravi *et al*. (2010) the methanolic extract of *A. annua* have the efficiency to reduce the concentration of total protein, carbohydrate and lipid in fifth instar larva of mulberry pyralid, *Glyphodes pyloalis.* The reduction of protein and carbohydrate content was observed in pyralid, *G. pyloalis* when treated with the essential oil isolated from *Lavandula angustifolia* (Yazdani *et al*., 2013)

The evaluation of toxic effect of *Annona squamosa* seed extracts on *S. litura* showed that there occurred changes in total protein in all stages of the insect (Boreddy *et al*., 2000). The reduction of protein content was noticed in *S. litura* larvae when treated with seed extracts of *Annona* and neem (Vijayaraghavan and Chitra, 2002). Jbiloua and Sayah (2008) studied the efficacy of methanolic seed extract of *P. harmala* on the metabolites of *T. castaneum* through the diet and noticed considerable reduction in protein and lipid contents in last-instar larvae. A considerable decrease in the concentration of total protein was noticed by Khosravi *et al*. (2011) in the larvae of *G.* p*yloalis* treated with *A. Annua* methanolic extract.

Neoliya *et al*. (2007) analysed the toxicity of Azadirachtin-rich commercial insecticides on fourth instar larvae of *H. armigera* and observed severe reduction of protein content in larvae. Sayed *et al*. (2011) studied the

insecticidal capability of *A. indica* and *C. colocynthis* extracts on *S. littoralis* larvae and noticed the decrease of total protein, glucose and lipid contents. Vijayaraghavan *et al.* (2010) conducted studies to prove the efficacy of the extracts of *Strychnos nuxvomica*, *Vitex negundo* and *Lippia nodiflora* on the nutritional parameters of leaf webber *C. binotalis* and found that serious reduction in protein, carbohydrate and lipid content of the larvae. Haung *et al.* (2004) reported that the treatment of Azadirachtin significantly lowered the protein expression in the larvae of *S. litura.*

Several literature reviews shows the effect of botanicals on the nutrients in haemolymph of insect pest. It was described that the treatment of acetone extract of *Melia azadarach* on 6th instar larvae of *Agrotis ipsilon* showed severe reductions in total proteins, lipids, and carbohydrates in haemolymph (El Shiekh, 2002; El-Wahab, 2002). Schmidt *et al.* (1998) evaluated the efficacy of methanolic extract of *Melia azedarach* on protein content of *Spodoptera littoralis* and *A. ipsilon* and it was observed that the haemolymph protein concentration in both the insects was reduced after the treatment of 6 days at 100 ppm.

Medhini *et al*. (2012) conducted studies on the effect of *Calendula officinalis* on the nutritional and physiological parameters of *S. litura* larvae by treating the larvae with the extracts of leaf and flower in various solvents and noticed significant reduction of total protein content in haemolymph and midgut of larvae. Schmidt *et al*. (1998) observed the decreased haemolymph protein composition in *S. littoralis* due to the treatment of azadirachtin. Annadurai and Rembold (1993) evaluated the interference caused by azadirachtin with protein synthesis in the desert locust. Li *et al*. (1995) studied the influence of azadirachtin in the haemolymph protein concentration of *S. litura.* Jadhav and Ghule (2003) tested the potency of azadirachtin on

C. cephalonica and supplied it through the diet and it decreased the total body and haemolymph proteins.

Treatment with *A. squamosa* seed extract exhibited significant reduction in protein content and created conspicuous variations in electrophoretic banding pattern of fourth instar of *S. litura* (Boreddy *et al*.,2000). Huang *et al*. (2004) observed altered pupal protein levels of *S. litura* when fed with azadirachtin contained diet and affected 10 protein bands were noticed in electrophoretic studies. Sharma *et al.* (2006 a) evaluated the exhibited diminution of protein profile and structural deformities of *Anopheles* larvae when treated with methanolic extract of *A. indica*. Sahayaraj and Kombiah (2010) reported that the impact of neem gold on *Cosmopolites sordidus* altered the normal physiological activity and induced the appearance of new polypeptides. Huang *et al*. (2007) conducted studies on the effect of azadirachtin on protein profile of *Ostrinia furnacalis* and noticed significantly affected six proteins bands by using two dimensional gel electrophoresis. Ethanolic extracts of *Ageratum vulgaris* and *Ageratum conyzoides* influenced the total head protein profile of *S. litura* (Renuga and Sahayaraj, 2009).

Jadhav (2009) reported the azadirachtin treatment for 24 h, reduced the free amino acid content in *C. cephalonica* but increased after 48 h, 72 h and 96 h due to either protein depletion or inhibition of amino acid incorporation into proteins. Senthilkumar *et al*. (2009) found that the treatment of botanicals reduced total protein, carbohydrate and lipids along with certain amino acids in *A. stephensi* larvae. Canavanine, the principal non-protein amino acid of certain leguminous plants disrupt tertiary and quaternary structure of the protein after incorporation of invitellogenin of female migratory locust (Rosenthal *et al*., 1989 a).

Effect of botanicals on the carbohydrate content of insect is species specific and also related to the larval physiology of species. Many literature

studies showed the activities of botanicals to interfere the glucose contents in insect pest and thereby prove their potency to control the pest. Sharma *et al*. (2011) studied on the treatment of petroleum ether extract of *A. annua* and methanolic extract of *A*. *indica* and concluded that the glucose levels were reduced in Culicine larvae. Shekari *et al*. (2008) observed severe reduction in protein and glucose content in third instar larvae of *Xanthogaleruca luteola* after treatment with *A*. *annua* extract. Similar findings were noticed by Rathi and Gopalakrishnan (2010) in *S. litura* showing reduced total proteins and carbohydrates due to the effect of methanolic extracts of *Lantana wightiana.* It was reported that the treatment of different plant extract caused severe reduction of carbohydrate content in *S. littoralis* larvae (Bakr *et al*., 2010).

Acheuk and Mitiche (2013) proved the efficiency of plant secondary metabolite alkaloids of *Pergularia tomentosa* to reduce both protein and carbohydrate contents of *Locusta migratoria* fifth instar larvae. The essential oil isolated from *Lavandula angustifolia* when treated on the pyralid, *G*. *pyloalis*, showed reduced protein and carbohydrate content (Yazdani *et al*., 2013). Abdul Razak and Sivasubramanian (2007) evaluated the impact of *Chelomenus sexmaculata* and *Chrysoperlacarnea* on the reduction of carbohydrate content in *Stephens* due to the impact of neem, pungam and madhuca. Reduction in total carbohydrate content of the midgut was noticed in *S. littoralis* with the treatment of extracts of *Azadirachta indica* and *Citrullus colocynthis* (Sayed *et al.,* 2011).

Shekari *et al*. (2008) analyzed the efficacy of *Artemesia annua* on the midgut of the third instar larva of *X. luteola* and noticed the decreased amount of glucose content in the midgut. Sharma *et al.* (2011) conducted the studies to evaluate the toxicity of *A. indica* on the mosquito larvae and observed the significant reduction in glucose content and it was mainly due to the utilization of reserve glucose in tissues as a result of insecticidal stress.

Similar effect was noticed by Sak *et al.* (2006) that the glycogen levels of juveniles and adults of *Pimpala turionella* decrease significantly when exposed to cypermethrin.

Senthilkumar *et al.* (2009) investigated the effect of water and ethanolic extract of *A. annua* on the third instar larvae of *A. stephensi* and noticed the reduction of carbohydrate content and suggested that reduction may be due to poor feeding and improper utilization of digested food and also it was reported that during treatment of *A. annua*, the alimentary canal was highly damaged in *Anopheles s*pecies (Sharma *et al*., 2006).

Koul (1999) carried out studies on the mode of action of neem extract on *S. litura* and suggested that the phyto extracts can block the alimentary canal and it leads to the inhibition of feeding thereby lowering the glucose level. Reduction in the carbohydrate level in the adult desert locust after inoculation with the entomopathogenic fungus *Metarhizium anisopliae* was observed (Seyoum *et al*., 2002). Shoukry *et al.* (2003) reported the decreased amount of carbohydrate content in haemolymph of *Plodia interpunctella* due to the toxic impacts of two volatile oils from leaves and stems of *Piper cubeba* and *Salvia officinalis* and three fixed oils from *Rumex dentatus* and *Trigonella foenumgraecum* seeds and *Acacia nilotica* leaves.

2.9.1 Efficacy of Botanicals on Enzyme activities.

According to the type of ingested food, several enzymes have played their own critical roles in insect digestion. Any kind of disruption caused in enzyme activities may lead to serious physiological disorders and death of the organism. The plant based active principles have the efficiency to induce changes in the enzymatic activities in insect pest and thereby controlling it.

Senthil-Nathan *et al*. (2006 a, b) conducted studies on the impact of *V. negundo* extract and neem seed kernel extract on *C. medinalis* larva in

combination with *Bacillus thuringiensis* through treated rice leaves and observed serious reduction in digestive enzyme activities such as protease, amylase and lipase and also reduced the lactate dehydrogenase activity. Furthermore, Lowery and Smirle (2000) reported that neem possess the capacity to disrupt enzyme synthesis in insects.

Many scientists showed decreasing activities of insect digestive enzymes due to the impact of plant allelochemicals and synthetic insecticides (War *et al*., 2003; Senthil-Nathan *et al*., 2008). The investigation by Zibaee *et al*. (2010) on sole and combined effect of *A. annua* and *Lavandula stoechas* on *Hyphantria cunea* revealed the drastic changes occurred in digestive enzyme activity. According to Senthil Nathan and Kalaivani (2005) the treatment of azadirachtin decreases the enzyme activity in larval midgut of *S. litura*. Timmins and Reynolds (1992) also reported the capability of azadirachtin to inhibit enzyme-secreting cells of the midgut wall of *M. sexta* and thereby to reduce the enzyme secretion.

Senthil-Nathan *et al.* (2006 c) noticed the suppressed activities of digestive enzymes like protease and lipase of *C. medinalis* as a result of the treatment with *V. negundo* and *A. indica.* Zibaee and Bandani (2010 a) conducted studies to analyse the mode of action of *Aannua* extract on digestive enzymes of *E. integriceps*.

Researchers proved that botanical insecticides may interfere with the production of certain types of proteases and disable them to digest ingested proteins (Senthil-Nathan *et al*., 2006 a, b and c; Zibaee and Bandani, 2010 a). Khosravi and Sendi (2013) conducted studies on the effects of azadirachtin on *G. pyloalis* and suggested that this active component lowered the activities of both proteases and α-amylase in the midgut, due to its feeding deterrence ability. The toxic impact of methanolic extract of *P. harmala* seeds on the amylase isoforms of *T. castaneum* showed reduced activities of the major

isoforms and inhibition of minor isoforms of amylase (Jbilou and Sayah, 2008).

Saleem and Shakoori (1987) reported the decreased level of *a*-amylase activity in the larval gut of the beetle *Tribolium castaneum* with the treatment of sublethal concentrations of pyrethroids. Ortego *et al*. (1999) conducted studies to evaluate the efficiency of terpenoids to interfere the activities of digestive enzymes and antioxidant enzyme of *Colorado potato* beetle, *Leptinotarsa decemlineata* larva, when larvae fed with treated potato leaves showed reduced esterase activity. The effects of neem derivative azadirachtin on the fourth instar larvae of *P. interpunctella,* showed serious reduction in amylase activity (Rharrabe *et al*., 2008).

The plant defense compounds present in extracts can act on insect gut enzymes and thereby reduce the activities of α-amylases and proteases (Ryan, 1990; Franco *et al*., 2002). It was observed by Khosravi *et al.* (2011) that the different concentrations of methanolic extract of *A. annua* at 48 hrs post-treatment in *G. pyloalis* larvae showed reduced protease activity when compared to control.

The inhibitory activities of lipase, α-amylase and cytochrome P450 and promoting effects of protease, carboxyl esterase and glutathione S-transferase with the treatment of fraxinellone, a significant naturally occurring compound isolated from Meliaceae and Rutaceae spp. were reported by Lv *et al*. (2014). Khosravi and Sendi (2013) evaluated the capacity of azadirachtin to reduce the protease activity and also to reduce the level of α and β-glucosidases in *G. pyloalis* treated with neem pesticide (achook). Zibaee *et al*. (2010) conducted studies on the effects of sublethal concentrations of different biopesticides treatment on *Hyphantria cunea* larvae and observed a serious reduction in the activity level of α- and β-glucosidases.

Shekari *et al.* (2008) reported the decreased α-amylase activity level after 24 hrs and sharply increased level after at 48 hrs in elm leaf beetle treated with *A. annua* extract. Similarly Hasheminia *et al.* (2011) noticed significant reduction of α-amylase activity in *Pieris rapae* treated with *A. annua* extract. War *et al*. (2014) recorded the synergetic effect of neem oil formulation and endosulfan against *H. armigera* and observed significant reduction in the activity of digestive enzymes, serine proteases and trypsin along with an increase in GST activity.

Zibaee and Bandani (2010 a) investigated the effect of different concentrations of *A. annua* extract on the adults of *E. integriceps* and showed that reduction in the activity of different glucosidases and this enzyme inhibition increased with enhancing the plant extract concentrations due to the disruption of consumption rates and food conversion efficiencies.

Rharrabe *et al*. (2007) reported the inhibition of amylase activity in *P. interpunctella* when treated with Harmaline and also stated that the cytotoxic effect on epithelial cells of the midgut may cause reduction of amylase activity. Adel *et al*. (2010) investigated the biochemical effects of *Artemisia monosperma* on the cotton leaf worm, *S. littoralis* and suggested significant inhibition in the activity of amylase and invertase enzymes; major reduction in the transaminase enzyme activity (both AST and ALT) and potent inhibitory effect of phosphatase enzyme activity (ACP and ALP).

The enzymes ACP and ALP are involved in different kind of metabolic activities such as growth and cell differentiation, permeability, absorption and transport of nutrients, protein synthesis, steroidogenesis and gonadal maturation (Ram and Sathyanesan, 1985). Many workers have studied on lysosomal marker enzyme ACP and suggested that it exists in the haemocytes and serum of bivalve (Mazorra *et al*., 2002; Wootoon and Pipe, 2003).

The higher toxicity of combined effect of neem oil formulation and endosulfan lead to inhibit the activities of detoxification enzymes in insect's body (Ortego *et al*., 1998; Scott *et al*., 2002). Anuradha and Annadurai (2008) recorded the mode of action of azadirachtin in insect pest, as it inhibits peristalsis, reduces enzyme production as food moves through gut, inhibits midgut cell replacement, and reduces feeding. Babu *et al.* (1996) also reported the efficiency of azadirachtin to increase the ACP activity in gut and decreased ATPase activity in *H. armigera*.

Several plant extracts such as *Apium graveolens, Ammi majus*, *Vinca rosea* and *Melia azedarach* were found to inhibit ACP activity in *Agrotis ipsilon* (Abo El-Ghar *et al*., 1995). Senthil-Nathan *et al*. (2004) conducted studies on the effect of neem extracts in *C. medinalis* and noticed considerable changes in acid phosphatases, alkaline phosphatases and adenosinetriphosphatases activities.

Younes *et al*. (2011) conducted studies to evaluate the effect of seven plant oils on some digestive enzymes in Khapra beetle, *Trogoderma granarium* and it suggested that the alkaline phosphatase (ALP) activity decreased with onion, garlic, rosemary and sunflower oil treatment and increased when peppermint, olive and camphor oils were used, whereas acid phosphatase (ACP) activity was completely lost. Zibaee and Bandani (2009) recorded severe decreased activity of α and β-glucosidases in *E. integriceps* when treated with *A. annua* extract. Similarly the effects of *Nigella sativa* and *Fagonia bruguieri* extracts on *S. gregaria* possess a great disruptive ALP activity in pest (Hamadah, 2009; Basiouny *et al*., 2010).

Al-Dali (2007) observed the influence of some neem limonoids on activities of the intestine acid and alkaline phosphatases in the grasshopper *Euprepocnemis plorans*. The leguminous plant compound canavanine treated *M. sexta* larva showed reduced lysozyme activity (Rosenthal and Dahlman,

1991; Rosenthal, 1998). Various solvent extractions of *A. visnaga* exhibited inhibitory and enhancing effects on ACP activity in different life stages of *S. gregaria* (Ghoneim *et al*., 2014).

Zibaee and Bandani (2010 a) conducted studies to evaluate the impact of *A. annua* extracts on the sun pest, *Eurygaster integriceps* and proved the suppression of ALP and ACP activities at different concentrations. The changes in LDH activity may lead to interfere the carbohydrate metabolism in fat body tissue and haemolymph of silkworm, *B. mori* when it was exposed to organophosphorus insecticides (Nath, 2000).

The active compounds present in *Aglaiaela eagnoidea* possess disturbances on nutritional physiology of both *H. armigera* and *S. litura* (Koul *et al*., 2005). A neem preparation called Neemazal can enhance the activity of ALP in haemolymph, but gradually inhibit the same in fat bodies of last instar nymphs of *S. gregaria* (Hamadah, 2009).

Senthil-Nathan *et al*. (2005) investigated the efficacy of azadirachtin and Nuclear Polyhedrosis virus individually and in combination against *S. litura* in reduction of gut enzyme activity such as acid phosphatase, alkaline phosphatase, ATPase and lactate dehydrogenase. Etebari *et al*. (2007) recorded the capability of pyriproxyfen residue to significantly reduce alkaline phophatase activity in silkworm larvae. The larvae of *S. litura* fed with azadirachtin treated *Ricinus communis* leaves possess a serious reduction in the amount of ALP and LDH in the midgut (Senthil-Nathan and Kalaivani, 2005).

Ayyangar and Rao (1990) observed the disruptive ACP activity in *S. littoralis* when treated with azadirachtin, which also caused inhibitory action on acid phosphatase (ACP) in *Musca domestica* (Saeed *et al*., 1987). Hassan (2002) also reported that certain botanical extracts disturbed the ACP

activity in *S. littoralis*. Similar effects were also reported by El-Sheikh (2002) in *A. ipsilon* subjected with the treatment of plant extracts and insect growth regulators. Senthil-Nathan *et al*. (2007) reported the capacity of dysoxylumtriterpenes to inhibit the activities of acid and alkaline phosphatases and ATPase in rice leaf folder Senthil-Nathan *et al*. (2006, d) recorded the effect of *M. azaderach* seed extract treatment on *Cnaphalocrocis medinalis* through rice leaves which resulted in reduction of ACP, ALP, ATPase and lactate dehydrogenase activities.

According to Curtis *et al.* (1972) Superoxide Dismutase (SOD) is one of the most important enzymes in the endogenous antioxidant defence system which scavenges the superoxide anion to form hydrogen peroxide and thus reducing the toxic effect through its radical. Kaur *et al.* (2014) conducted studies on the effect of plant extracts against the larvae of *Bactrocera cucurbitae* treated with plant extracts and showed the induced activity of SOD after the treatment for 48 hrs and 72 hrs; increased catalase activity after 48 hrs and 72 hrs of treatment and decreased or increased esterase activity at different time periods. Krishnan and Kodrik (2006) conducted studies on larvae of *S. littoralis* fed with potato leaves containing high content of allelochemicals and found increased activity of superoxide dismutase (SOD) in midgut tissue.

CHAPTER III
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Experimental insect: *Orthaga exvinacea* Hampson

Phylum	:	Arthropoda
Class	:	Insecta
Order	:	Lepidoptera
Super Family	:	Pyraloidea
Family	:	Pyralidae
Sub Family	:	Epipaschiinae
(Other Name	:	*Balanotis exvinacea*)

Orthaga exvinacea commonly called as the mango leaf-webber gained serious pest status in mango crop. The most gregarious larvae cause complete defoliation of leaves and thereby reduce the mango crop yield. (Plate III.1 Figure 1a)

Life Cycle

Life cycle of *O. exvinacea* consists of egg, larval stage of 6 instars, a prepupal stage, pupal stage and adult stage (Plate III.1 and Figure 2). The entire life cycle of this species may take 45 to 52 days. The duration of each developmental stage may vary according to changes in the availability of food, temperature and other climatic factors (Srivastava *et al.*, 1982; Rafeeq and Ranjini, 2011). At summer season there occurs six larval instars and in other seasons the larval period is prolonged with seven instars. According to Nair (1986) there are 5 to 7 larval instars in *O. exvinacea*.

Egg: Female moth lays between 60 to 400 eggs on mango leaves, which are creamy whitish in colour, oval, flattened, ordinarily smaller than 0.5 mm and not sticky. The colour of the eggs slowly changes to reddish and then to dark brown and hatch in 2 to 4 days. During summer when temperature rises above 32^0C, the eggs fail to hatch (Plate III.1 Figure 2b).

Larvae: The young caterpillars feed on leaf surface by scrapping. Later, they make web of tender shoots and leaves together and feed within. The larval stage lasts about 27 days, and the prepupal stage which is usually inactive, lasts for 3 days. The size of first instar larvae is about 2- 2.5 mm in lengthand 3-4 days of larval duration. The second instar larvae are larger than first instar, about 2.5 to 3 mm in length and duration of instar lasts for 4 days. In third instar, length of the larvae almost doubled and reached 4.5 to 6 mm and becomes dark yellow or greenish in colour and the instar lasts for 3-4 days. The fourth instar is more or less similar to third instar, gregarious in feeding and attained a length of 8 mm to 1 cm and it has about 4 days duration. The fifth instar larvae have about 2 cm length and 4-5 days of instar duration. It feeds voraciously on whole leaf lamina instead of scraping the leaf surface.The sixth instar is the active feeding larval stage and they feed all the green parts of the leaf and living only the midrib and this stage last about 5 days of instar duration and attains a length of 2.2 cm. The seventh instar are also voracious and approximately 2.5 to 3 cm in length and 3 mm in width and have maximum 5 days of instar duration (Plate III.1 Figure 2c).

Prepupal stage: During prepupal stage the feeding is stopped. They are bulky, sluggish in nature and pale brown in colour. The construction of cocoon is started in this stage and it pupates with its own silken webbing (Plate III.1 Figure 2c).

Pupae: The pupa is brown in colour and according to size of the pupae it is possible to predict its sex, large sized pupa indicate female and smaller one

male. Generally the pupal stage lasts for about 10 to 14 days and this duration also differs between male (10-13 days) and female (12-14 days) (Plate III.1 Figure 2d).

Adult: Adults moths are 8-10 mm in length with 24-30 mm wing span. The outer half of their forewing is dark grey or black in colour. While the hind wing is pale-grey or brownish in colour. Females are bigger in size with stout abdomen and males are smaller than females. Adult moths feed on nectar of flowers generally but in lab they are fed with 50% honey: 50% water, which are soaked in cotton wool. Adult moths have 2-3 days of pre-oviposition period and they can mate more than once. Adult male has 5 days of life span and female has 6 days life span (Plate III.1 Figure 2a).

3.1.2 Experimental plants

The fresh leaves of both experimental plants *Clerodendrum infortunatum* and *Chromolaena odorata* selected for the present study were collected from various regions of Kozhikode and Palakkad districts of Kerala mainly during the period from June to September. The plants were identified in the herbarium of Department of Botany, Malabar Christian College, Calicut.

3.1.2.1 *Clerodendrum infortunatum* L.

(Common name in English: Hill glory bower. Hindi: Titabhamt. Malayalam: Periyilam) *Clerodendrum infortunatum* is a gregarious shrub belonging to the family *Lamiaceae* (Plate III.2 a). This species is native to tropical regions of Asia including India, Bangladesh, Myanmar, Pakistan and Srilanka. It grows commonly in barren areas. The plant has an erected stem about 0.5–4 m high, without any branches and has simple and circular leaves, up to 10 inch long and 8 inch broad, arranged opposite, ovate to oblong or elliptic and hairy. Flowers occur in large, terminal erect panicles

with persistent calyx and are white with purplish pink in colour, and has black drupe and totally covered by the enlarged red calyx.

The major phytochemicals present in *Clerodendrum infortunatum* are tannins, alkaloids, terpenoids, glycosides, sterols, sugars, flavanoids and saponins. The compounds scutellarin and hispidulin-7 O-glucuronide are present in the leaf and novel crystalline compounds such as clerodolone, clerodone, clerodol and a sterol designated as clerosterol have been isolated from the root. The isolated pure compounds belonging to clerodane diterpenoids showed very high antifeedant activity against *Helicoverpa armigera* larvae (Kato *et al*., 1972). It was also reported that a bitter active principle clerodin ($C_{l3}H_{l8}O_3$) has been isolated from the petroleum ether extract of the air-dried leaf powder possess anthelmintic properties (Valsala, 2004). Koul (1982) showed 100% antifeedant activities of diterpenes and among most active compounds were those with a clerodane skeleton, such as dihydroclerodin, and clerodinhemiacetal from *Clerodendrum divaricate*.

The leaves and roots of *C. infortunatum* are also well-known natural health remedies in Ayurvedic practices and Siddha medicine. It has herbal remedy for fever and skin diseases, asthma, alopecia, cough, rheumatism, diarrhoea and also known to have hepato-protective and antimicrobial activity.

3.1.2.2 *Chromolaena odorata* (L.) King & Robins.

(Common name in English: Christmas Bush: Hindi: Tivra Gandha; Malayalam: Communist pacha.)

Chromolaena odorata belonging to *Asteraceae* family (Plate III.2 b) has long been referred to as *Eupatorium odoratum* and then its name changed to *Chromolaena odorata* by King and Robinson (1970). It is local to North America, and has been acquainted to South America, West Africa, tropical

Asia and parts of Australia. In India, distributed in southwestern and north western areas where the heavy rainfall was reported.

C. odorata is a very fast growing perennial herb with multi-stemmed bush and 2.5 m tall in open territories and have the maximum growth up to 10 m. The leaves are simple and very hairy, opposite-decussate, carried by a petiole (1 to 3 cm) and without stipules and it produces an aromatic odour when crumpled. Leaves are 4–10 cm long and 1–5 cm wide in size. The flowers are tubular and white to pale pink in colour and flowers are in panicles of 10 to 35 flowers that form at the ends of branches. Fruits are narrow and elongated achene with somewhat hairy.

The different solvent extracts of *Chromolaena odorata* leaves possessing major phytochemicals are steroids, terpenes (monoterpenes, diterpenes, sesquiterpines and triterpenes), alkaloids, flavonoids, tannins, glycosides and saponins. The observed toxicity of *Chromolaena odorata* was probably due to the relative presence of the different toxic phytochemicals (Prasad *et al*., 2005). Thirty-three components were identified from the volatile oil of *C. odorata* and from these, terpenoid compounds are major components of the volatile oil. The reported main terpenic components are trans-caryophyllene, d–cadinene, a-copaene, caryophyllene oxide, germacrene-D and a –humulene (Bing *et al*., 2003).

Large spectra of biological activities exhibited by the phytochemicals present in *C. odorata* are antihelmintic and antimalerial activity, analgesic activity, antipyretic and antispasmodic properties, antimicrobial activity, insecticidal activity, fungicidal activity, antigonorrhoeal activity and wound healing effect. The young leaves are crushed, and the subsequent fluid can be used to treat skin wounds. Hence it is also used as a traditional medicine in Indonesia, Thailand, Malaysia and parts of Africa including Nigeria.

3.1.3 Chemicals and Equipments

3.1.3.1 Chemicals: - Lab chemicals and equipments were purchased from local suppliers.

1. Acetone
2. Acrylamide
3. Alcohol
4. Amino acid standard kit
5. 4-Aminophenazone
6. Ammonium persulphate
7. Anisaldehydesulphuric acid
8. Anthrone
9. Araldite resin
10. B- mercapto ethanol
11. Benzene
12. Bisacrylamide
13. Bouin's fixative
14. Bovine serum albumin
15. Bromophenol Blue
16. Cadmium acetate
17. Calcium chloride
18. Casein
19. Chloroform
20. Citric acid
21. Coomassie brilliant blue
22. Copper sulphate
23. Diethyl ether

24. 3,5 Dinitrosalicylic acid
25. Disodium hydrogen phosphate
26. Disodium phenyl phosphate
27. DPX
28. Draggendorff's reagent
29. EDTA
30. Eosin
31. Ethanol
32. Ethyl acetate
33. Ferric chloride
34. Folin- Ciocalteau reagent
35. Formic acid
36. Glacial acetic acid
37. Glucose
38. Glutaraldehyde
39. Glycerol
40. Glycine
41. Haematoxylin
42. n- Hexane
43. Honey
44. Hydrochloric acid
45. L-Tyrosin
46. Maltose
47. Methanol
48. Methyl benzoate
49. Monosodium hydrogen phosphate

50. Ninhydrin
51. Osmium tetroxide
52. Phenol
53. Potassium chloride
54. Propylene oxide
55. Pyrogallol
56. Sodium bicarbonate
57. Sodium carbonate
58. Sodium chloride
59. Sodium duodecylsulphide
60. Sodium hydroxide
61. Sodium potassium tartarate
62. Sodium tungstate
63. Starch
64. Sulphuric acid
65. TEMED
66. Toluene
67. Toluidine blue
68. Trichloro acetic acid
69. Tris- Base
70. Tris- HCI
71. Uranyl- lead acetate strain
72. wax
73. Xylene

3.1.3.2. Equipments

1. Acquity H class (Waters) Ultra Performance LC system coupled with Xevo G2 (Waters) Quadrapole – Time-of-Flight.
2. Boiling tubes
3. CAMAG HPTLC system
4. Capillary tube
5. Centrifuge
6. Centrifuge tubes
7. Culture bottles
8. Deep freezer
9. Digital camera
10. Dissection set
11. Distillation Unit
12. Drier
13. Electrophoresis Unit (Genei)
14. Eppendorf tubes
15. Glass column (90×5)
16. Glass slides and cover glass
17. Hot plate
18. Incubator
19. Micropipettes
20. Microtome
21. Olympus light microscope
22. Oven
23. Petri dish
24. Pipettes

25. Plastic trough
26. Reagent bottles
27. Rearing cage
28. Slide warming table
29. Soxhlet apparatus
30. Test tubes
31. Tissue Homogenizer
32. TLC chamber
33. TLC plate
34. Transmission electron microscope
35. UV spectrophotometer
36. Visible Spectrophotometer
37. Vortex mixer
38. Watch glass
39. Water bath
40. Weighing balance

3.2 METHODS

3.2.1 Collection and culturing of *Orthaga exvinacea*

The pupae and larvae of *O. exvinacea* were collected from the infected mango field by hand picking method and maintained in laboratory conditions. The larvae were reared in plastic troughs covered with muslin clothes and kept inside rearingcages, the sides of which were closed with wire gauze (Plate III.1 Figure 1b). The culture of *O. exvinacea* was maintained in laboratory under optimum conditions of temperature at 28± 3º C and relative

humidity about 70-85%. Fresh mango leaves were given for feeding till the pupation of the larvae.

After pupation of larvae, the pupae were collected from the rearing cages and transferred into plastic trough and kept it in adult emerging cage. Adult moths emerged were sorted out for their sexes and kept in plastic jars in the ratio of 1:1 and closed the jars with cotton clothes. The sorted out adults were fed with 50% honey and within 2-4 days creamy white eggs were laid on the sides as well as on the covering clothes of the plastic jars. After one day, the creamy colour changed to brown and then became black which indicates ready for hatching within 4 days.When the eggs hatched, young larvae were fed with fresh tender mango leaves.The larvae in initial stages were kept in 1500 ml of capacity containers (18×12 cm) and later they were transferred to plastic troughs (40×13cm) covered with muslin cloths (Plate III.1 Figure 1b).

3.2.2 Preliminary phytochemical studies and selection of appropriate polar solvent for extraction.

The selection of solvent for the complete phytochemical extraction is the major step in phytochemical studies. Five different polar solvents (Chloroform, ethyl acetate, methanol, ethanol and water) were selected for the massive soxhlet extraction of the plant materials. To select the most appropriate solvent, HPTLC studies were carried out and confirmed the presence of maximum yield of compounds with that particular solvent. The preliminary toxicity evaluation was also performed with five different polar leaf extracts by testing their bioactivity on *O. exvinacea* to confirm the selection of solvent.

The preliminary toxicity screening was carried out by using five different solvent extracts and tested their toxicity against *O. exvinacea* larva and thereby fixing the most appropriate solvent for further extraction process.

The preliminary toxicity evaluation was carried out by topical application method. For this, 75 µl of each solvent extracts were applied topically on both dorsally and ventrally of each individual insects using micropipette. After application of extract, the insects were fed with fresh mango leaves and the experiment with each solvent extract was repeated thrice with each set of treatment consisting of 8 larvae. A control set was also maintained with each experiment set using the solvent alone for treatment. The observations on mortality in each set were noticed after 6 hrs, 12 hrs and 24 hrs of exposure and the treatment with maximum mortality within 24 hrs of exposure was considered as the most active solvent extract against this organism and this solvent was fixed as the most suitable solvent for further extraction process.

3.2.3 Preparation of leaf extracts

Fresh leaves of both plants *Clerodendrum infortunatum* and *Chromolaena odorata* were collected from the field, washed and shade dried at room temperature (28 ± 2ºC) for about one month. These dried leaves were ground into fine powder with an electric mixer grinder and sieved through a muslin cloth. This powder was used for preparing solvent extracts. 50 gm of leaf powder was extracted using 500 ml methanol in Soxhlet apparatus at 70-80ºC temperature.

3.2.3.1 Preparation of stock solution for histopathological and biochemical studies:-

The extract was allowed to evaporate in a pre-weighed petridish in an oven at 40-50ºC. After complete evaporation of solvent, the weight of dried extract was taken and it was dissolved in appropriate volume of methanol to prepare 10% stock solution and stored in air-tight glass containers in a refrigerator. From this stock, different desirable concentrations of botanicals (1%, 2%, 3%, 4% and 5%) were prepared by diluting with methanol.

3.2.3.2 Mode of application- Food treatment method

The application of botanicals on the larvae was carried by food treatment method. For this fresh mango leaves were uniformly smeared with equal volume (1.0 ml) of different concentrations of extract and kept open at room temperature for the complete evaporation of the solvent methanol.This treated leaves were provided to the sixth instar larvae for 48 hrs and control larvae were provided with mango leaves treated with same volume of methanol alone.

3.2.4 Separation and characterization of toxic principles present in botanicals

3.2.4.1 Column chromatography

To study the presence of active components in both leaf extracts, large quantities of different fractions had to be separated from the leaf extracts. For this, Column chromatography was quite apt technique for getting higher yield in eluted fractions. Large quantity of leaf extracts of both plants were prepared by using Soxhlet apparatus having 5 L capacity. 157 gm of leaf powder of *C. infortunatum* and 187.73 gm of leaf powder of *C. odorata* were used for preparing the extracts.

a. Packing of column and selection of mobile phase

Glass column (90×5) was packed with the activated adsorbent Silica gel (230-400 mesh at 120^0 C) made into slurry with toluene solvent. For a successive fractionation, the appropriate solvents should be selected as mobile phase in column. For this, a preliminary profiling was done using Thin layer chromatography. On the basis of TLC profile, different solvent mixtures were prepared and run through the column as mobile phase. The solvent mixture

toluene: ethyl acetate in the ratio of 7:3 was used as mobile phase for TLC profiling of *C. infortunatum* and in the ratio 9:1 for *C. odorata.*

b. Adsorbing the solvent extract and column running

The concentrated extracts of *C. infortunatum* and *C. odorata* were adsorbed on activated silica gel (230-400 mesh) and loaded in the top of gel packed column. For separating the fractions from *C. infortunatum,* the column was eluted with 300 ml each of different solvents such as petroleum benzene, toluene, chloroform, ethyl acetate, ethanol, methanol and distilled water. And for *C. odorata*, column was eluted with 300 ml each of solvents such as toluene (100%), different mixture of toluene : ethyl acetate (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7 and 2:8 ratio), ethyl acetate, acetone, propanol, ethanol, methanol, distilled water and 2% acetic acid.

c. Collection of eluted fractions

After separation, fractions were eluted out through the outlet of the column and these were collected in different boiling tubes having 30 ml of capacity. Total of 68 fractions were collected from *C. infortunatum* and 135 fractions were collected from *C. odorata.* The fractions with similar chemical profiles were pooled based on TLC analysis. After clubbing, the fractions were allowed to evaporate in a pre-weighed petridish in an oven at 50ºC. After complete evaporation of solvent, 10% stock solution was prepared from the weighed extract using appropriate solvent (methanol or acetone).

3.2.4.2 Toxicity evaluation by topical application method

a. Preliminary toxicity screening of clubbed fractions

The preliminary toxicity screening tests of clubbed fractions were carried out to select the most toxic fraction for determining LD_{50} value. For this, 75 µl of each clubbed fractions (10%) were applied topically on both

dorsally and ventrally of each individual insects using micropipette. After application of extract, the insects were fed with fresh mango leaves and the experiment was repeated thrice with each set of treatment consisting of 8 larvae. A control set was also maintained with each experiment set using the solvent alone for treatment. The observations on mortality in each set were noticed after 12 hrs, 24 hrs and 36 hrs of exposure and the maximum mortality exhibited within 36 hrs of fractions were selected for LD_{50} analysis.

b. Determination of LD_{50} value of most toxic fractions

Toxicity evaluation was done by the application of 75 µl of selected fraction of the extract on larvae by using micropipette. Different concentrations (6%, 7%, 8%, 9% and 10%) were prepared from the stock of active fractions by diluting with acetone and methanol. 1% soap solution was used as wetting agent. In the present study, required number of larvae (10 insects / set) was immobilized by chilling them for a few minutes. With the help of a micropipette, 75 µl of each of different concentrations were mixed with 10 µl of wetting agent and applied on both dorsal and ventral side of larvae. Same volume of dissolving medium (methanol and acetone) was applied on the control set of larvae. The larvae were observed after 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs intervals. Those that did not move or respond on gentle touch even after 1 h were considered as dead. All experiments were replicated 5 times. Percentage of mortality was calculated with the formula:

$$\text{Percentage of mortality} = \left(\frac{\text{No. of dead insect}}{\text{Total No. of treated insects}}\right) \times 100$$

The lethal dosage required for 50% mortality or LD_{50} values of both active fractions F2 and F5 were calculated by Probit analysis method (Finney, 1971).

3.2.4.3 Identification of most toxic compounds

3.2.4.3.1 Preliminary phytochemical screening of most toxic fractions by HPTLC

For the categorizing of active compounds present in most toxic fractions (F2 of *C. infortunatum* and F5 of *C. odorata*), fractions were analyzed by CAMAG HPTLC system. After the separation, HPTLC plates were subjected to qualitative tests for secondary metabolites. The presences of different types of compounds were identified by using various visualizing reagents and by calculating R_f values of different bands appeared.

a. **Test for phenols: -** To detect the presence of phenols, 10% alcoholic ferric chloride solution were sprayed on the HPTLC plates and read under visible light.

b. **Test for flavonoids: -** Sprayed 1% Aluminium chloride in ethanol on the HPTLC plates and viewed the plates under long wave UV (366 nm) lamp.

c. **Test for terpenes:-** Anisaldehyde- H_2SO_4 solution (ANS): concentrated H_2SO_4 (1.0 ml**)** is added to a solution of 0.5 ml anisaldehyde in 50 ml acetic acid and mixed thoroughly. After spraying this mixture on the chromatogram, the plates are heated at 100-105°C until the spot attained maximum colour intensity and read under visible light.

3.2.4.3.2 GCMS analysis

GC-MS analysis of most toxic fractions F2 separated from *C. infortunatum* and F5 from *C. odorata* were successfully carried out by using Agilent GC – 7890 A system with 5975C Mass Selective Detector under the following condition: the column sized -DB – 5MS – 30 m x

0.25mm x 0.25µm and Helium as carrier gas at 1.0/min flow rate and the column oven temperature was 40^0 C for 5 min. – 5 ^{0}C/min to 280 ^{0}C- hold for 10 min.

3.2.4.3.3 LC- Q- TOF and MS/MS Analysis

The most toxic fractions were also analyzed by Liquid Column-Quadrapole-Time of Flight (LC-Q-TOF). LC-Q-TOF analysis was carried out by using Acquity H class (Waters) Ultra Performance LC system coupled with Xevo G2 (Waters) Quadrapole – Time-of-Flight with the mobile phaseof mixture of water and formic acid (0.1%) . The column used for analysis is BEH C18 column (50 mm × 2.1 mm × 1.7 µm) and at 0.4 ml/ minute flow rate. To check the mass accuracy of the compounds, experimentally observed mass has been subtracted from theoretically calculated mass (m/z) from the identified compounds and this value should less than 2 m/z.

3.2.5 Histopathological studies

3.2.5.1 Light microscopic and morphometric studies of midgut tissue.

Newly moulted sixth instar larvae were used for the experiment. Fresh mango leaves were treated with five different concentrations (1%, 2%, 3%, 4% and 5%) of plant extracts and allowed to air dry for a few minutes. This treated leaves were fed to pre-starved experimental larvae for 48 hours. The control set was maintained with feeding larvae with methanol treated leaves. After 48 hours of feeding, the larvae were dissected out in insect ringer solution and the midgut tissue, cleared from adhering trachea was fixed in the alcoholic Bouin's fluid for 4 hrs. The tissue was then washed in 70 % alcohol and processed for making wax blocks. Following standard histological procedures serial section of 5 micron thickness were prepared and stained with Delafield's Haematoxylin and Eosin stains, mounted in DPX and examined under Olympus CX21i microscope.

The botanical effects on different cells were noted by changes occurred in their normal histological structure and the images were photographed. Microphotographs taken were analyzed for the morphometric studies. Height and width of columnar cells and nucleus were measured from five different regions of same section by using Olympus Magnus-pro software.

3.2.5.2 Ultrastructural studies by TEM

From the observations on light microscopic studies on the effect of different concentrations of botanicals (1%, 2%, 3%, 4%, 5%), the highly effective 4% of *C. odorata* and 5% of *C. infortunatum* were selected for the ultrastructural studies. The pre-starved experimental sixth instar larvae were fed with respective 4% and 5% botanical treated mango leaves and the control larvae were fed with methanol treated leaves. After feeding for 48 hours, the larvae were sacrificed to collect midgut tissue and fixed in 3% glutaraldehyde in 0.1M phosphate buffer fixative for 48 hours.These fixed tissues were washed with phosphate buffer and incubated for 90 min with 1% osmium tetroxide. Washed and post fixed tissues were dehydrated in the series of alcohol (70%, 90%, 95% and 100%) and two changes of propylene oxide, then the tissues were infiltrated over night in 1: 1 ratio of propylene oxide-resin mixture and embedded in araldite molds and kept at 60^0C for 48 hours. Semi and ultrathin sections were cut using ultra microtome (Leica EMU C6). Semithin sections (5-10 nm thickness) were stained with 1% Toluidine blue and observed under Olympus CX21i microscope and photographed. Ultrathin sections were stained with Uranyl-lead acetate stain and examined under Transmission Electron Microscope (Hitachi H500 TEM) and the images were taken.

3.2.6 Bioassay

3.2.6.1 Determination of total protein concentration

a. Midgut

From the laboratory reared culture, newly moulted sixth instar larvae were used for the experiment. Fresh mango leaves were treated with different concentrations (1%, 2%, 3%, 4%, and 5%) of the leaf extracts and allowed to air dry for complete evaporation of solvent. This treated leaves were fed to pre-starved experimental larvae for 48 hours. The control set contained the larvae fed with methanol treated leaves. The experiments were replicated five times for each concentration and each set contained 3 larvae. After 48 hours, larvae were sacrificed to collect the midgut tissue, water adhered to the tissue was blotted with filter paper, weighed and homogenized with 1 ml of insect ringer solution. Protein was precipitated from the tissue homogenate with 80% ethanol, centrifuged and the residue was dissolved in 1 ml of 0.1 N sodium hydroxide by boiling. The total protein concentration in each sample was estimated spectrophotometrically by the method of Lowry *et al.* (1951).

b. Fat body

For the quantitative estimation of protein in fat body tissue the larvae fed with the same five different concentrations of botanical treated mango leaves and a control set of larvae fed with methanol treated leaves were taken. Five replicates of samples were prepared for each concentration and a set of sample contained the fat body tissue from 3 larvae. After 48 hrs of feeding the larvae were sacrificed to collect fat body tissue, cleared from trachea and water adhered was removed using filter paper. Each sample set was weighed and homogenized with 1ml of ringer solution. 80 % ethanol is used for precipitating the protein from homogenized samples, centrifuged and the residue was dissolved in 1 ml of 0.1 N Sodium hydroxide by boiling. The

protein in the sample was estimated by method of Lowry *et al.* (1951) using visible spectrophotometer.

Procedure

For the determination of protein in midgut tissue and fat body, 1 ml of protein sample was thoroughly mixed with 5 ml of alkaline copper reagent and was allowed to incubate at room temperature for 10-15 minutes. Then added 0.5 ml of diluted folin-ciocaltaeu reagent (1:1) and kept for another 30 min of incubation at room temperature. The blue colour developed was read in a visible spectrophotometer at 650 nm against a reagent blank. A set of BSA standard was also carried out in a similar manner.

c. Haemolymph

Experimental sets and control set of sixth instar larvae were prepared in similar manner as in the midgut and fat body estimation. 0.2 ml of haemolymph sample was collected by amputing thoracic legs and using capillary tube or micropipette and it was quickly transferred into pre-chilled Eppendorf tubes (kept among ice cubes) containing 0.5 ml of 10 % sodium tungstate solution to prevent melanization. Five sets of samples were collected for each concentration and the total protein in the haemolymph samples were estimated using spectrophotometer by the method of Lowry *et al*. (1951).

Procedure

To the collected haemolymph, 0.5 ml of 0.66 N sulphuric acid was added and centrifuged for 5 minutes. The residue was dissolved in 1 ml of 0.1 N Sodium hydroxide. This protein sample was used for estimating the total protein content as described in section 3.2.6.1.b.

The percentage reduction in protein content in midgut tissue, fat body and haemolymph were estimated by using the formula:

$$Percentage\ reduction\ in\ protein\ content = \left(\frac{Control - Test}{Control}\right) \times 100$$

3.2.6.2 Protein profile

The protein profiling of midgut tissue, fat body and haemolymph was performed by Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis (SDS – PAGE) according to Laemmle (1970). The control and experimental sets of larvae were prepared similarly as in the case of protein estimation (section 3.2.6.1 a, b, c). Each set contained 10 larvae for both experimental and control. After 48 hrs of feeding, haemolymph samples were collected from the larvae and transferred into phosphate buffer containing tubes which were pre-chilled and stored in freezer (-20^{0}C). The larvae of each set were dissected out in insect ringer separately; the midgut tissue and fat body were collected and weighed. 100 mg of tissue was homogenized in 1 ml of phosphate buffer (pH 7), centrifuged and supernatant was taken for analysis. The protein samples were mixed with equal volumes of phosphate buffer. Samples were heated in micro-centrifuge tubes for 3 min at 95º C in a water bath. The denatured sample can be stored at -20º C for future use. The electrophoretic analysis for diverse protein samples was carried out using 5 % of stacking gel and 12 % of resolving gel. The resolved proteins were visualized by Coomassie brilliant blue (R-250) staining as per standardized protocol for Laemmle's method. Substantial changes in the SDS – PAGE protein profile was observed in control and treated ones and photographed. Molecular weight determination of protein was carried out and documented using gel documentation system.

3.2.6.3. Determination of total free amino acid concentration in different tissues

a. Midgut and fat body tissue

Treatment of the experimental and control larvae and preparation of tissue sample was similar to the method described in 3.2.6.1. After precipitation of protein and centrifugation, the supernatant was collected from the homogenized midgut tissue and fat body during total protein estimation as described in section 3.2.6.1 a, b. The total free amino acids in these samples were analyzed by Lee and Takahashi method (1966) using spectrophotometer.

b. Haemolymph

Haemolymph samples were collected in pre-chilled tubes and were kept in ice cubes to prevent melanization. After protein precipitation of the samples by using 80 % ethanol, the samples were again centrifuged and the supernatant was collected and total free amino acid concentration in the samples were detected using the method of Lee and Takahashi (1966) by spectrophotometry.

Procedure

The supernatant left after protein precipitation and centrifugation was used for estimating the free amino acid concentration. For the estimation, 0.5 ml of sample supernatant was thoroughly mixed with 0.5 ml of ninhydrin-cadmium acetate reagent and shaken well. To this 5 ml of distilled water was added and this mixture was boiled in a water bath for 20 min. Then cooled and allowed to stand for 15 min and the colour developed was measured at 540 nm in a spectrophotometer. Different concentrations of glycine solution were used as standards in similar manner.

The percentage reduction was calculated in midgut tissue, fat body and haemolymph by the equation

$$Percentage\ reduction\ of\ free\ amino\ acid\ content = \left(\frac{Control - Test}{Control}\right) \times 100$$

3.2.6.4 Estimation of carbohydrate content in different tissues

a. Midgut and fat body tissue

Treatment of botanicals on both the experimental and control larvae was similar to the method described in 3.2.6.1 a. After precipitation of protein and centrifugation, the supernatant was collected from the homogenized midgut tissue and fat body as described in section 3.2.6.1.a, b. These samples were poured to petriplates and kept in oven at 50^0c for evaporating the water content. After complete evaporation, remaining white coloured powder was dissolved in 1 ml of distilled water and centrifuged again. These samples were used for the estimation of carbohydrate content by Anthrone method (Plummer, 1971) using visible spectrophotometer.

b. Haemolymph

Haemolymph samples were collected from the larvae by amputing its thoracic legs and immediately transferred into pre-chilled tubes which were kept among ice cubes to prevent melanization. After protein precipitation of the sample by using 80 % ethanol, the supernatant was poured in to petriplate and was kept in oven for evaporating the water content. After complete evaporation, remaining white powder was dissolved in 1 ml of distilled water, centrifuged again and the estimation of carbohydrate content was carried by Anthrone method (Plummer 1971).

Procedure

To 0.5 ml of sample taken in a test tube, added 0.5 ml of distilled water and 4 ml of Anthrone reagent and mixed well. Then boiled the mixture for 10 minutes in water bath and allowed to cool in room temperature. The samples were measured at 620 nm in visible spectrophotometer. Different concentrations of glucose was used as standards.

The percentage reduction of carbohydrate content in midgut, fat body and haemolymph were calculated by

$$Percentage\ reduction\ of\ carbohydrate\ content = \left(\frac{Control - Test}{Control}\right) \times 100$$

3.2.7 Determination of enzyme activities in different tissues

Tissue preparation

The activity of digestive enzymes protease and amylase and antioxidant enzymes alkaline phosphatase (ALP), acid phosphatase (ACP) and superoxide dismutase (SOD) in midgut tissue, fat body and haemolymph were estimated for different time periods of 24 hrs, 48 hrs, and 72 hrs. For this, the sixth instar larvae were fed with botanical smeared mango leaves with 5% concentration of each leaf extract. After feeding for 24 hrs, 48 hrs, and 72 hrs, each set of treated larvae were dissected out and midgut tissue, fat body and haemolymph were collected as mentioned in section 3.2.6.1 a. b. c. A set of control larvae was maintained with each set of experiment.

3.2.7.1 Determination of total protease enzyme activity

The midgut and fat body tissue was homogenized at 4^0C by using ice cold distilled water and used for protease activity assay by the method of Furne *et al*. (2005).

Reagents used

1) Phosphate buffer- 0.1M, pH (7.6).

2) Trichloroacetic acid (w/v) -8%.

3) Substrate- Casein (w/v)- 1%, (7pH)

4) Standard solution of L-Tyrosine (w/v): - 0.03 %.

Procedure

250 µl of the substrate (1% casein) solution, 250 µl of 0.1M phosphate buffer (pH 7.6), 100 µl of crude enzyme extract (homogenate) were taken in a test tube. It was mixed well and incubated the tubes at 37°C for 1 hr. After 1 hr the reaction was stopped by adding 600 µl of 8% TCA solution and then kept in ice for 1 hr. After 1 hr the sample was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for the determination of enzyme activity and it was read at 280 nm. The soluble protein from the enzyme extract was determined by Lowry's method. A set of tyrosin standards were also carried out in similar manner along with the blank containing only the reagent.

The protease activities were expressed as IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.7.2 Determination amylase enzyme activity

The midgut and fat body tissue was homogenized at 4^0C by using ice cold distilled water and used for amylase activity assay. Amylase enzyme activity was assayed according to the Bernfeld (1955) method in which the increase in reducing power of buffered starch solution was measured.

Reagents used

1) Substrate- Starch solution (1%)

2) NaCl Solution (1%)

3) 0.1M phosphate buffer (pH 7).

4) Standard Maltose Solution: 0.1%

Procedure

1 ml of 1% starch solution (substrate), 1 ml of 0.1M phosphate buffer (pH 7.0), 1ml of 1% NaCl and 1 ml of enzyme extract solution were taken in a test tube. The test tube was incubated for 1 hr at 37°C. After 1 hr, the reaction was stopped by the addition of 0.5 ml of 3, 5 dinitrosalicylic acid. The absorbance was recorded at 540 nm. The concentration was detected from standard curve prepared by using maltose monohydrate. The soluble protein (mg/g) from the enzyme extract was determined by Lowry's method. The different concentrations of standards were prepared from maltose monohydrate and treated in similar manner along with the blank.

The amylase activities were expressed as IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.7.3 Determination of acid phosphatase (ACP) enzyme activity

Midgut and fat body tissue samples were prepared by homogenizing in citrate buffer and were used for acid phosphatase estimation by Kind and King Method (1954) in which 4-aminophenazone was used to determine the phenol liberated instead of the Folin-Ciocalteau reagent.

Reagents

1. Disodium phenyl phosphate – 218 mg/100 ml
2. Citric acid – sodium citrate buffer – pH- 4.9
3. Buffered substrate
4. Sodium hydroxide – 500 mmol/l
5. Sodium bicarbonate – 500 mmol/l
6. 4-Aminophenazone – 6 g/l
7. Potassium ferricyanide – 24 g/l
8. Stock phenol standard – 1 g crystalline phenol/l
9. Working standard – 10 µg phenol/ml

Procedure

A mixture of 1 ml of citric acid-sodium citrate buffer and 1 ml of disodium phenyl phosphate solution was used as buffered substrate and was incubated for few minutes in a water bath at 37ºC. For experiment samples 0.1 ml of enzyme sample was added to this and allowed the tubes for incubation for an hour. After incubation completed, 0.8 ml of sodium hydroxide and 1.2 ml sodium bicarbonate were added to both experiment and control samples and then added 0.1 ml enzyme sample to the control. To both of these, 1 ml of 4-amino phenazone and 1 ml of potassium ferricyanide were added, after thorough mixing, colour developed was read at 520 nm against a reagent blank. A set of phenol standards were also carried out in a similar manner.

ACP activities were expressed as IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.7.4. Determination of alkaline phosphatase (ALP) enzyme activity

The midgut and fat body tissues were homogenized with bicarbonate buffer and the activity of ALP was analyzed using the method of King and Armstrong (1934) in which disodium phenyl phosphate was used as the substrate. Disodium phenyl phosphate is hydrolyzed by alkaline phosphatase liberating phenol, which reacts under alkaline condition with Folin-phenol reagent to form blue colour, which was estimated colorimetrically at 680 nm.

Reagents

1. Bicarbonate buffer – 0.1 M, (pH 10)
2. Substrate – 0.01 M disodium phenyl phosphate salt solution
3. Folin - phenol reagent
4. Sodium carbonate – 10 %
5. Standard phenol solution – 5 µg/ml.

Procedure

An incubation mixture was prepared with bicarbonate buffer (150 µmoles) and substrate-disodium phosphate (10 µmoles) in distilled water (2.9 ml) and was preincubated for 10 min at 37ºC. Then enzyme sample (0.2 ml) was added to this and subjected for incubation at 37ºC for 15 min. The reaction was stopped by the addition of 1.0 ml of Folin-phenol reagent. The whole suspension was centrifuged and the supernatant was taken. To this 2 ml of 10 % sodium carbonate (2.0 ml) was added and the solution was incubated at 37ºC for 10 minutes. A set of phenol standard solutions were also treated with Folin-phenol reagent and sodium carbonate. The blue colour developed was read at 680 nm.

ALP enzyme activities were expressed as IU/mg in the case of midgut tissue and fat body whereas IU/ml for haemolymph samples.

3.2.7.5 Determination of superoxide dismutase (SOD) enzyme activity

The midgut and fat body tissue homogenate was prepared in Tris-HCI buffer (0.01 M, pH 7.5) containing 0.25 M sucrose and the enzyme was assayed according to the method of Marklund and Marklund (1974).

The degree of inhibition of auto-oxidation of pyrogallol, at an alkaline pH, by superoxide dismutase was used as a measure of the enzyme activity.

Reagents

1. Tris - HCl buffer, (0.1M; pH 8.2) containing 2 mM EDTA (w/v).
2. Tris - HCl buffer, (pH 7.5; 0.05 M).
3. Absolute ethanol
4. Chloroform
5. Pyrogallol working solution - 0.5 ml of the stock solution was diluted to 5 ml with 0.05 M Tris-HCl buffer to give a 2 mM working solution and use immediately.

Procedure

To 0.5 ml of each of midgut and fat body tissue homogenate and haemolymph, 0.25 ml cold absolute ethanol and 0.15 ml chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged for 15 min at 13,000 rpm and 0.5 ml of supernatant was used for the assay. The reaction mixture for auto - oxidation consisted of 2 ml Tris - HCl buffer (pH 8.2), 0.5 ml 2 mM pyrogallol and 2.0 ml water. Initially, the rate of auto - oxidation of pyrogallol was noted at an interval of 1 min for 3

min. This was considered as 100% auto-oxidation. The assay mixture for the enzyme contained 2 ml Tris - HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol and 0.5 ml aliquots of the enzyme and preparation was made to a final volume of 4.5 ml with distilled water. The blank was prepared simultaneously, which contained 2.0 ml of Tris-HCl buffer (pH 8.2) and 2.5 ml of distilled water. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The percentage inhibition in the auto-oxidation of pyrogallol in the presence of cell extract was converted to units of inhibition.

The amount of enzyme required for 50% inhibition of pyrogallol auto-oxidation is considered as 1 unit of enzyme activity. The enzyme activity was expressed as IU/mg protein for midgut and fat body whereas IU/ml for haemolymph samples.

3.2.8 Statistical analysis

Histomorphometric studies were statistically analyzed with ANOVA. Results of quantitative estimation of protein, amino acids, carbohydrate and enzyme activities were subjected to statistical analysis by using ANOVA and post hoc test (Scheffe test) with SPSS 16 package. Results with $P<0.05$ were considered to be statistically significant. In toxicity studies, mean percentage of mortality was calculated and also the toxic effects of leaf extracts were calculated by Probit analysis (Finney, 1971) by using the software SPSS.

CHAPTER IV

PHYTOCHEMICAL SEPARATION, TOXICITY EVALUATION AND COMPOUND IDENTIFICATION OF *CLERODENDRUM INFORTUNATUM* AND *CHROMOLAENA ODORATA*

4.1 Introduction

The plant possessing diverse group of insecticidal substances has created an extraordinary interest in phytochemical research in recent years to develop different potential sources for natural insect control agents. Phytochemistry deals with the study of natural products which are derived from plants. It includes large spectra of organic compounds that are elaborated and accumulated by plants and also deals with their chemical structures, biosynthesis, biological function and natural distribution. Major steps needed for the studies concerned include extraction, separation and identification of active compounds in plants.

Today, plant producing secondary metabolites and their substances has received considerable attention in the research to find new molecules having insecticidal activity, feeding deterrence, repellence, reproduction retardation and growth regulation against various insect species. (Rice and Coats, 1994; Isman, 2000). Generally, secondary metabolites are biologically active ingredients dispersed in different plant parts and classified as three major groups especially phenols, alkaloids and terpenes (Schoonhoven *et al*., 1998).

Plants producing phenolic compounds are one of the most abundant groups of substances having more than 8,000 recognized structures and broadly spread all over the plant kingdom (Harborne, 1989). They structurally consist of a hydroxyl group (-OH) bonded directly to an aromatic

hydrocarbon group and are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. Phenolic compounds also exhibit many biological activities like attraction of pollinators and seed dispersers and have antifeedant action on herbivores (Taiz and Zeiger, 2013).

Alkaloids are naturally occurring chemical compounds that contain basic nitrogen atoms. They are found in many plants, comprising the largest single class of secondary plant metabolites, with more than 5,500 identified compounds (Okwu, 2005). They are also reported as biologically very active constituent and contribute towards plant defenses against phytophagous animals and possess insecticidal activity with varying mode of actions (Aniszewski, 2007).

Terpenoids or isoprenoids are diverse group of naturally occurring organic compounds derived from five-carbon isoprene units arranged and expressed in thousands ways. Terpenoids are largest group of secondary metabolites, comprising more than 40,000 distinct molecules and are ecologically important for the defense functions in plants (Garcia and Carril, 2009). Terpenoids are also possessing insecticidal activities like causing larval mortality, significant growth inhibition, repellent and fumigant activity and these plant terpenoids along with phenols, flavonoids, steroids play a prominent role in plant defenses against phytophagous insects (Hartmann, 1996; Bruneton, 1999; Cox, 2004; Kubo, 2006).

Selection of appropriate phytochemical extraction technique is an important step to achieve the maximum extraction of components from the plant. The solubility of phytochemicals possess difference in different solvents (Cowan, 1999). So extraction is the initial and major step in the phytochemical processing for the recovery of bioactive constituents from plant materials (Karimi *et al*., 2011).The phytochemical extraction of active components from the plants depends upon the polarity of the solvents (Ghosh

et al., 2012). Generally, the polarity of solvents range from the most polar solvents like water, acetic acid, ethylene glycol etc. to the most non polar especially pentane, hexane, petroleum ether etc. and this wide range help to choose the most convenient solvent for the successive extraction purpose. Generally polar solvents will always extract polar molecules and non-polar solvents extract non-polar molecules. Many researchers reported the greater efficiency of methanol for maximum phytochemical extraction (Dhawan and Gupta, 2017; Prasad *et al.*, 2005).

Phytochemical studies are directly related to successful exploitation of different phytochemical techniques. Selection of most appropriate separation technique is a major step in phytochemical analysis. The maximum yield of separation is required for toxicity evaluation of phytochemicals present in botanicals. The application of column chromatographic technique is the most convenient method to get maximum yield of separated group of compounds. Gas chromatography-mass spectrometry (GC-MS) is an analytical method to identify the volatile compounds present in samples. Liquid chromatography-mass spectrometry (LC-MS and LC-MS/MS) is also an analytical method to identify, characterize and quantify the known and unknown compounds in samples.

The present investigation include the selection of suitable solvent for the complete extraction on the basis of their HPTLC profile and toxicity screening, the fractionation of extracts by using column chromatography, evaluation of toxicity of most active fractions separated from both botanicals by LD_{50} determination (topical application method) and identification of responsible group of compounds present in most toxic fractions by GCMS, LC-MS and LC-MS/MS systems.

4.2 Materials and methods

Methods used for preliminary phytochemical studies and selection of appropriate polar solvent for extraction were explained in section 3.2.2. Methods for fractionation by using column chromatography, toxicity evaluation of separated fractions and characterization of toxic principles present in most toxic fractions by HPTLC were discussed in sections 3.2.4.1, 3.2.4.2 and 3.2.4.3.1 respectively. The identification of compounds by using GC-MS, LC-Q-TOF and LC-MS/MS were described in sections 3.2.4.3.2 and 3.2.4.3.3.

4.3 Results

4.3.1 Selection of solvent

The selection of solvent for successful Soxhlet extraction was confirmed on the basis of HPTLC profile and results obtained from toxicity screening of five different polar solvent extracts of *C. infortunatum* and *C. odorata*. The maximum phytochemical extraction was confirmed by the maximum spots expressed in the profile of HPTLC of both leaf extracts (Table IV.1 and IV.2). The toxicity screening of different solvent extracts revealed the maximum toxicity of extracted phytochemicals and control (solvent alone) in different solvents (Table IV.3).

4.3.1.1 HPTLC analysis of different polar solvent extracts of *Clerodendrum infortunatum* and *Chromolaena odorata*.

The HPTLC analysis of different polar solvent extracts (chloroform, ethyl acetate, methanol, ethanol and water) of *C. infortunatum* revealed the maximum extraction efficiency of different solvents (Plate IV.1 and Table IV.1).

The HPTLC chromatogram of solvent extracts of *C. infortunatum* showed the varying number of spots corresponding to different polar solvents. The profile of chloroform extract expressed 11 spots under visible light (Plate IV.1). Similarly, 8 spots in ethyl acetate, 12 spots in methanol, 7 spots in ethanol and 4 spots in water extract were noticed (Table IV.1).

In the case of *C. odorata*, 12 spots were found in the profile of chloroform extract (Plate IV.2). The profile of ethyl acetate extract showed 10 spots, 14 spots for methanolic extract, 9 spots for ethanol and only 1 spot was observed in the profile of water extract (Table IV.2). In both leaf extracts, the maximum number of spots were noticed in methanolic extract followed by chloroform, ethyl acetate, ethanol and water. The HPTLC profiling of methanolic solvent extract revealed the extracting efficiency of methanol with maximum separated components.

4.3.1.2 Toxicity screening of different polar solvent extracts of *Clerodendrum infortunatum* and *Chromolaena odorata*.

For selecting the most appropriate solvent for soxhlet extraction, the toxicity of different polar solvent extracts of *C. infortunatum* and *C. odorata* were tested on the sixth instar larvae of *O. exvinacea* (by topical application) and observed the mortality at different intervals (6 hrs, 12 hrs and 24 hrs). The mean percentage mortality of both experimental and control were calculated and was given in Table IV.3.

The topical application of different solvent extracts of *C. infortunatum* on the larvae showed the efficacy of solvent extract to cause death of the larvae. The mean percentage mortality noticed after 24 hrs of exposure of different solvent extracts (Chloroform, ethyl acetate, methanol, ethanol and water) of *C. infortunatum* were 100%, 59.25%, 81.48%, 62.96% and 33.33% respectively whereas in the case of *C. odorata* the mortality

percentage were 100 %, 70.37 %, 92.59 %, 66.67 % and 40.74 % respectively for the corresponding solvent extracts. In both botanical exposures, chloroform possessed the maximum mortality followed by methanol, ethanol, ethyl acetate and water. It was also noticed that the percentage mortality of 51.85 %, 37.04 % and 25.92 % in the case of control due to exposure of chloroform, ethyl acetate and ethanol respectively. The control treated with methanol and water extracts did not exhibit any mortality during the different hours of treatment and these indicated that these solvents were least toxic to the insect.

The results of HPTLC analysis and toxicity screening revealed that methanol was the most suitable solvent for the phytochemical extraction by using soxhlet apparatus. The abundance of spots expressed in HPTLC profile of methanolic extracts proved its maximum separation efficiency and also the toxicity screening confirmed the least toxicity of the methanol. On the basis of these observations methanol was selected as best extraction solvent for the entire phytochemical study.

Table IV.1 Rf values of separated spots calculated from the HPTLC profile of different polar solvent extracts of *Clerodendrum infortunatum*.

Chloroform		Ethyl acetate		Methanol		Ethanol		Water	
No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values
11	0.05 0.08 0.15 0.23 0.34 0.43 0.57 0.71 0.78 0.90 096	8	0.03 0.14 0.23 0.42 0.55 0.67 0.77 0.91	12	0.04 0.08 0.15 0.21 0.26 0.34 0.43 0.57 0.72 0.78 0.87 0.98	7	0.03 0.14 0.23 0.57 0.68 0.85 0.91	4	0.02 0.07 0.12 0.77

Table IV.2 Rf values of separated spots calculated from the HPTLC profile of different polar solvent extracts of *Chromolaena odorata*.

Chloroform		Ethyl acetate		Methanol		Ethanol		Water	
No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values	No. of spots	Rf values
12	0.02 0.09 0.19 0.22 0.28 0.37 0.43 0.46 0.59 0.79 0.88 0.99	10	0.01 0.07 0.10 0.17 0.25 0.34 0.43 0.54 0.74 0.82	14	0.02 0.09 0.16 0.19 0.21 0.27 0.35 0.40 0.44 0.55 0.64 0.75 0.83 0.95	9	0.05 0.09 0.15 0.22 0.32 0.41 0.52 0.72 0.80	1	0.01

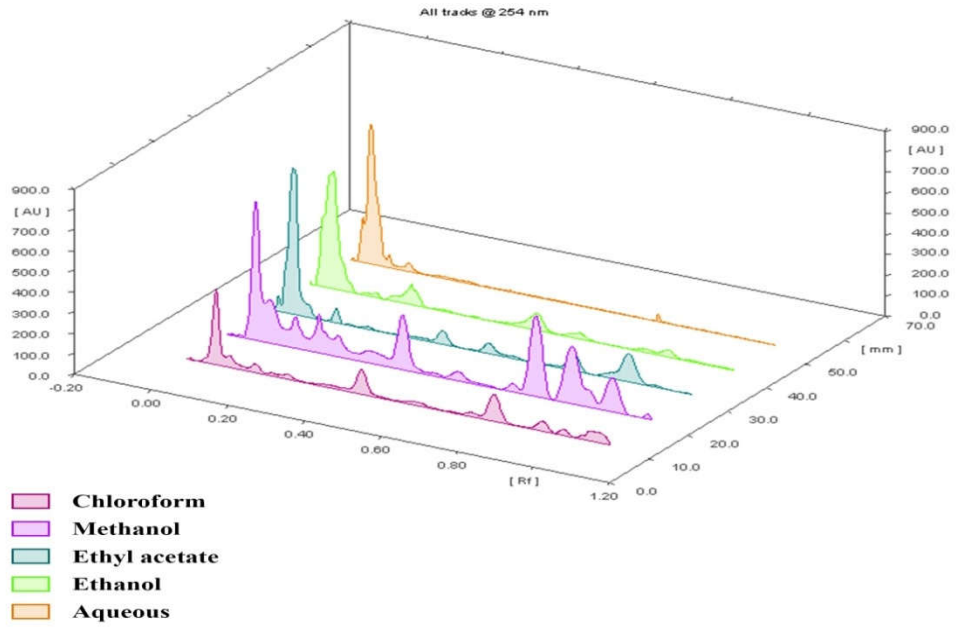


Figure IV.1 Densitometric chromatogram of different solvent extracts of *Clerodendrum infortunatum*.

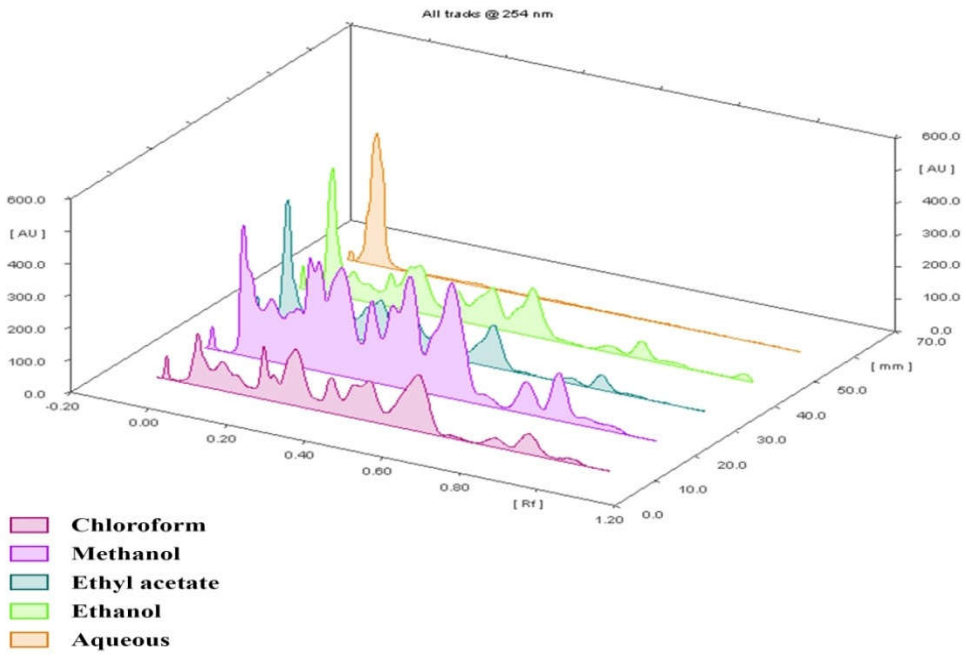


Figure IV.2 Densitometric chromatogram of different solvent extracts of *Chromolaena odorata*.

Table IV.3. Showing the mean percentage mortality of *Orthaga exvinacea* larvae exposed to different solvent extracts.

Different solvent extracts	Treatment groups	Mean percentage mortality (%)		
		6 hrs	12 hrs	24 hrs
Chloroform	*C. infortunatum*	44.44	85.19	100
	C. odorata	48.15	96.30	100
	Control	14.81	37.04	51.85
Ethyl acetate	*C. infortunatum*	22.22	33.33	59.25
	C. odorata	29.68	48.15	70.37
	Control	11.11	25.93	37.04
Methanol	*C. infortunatum*	51.85	66.67	81.48
	C. odorata	55.56	88.90	92.59
	Control	0	0	0
Ethanol	*C. infortunatum*	29.63	40.74	62.96
	C. odorata	33.33	51.85	66.67
	Control	3.70	18.52	25.92
Water	*C. infortunatum*	7.41	22.22	33.33
	C. odorata	14.81	37.04	40.74
	Control	0	0	0

Values presented are mean percentage mortalities of 3 replicates

4.3.2 Fractionation of methanolic extracts and toxicity evaluation of active fractions

Fractionation of methanolic extracts of both *C. infortunatum* and *C. odorata* were carried out by column chromatographic method. Total 68 eluted fractions of *C. infortunatum* and 135 eluted fractions of *C. odorata* were collected and on the basis of their similar TLC profile it was clubbed into 3 and 12 major fractions for *C. infortunatum* and *C. odorata* respectively and the details were given in Table IV.4 and IV.5. A preliminary toxicity screening test was carried out to select the most active fractions from both botanicals and further toxicity evaluation of these selected active fractions were carried out by LD_{50} calculation by Probit analysis (Finney, 1971) and details were presented in Tables IV.6, IV.7, IV.8 and IV.9.

4.3.2.1 Column chromatography

- **Preliminary profiling of leaf extracts by TLC**

TLC analysis of methanolic leaf extracts of both *C. infortunatum* and *C. odorata* were carried out and this profile helped the selection of appropriate solvent mixture as mobile phase in column. The solvent mixture used to get good separation of bands in TLC indicated the polarity range of solvent which is to be used as mobile phase in column. The different ratios of toluene: ethyl acetate mixtures (7:3 for *C. infortunatum* and 9:1 for *C. odorata*) were used in TLC of both botanical profiling (Plate IV.3 and Plate IV.4).

- **Clubbing of eluted fractions**

As result of successful phytochemical fractionation, a large number of eluted fractions were collected from the column and the number of fractions were reduced by clubbing the fractions on the basis of their similar TLC profile. Total 68 eluted fractions were collected from the leaf extract of *C. infortunatum* and those fractions with similar TLC profiles were pooled into 3 major fractions (Table IV.4). Solvent mixtures used in column to separate these major fractions were ethanol and methanol for fraction F1, methanol alone for F2 and mixture of methanol and water for F3 (Table IV.4). In the case of *C. odorata*, a total of 135 fractions were collected from the extracts and according to the similar TLC profiles they were clubbed into 12 major fractions (Table IV.5). Different ratios of toluene: ethyl acetate solvent mixtures were used to separate fractions F1 to F8, toluene: ethyl acetate and ethyl acetate alone were used to separate fraction F9, ethyl acetate and acetone for F10, acetone alone for F11 and acetone and propanol for F12 (Table IV.5).

Table IV.4. The details of clubbed or pooled fractions separated from *Clerodendrum infortunatum* extract.

Fraction number	Clubbed series of sub fractions	Mobile phase used in column	Mobile phase used in TLC
F1	40 D to 54 E	Ethanol ; Methanol	Toluene : Ethyl acetate (8:2)
F2	55 E to 60 F	Methanol	Toluene : Ethyl acetate (8:2)
F3	61 G to 68 G	Methanol ; water	Toluene : Ethyl acetate (8:2)

Table IV.5. The details of clubbed or pooled fractions separated from *Chromolaena odorata* extract.

Fraction number	Clubbed series of sub fractions	Mobile phase used in column (Solvent mixture)	Mobile phase used in TLC
F1	17 B to 22 C	Toluene : Ethyl acetate (8:2 and 7:3)	Toluene : Ethyl acetate (8:2)
F2	33 D to 36 D	Toluene : Ethyl acetate (6:4)	Chloroform : Methanol (8:2)
F3	37 D to 42 E	Toluene : Ethyl acetate (6:4 and 5:5)	Chloroform : Methanol (8:2)
F4	43 E to $51_{(a)}$ F	Toluene : Ethyl acetate (5:5 and 4:6)	Chloroform : Methanol (8:2)
F5	$51_{(b)}$ F to 55 F	Toluene : Ethyl acetate (4:6)	Toluene : Ethyl acetate (9:1:2)
F6	56 F to 62 G	Toluene : Ethyl acetate (4:6 and 3:7)	Toluene : Ethyl acetate (9:1)
F7	63 G to 69 G	Toluene : Ethyl acetate (3:7)	Toluene : Ethyl acetate (9:1)
F8	70 H to 74 H	Toluene : Ethyl acetate (2:8)	Toluene : Ethyl acetate: methanol (8:2:1)
F9	75 H to 84 I	Toluene : Ethyl acetate (2:8) and Ethyl acetate (alone)	Toluene : Ethyl acetate: methanol (8:2:1)
F10	85 I to 93 J	Ethyl acetate and acetone	Toluene : Ethyl acetate: methanol (7:3:1)
F11	94 J to 98 J	Acetone	Toluene : Ethyl acetate: methanol (7:3:1)
F12	99 J to 103 k	Acetone and propanol	Toluene : Ethyl acetate: methanol (7:3:1)

4.3.2.2 Preliminary toxicity screening test for identifying the most toxic fractions

Preliminary toxicity screening tests of all clubbed fractions were carried out to select the most toxic fraction to evaluate the toxicity by LD_{50} analysis. Table IV.6 showed the mean percentage mortality at different time (12 hrs, 24 hrs and 36 hrs) of exposure to *C. infortunatum* and *C. odorata* by topical application method. All major fractions separated from *C. infortunatum* (F1, F2 and F3) possessed 50% above mortality and in the case of F2, 98.15% mortality was noticed (Table IV.6). Whereas for *C. odorata*, majority of fractions exhibited above 50% mortality except the cases of F1, F2, F4 and F11. Out of 12 major fractions screened, fraction F5 possessed 100% mortality at 36 hrs of exposure (Table IV.6**)**.

From the results of preliminary toxicity screening of major fractions separated from both leaf extracts, it was revealed that fraction F2 of *C. infortunatum* and F5 of *C. odorata* showed the maximum mean percentage mortality and these fractions were selected for further toxicity analysis by determining LD_{50}.

Table IV.6. Mean percentage mortality of major fractions separated from *Clerodendrum infortunatum* and *Chromolaena odorata* on *Orthaga exvinacea* larvae.

Botanical	Fractions	Mean Percentage of mortality (%)		
		12 h	24 h	36 h
C. infortunatum	F1	24.07	59.26	61.11
	F2	37.04	85.19	98.15
	F3	25.93	61.11	77.78
C. odorata	F1	0	12.96	33.33
	F2	9.26	22.22	42.59
	F3	1.85	25.93	57.41
	F4	12.96	31.48	42.59
	F5	40.74	88.89	100
	F6	20.38	31.48	50.00
	F7	14.81	40.74	62.96
	F8	12.96	27.78	53.70
	F9	25.93	61.11	87.04
	F10	18.52	35.19	55.56
	F11	14.81	33.33	42.59
	F12	35.18	53.70	74.07
	Control	00	00	00

Values presented are mean values of 3 replicates

4.3.2.3 Toxicity analysis of most toxic fraction by LD_{50} calculation

On the basis of results obtained from preliminary toxicity screening analysis, the maximum mortality caused fractions F2 of *C. infortunatum* and F5 of *C. odorata* were selected for LD_{50} analysis. The mean percentage mortality of fractions F2 and F5 were calculated up to 72 hrs and showed in Table IV.7 and Table IV.8 and the LD_{50} values by probit analysis were also presented in Table IV.9.

The five different concentrations (60 µg, 70 µg, 80 µg, 90 µg and 100 µg) of fraction F2 of *C. infortunatum* were topically applied on the sixth instar larvae of *O. exvinacea* and observed the mortality up to 72 hrs. The mean percentage mortality at 72 hrs noticed were 40%, 52%, 77.5%, 95% and 97% corresponding to different concentrations applied (Table IV.7). In the case of

C. odorata, application of different concentrations (60 µg, 70 µg, 80 µg, 90 µg and 100 µg) of fraction F5 exhibited 46 %, 60 %, 72.5 %, 96.5 % and 100 % mortality at 72 hrs respectively (Table IV.8).

Table IV.7 The mean percentage mortality of *Orthaga exvinavea* larvae exposed to the fratction F2 of *Clerodendrum infortunatum*.

Concentration (µg)	Mean percentage mortality (%)					
	12 hrs	24 hrs	36 hrs	48 hrs	60 hrs	72 hrs
60	5	13	20.5	26	30	40
70	12.5	23	32.5	42	44	52
80	19	40.5	56	64	71	77.5
90	39	56	74.5	80	88	95
100	51	67	81	89.5	94	97
Control	0	0	0	0	0	0

Values presented are mean percentage mortalities of 5 replicates

Table IV.8 The mean percentage mortality of *Orthaga exvinavea* larvae exposed to the fraction F5 of *Chromolaena odorata*

Concentration (µg)	Mean percentage mortality (%)					
	12 hrs	24 hrs	36 hrs	48 hrs	60 hrs	72 hrs
60	5	8.5	15.37	23.5	30.37	46
70	15.5	26.5	34.5	40	46.5	60
80	20.5	31	39	54	62.5	72.5
90	42	63	77.5	87.5	92.5	96.5
100	63	84.5	92.5	99.5	100	100
Control	0	0	0	0	0	0

Values presented are mean percentage mortalities of 5 replicates

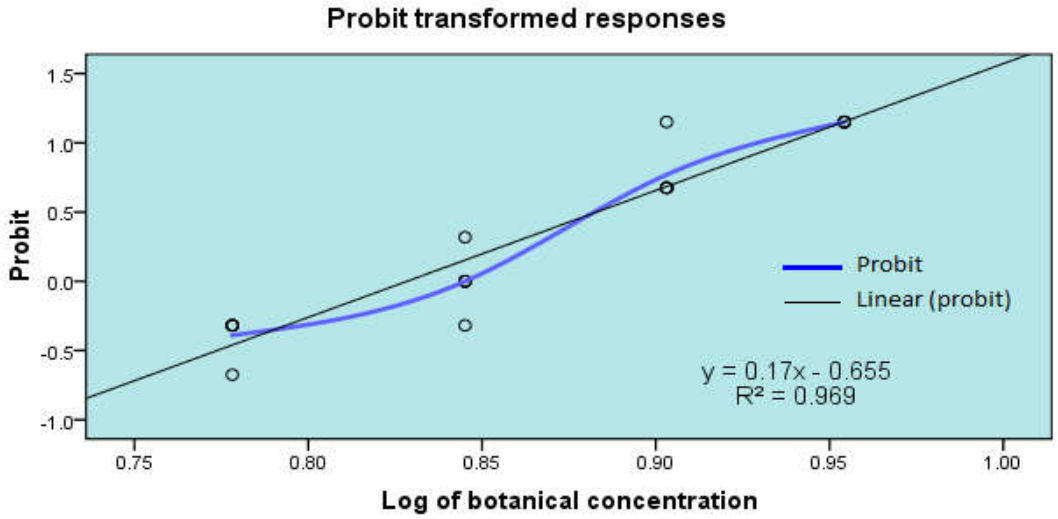
The probit analysis of concentrations applied on the larvae showed the LD_{50} values of both fractions. The LD_{50} value of fraction F2 from *C. infortunatum* was 67.40 µg (6.740 %) and for *C. odorata*, fraction F5 showed the value of 65.74 µg (6.574 %) (Table IV.9).

`The graphic representation of toxicity exhibited by the fraction F2 from *C. infortunatum* displayed the regression equation which correspond to

the probit mortality Y= 0.17 and log concentration of X = x-0.655 (Figure IV.3). In case of F5 from *C. odorata*, the graph expressed the regression equation which correlated with probit mortality Y= 0.157 and log concentration x= x – 0.535 (Figure IV.4).

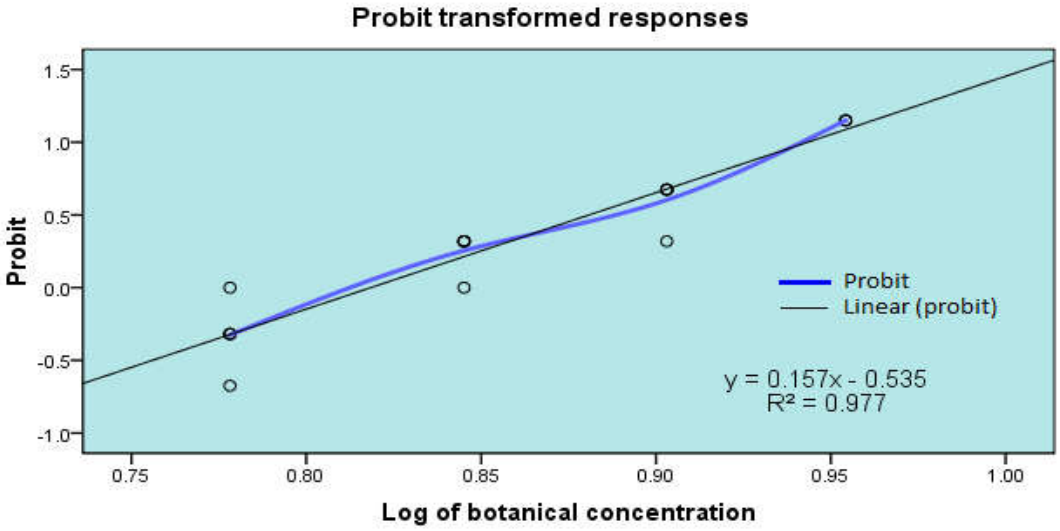
Table IV.9 Table showing the toxicity of major fractions F2 and F5 separated from the leaf extracts of both *Clerodendrum infortunatum* and *Chromolaena odorata*.

Fractions	LD_{50} Value (%)	95% Confidence limits		Chi square value
		Lower limit	Upper limit	
F2 (*C. infortunatum*)	6.740	6.344	7.056	6.323
F5 (*C. odorata*)	6.574	6.056	6.952	7.996



LD_{50} value = **6.74**

Figure IV.3. Probit analysis graph showing LD_{50} value of fraction F2 of *Clerodendrum infortunatum*.



LD_{50} value = **6.57**

Figure IV.4. Probit analysis graph showing LD_{50} value of fraction F5 of *Chromolaena odorata*.

4.3.3 Identification of compounds in most toxic fractions

4.3.3.1 HPTLC analysis

The HPTLC analysis was carried out to detect the presence of toxicity causing categories of secondary metabolites (Phenols, Flavanoids and Terpenes) and this analysis further helped to identify the compounds by GCMS and LCMS analysis. By spraying different visualizing reagents on HPTLC plates, presence of phenols, flavonoids and terpenes were detected (Plate IV.5). On the basis of the intensity of bands appeared, their R_f values were calculated.

a. Phenols:-The HPTLC profile after spraying visualizing reagent showed green and dark brown spots which indicated the presence of phenolic compounds. In *C. infortunatum* (F2) mainly two spots were noticed with R_f values of 0.41 and 0.46 respectively and in the case of *C. odorata* (F5) five

spots with different R_f values of 0.46, 0.54, 0.59, 0.64 and 0.75 were observed (Plate IV.5 a, arrows).

b. Flavonoids:-When the visualizing reagent aluminium chloride was sprayed on the HPTLC plates and read under long wave UV (366nm) lamp, 3 fluorescent spots were noticed in the most toxic fraction (F5) of *C. odorata* with R_f values of 0.45, 0.51 and 0.56. But in *C. infortunatum* (F2) there were no such clear fluorescent spots to suggest strongly the presence of flavonoids (Plate IV.5 b, arrows).

c. Terpenes: -Spraying with ANS visualizing reagent, resulted in the appearance of different colour spots at different R_f values. Appearance of light brownish and pinkish violet spots indicated the presence of terpenes. Terpenes were present in both F2 fraction of *C. infortunatum* and F5 fraction of *C. odorata* respectively. Five different spots with R_f values of 0.1, 0.13, 0.16, 0.20 and 0.24 were noticed in F2 and in F5, different spots with Rf values of 0.1, 0.12, 0.14, 0.20, 0.24, 0.29, 0.35, 0.39, 0.51 and 0.60 were observed (Plate IV.5 c, arrows).

4.3.3.2 Compound identification by GCMS

To identify the volatile compounds present in the most toxic fractions F2 of *C. infortunatum* and F5 of *C. odorata*, these fractions were analyzed by GCMS and the details regarding retention time, compound name, molecular formula and molecular mass were presented in Table IV.10 and Table IV.11. Total 8 constituents were identified in fraction F2 of *C. infortunatum* and 13 constituents were identified in fraction F5 of *C. odorata*.

Different peaks of GCMS-chromatogram of both fractions indicated the mass abundance in different retention time (Figure IV.5 and Figure IV.6). Out of 8 compounds identified in fraction F2, 6 compounds were already

reported as biologically active (Table IV.10, highlighted ones). Similarly, activities of 7 compounds were already reported out of 13 compounds identified in fraction F5 (Table IV.11, active compounds highlighted).

Biologically active volatile compounds identified in fraction F2 from *C. infortunatum* are Phenol, 2,4-bis (1,1-dimethylethyl); n-Hexadecanoic acid; 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester; squalene; psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy and digitoxin. In the case of *C. odorata*, active compounds in F5 are Phenol, 2,4-bis(1,1-dimethylethyl)-; Cholestan-3-one, cyclic 1,2-ethanediyl aetal (5β); Hexadecanoic acid, methyl ester; 1-Monolinoleoylglycerol trimethylsilyl ether; 10,13-Eicosadienoic acid,methyl ester; 1,2-Benzenedicarboxylic acid, diisooctyl ester; Squalene.

Table IV.10. Details of compounds identified in F2 fraction of *Clerodendrum infortunatum* by GCMS analysis.

Sl No	RT value	Name of compound	Molecular formula	Molecular mass (g/mol)
1	27.553	Phenol, 2,4-bis (1,1-dimethylethyl)	$C_{17}H_{30}OSi$	278.511
2	34.368	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43
3	35.314	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	$C_{16}H_{22}O_4$	278.3435
4	39.882	Zeaxanthin	$C_{40}H_{56}O_2$	568.88
5	50.892	Squalene	$C_{30}H_{50}$	410.718
6	53.304	psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	$C_{42}H_{64}O_2$	600.972
7	54.928	Digitoxin	$C_{41}H_{64}O_{13}$	764.95
8	55.668	Fucoxanthin	$C_{42}H_{58}O_6$	658.92

Table IV.11. Details of compounds identified in F5 fraction of *Chromolaena odorata* by GCMS analysis.

Sl No	RT value	Name of compound	Molecular formula	Molecular weight
1	27.330	Octanoic acid, 6,6-dimethoxy-, methyl ester	$C_{11}H_{22}O_4$	218.2900
2	27.572	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{17}H_{30}OSi$	278.511
3	33.589	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5β)-	$C_{29}H_{50}O_2$	430.706
4	34.624	Cholest-5-en-3-one	$C_{27}H_{44}O$	384.638
5	36.612	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.457
6	40.090	1-Monolinoleoylglycerol trimethylsilyl ether	C27H54O4Si2	498.89
7	43.280	10,13-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322.525
8	43.490	Methyl 15-hydroxy-9,12-octadecadienoate	$C_{19}H_{34}O_3$	310.471
9	44.023	Ethyl 6,9,12,15-octadecatetraenoate	$C_{20}H_{32}O_2$	304.467
10	46.868	9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	$C_{27}H_{52}O_4Si_2$	496.870
11	47.086	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390.556
12	51.159	Squalene	$C_{30}H_{50}$	410.718
13	53.321	(1,6-Dimethyl-3,4-dihydro-1H-pyrrolo[1,2-a]pyrazin-2-yl)(3,4,5-trimethoxyphenyl)methanone	$C_{19}H_{24}N_2O_4$	344.405

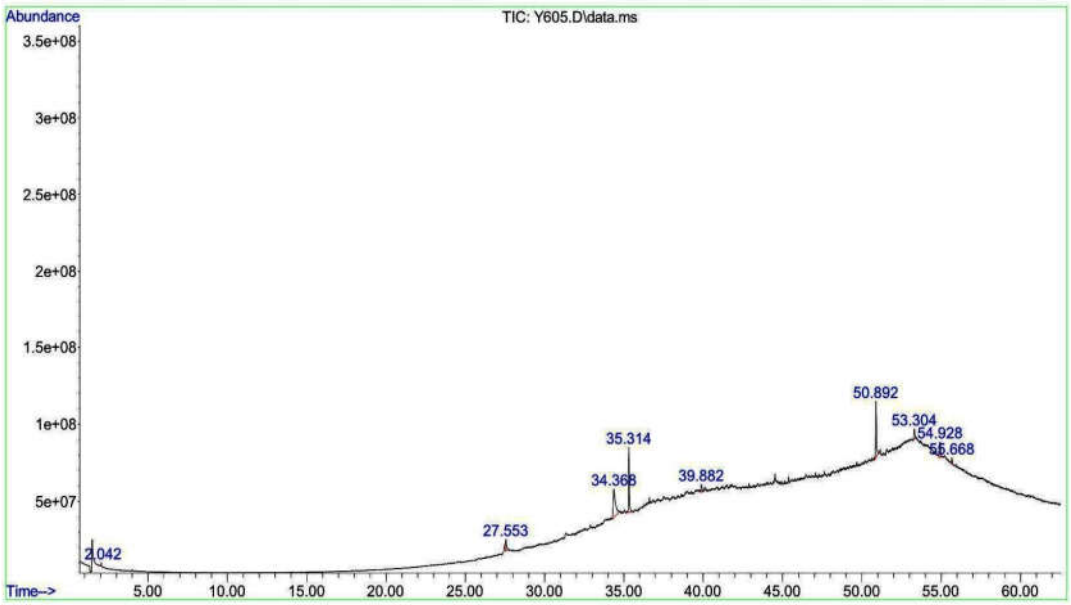


Figure.IV.5. GCMS-Chromatogram of F2 fraction separated from *Clerodendrum infortunatum* extract.

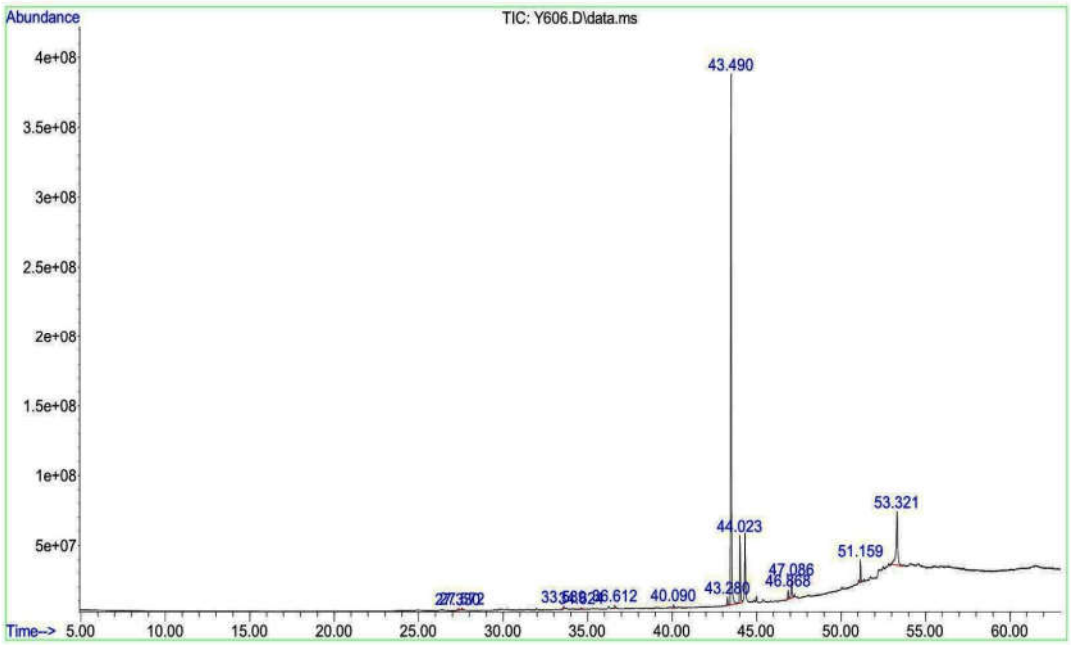


Figure IV.6 GCMS-Chromatogram of F5 fraction separated from *Chromolaena odorata* extract

4.3.3.3 Compound identification by using LC-Q-TOF and LC-MS/MS

The GCMS analysis helped only to identify the volatile constituents in fractions. So for further identification of compounds, LC-Q-ToF and LC-MS/MS analysis of both F2 and F5 fractions were carried out. Total Ion current Chromatogram (TIC) of fraction F2 and F5 indicated the mass abundance in different retention time (Figure IV.7 and IV.8). On the basis of TIC, different mass spectrums at different retention time were selected for LC-MS/MS analysis. The possible elemental compositions of masses obtained at different retention time in both ionization modes (positive and negative) were analyzed and from this wide range of elemental compositions, most repeated ions were randomly selected for LC-MS/MS analysis.

Total 8 compounds were further identified from the fraction F5 of *C. odorata* in negative ionization by LC-MS/MS. But in the case of fraction

F2 from *C. infortunatum*, there was no occurrence of compound in selected ions from the elemental compositions obtained. The details of identified compounds regarding their name, molecular formula, calculated mass and error (m/z) were expressed in the Table IV.12. The mass error indicated the mass accuracy of compounds and this value should occur less than 2 (< 2 m/z). The MS/MS fragmentation patterns and the structure of compounds were also displayed in Figure IV.9 to IV.16. Most of the compounds identified from fraction F5 were reported as biologically active and the details were given in discussion part.

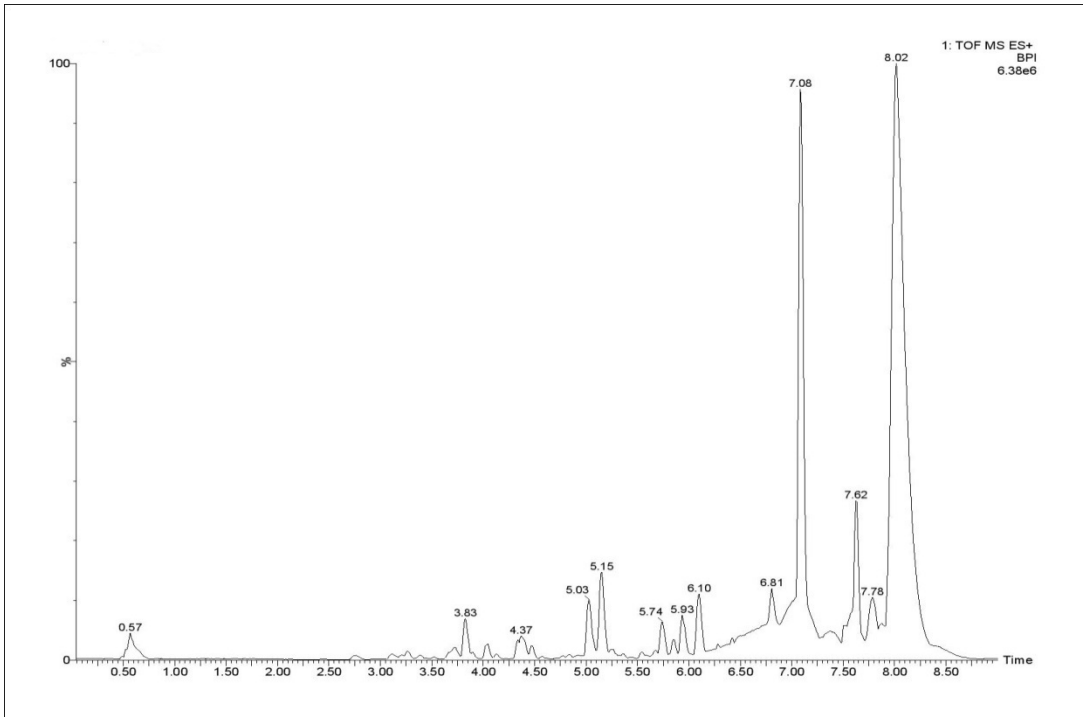


Figure IV.7 Showing Total Ion Chromatogram (TIC) of most toxic fraction F2 of *Clerodendrum infortunatum* extract.

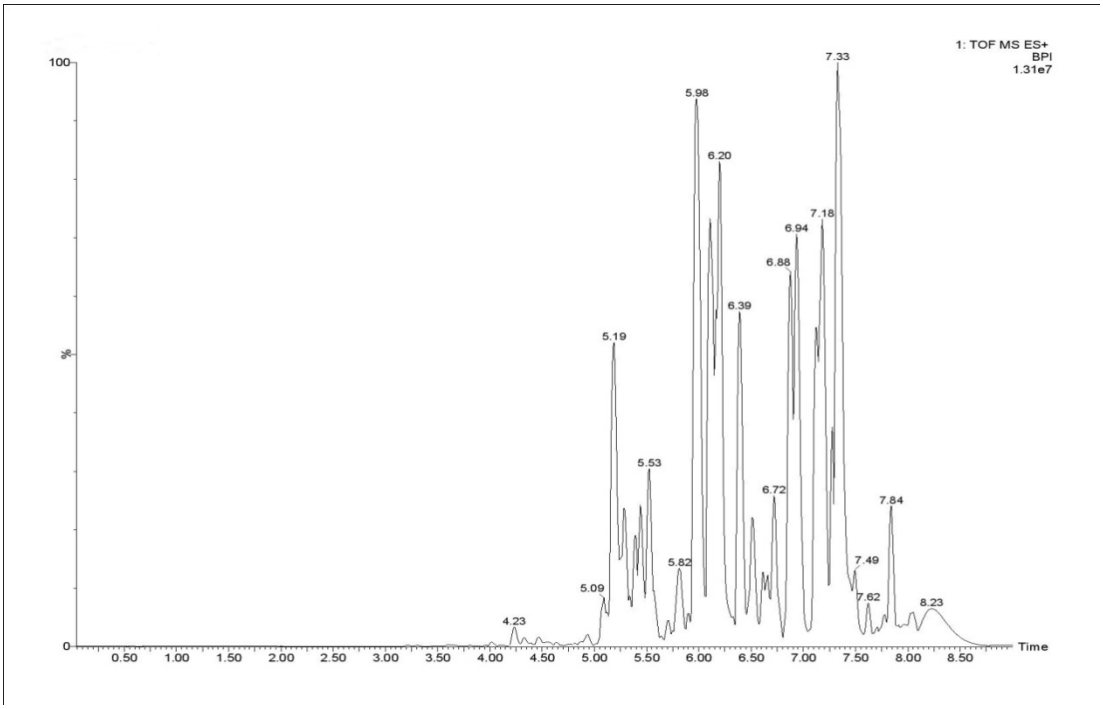


Figure IV.8 Showing Total Ion Chromatogram (TIC) of most toxic fraction F5 of *Chromolaena odorata*.

Table IV.12. Table showing the details of compounds identified from fraction F5 by LC-MS/MS.

Fractions	Compound name	Molecular formula	Calculated mass (m/z)	Error (m/z)
F5 (*Chromolaena odorata*)	**2,3-Bis(3,4-dihydroxyphenyl) succinic acid**	$C_{16}H_{13}O_8$	334.441	-1.379
	Hesperetin	$C_{16}H_{13}O_6$	302.546	-1.474
	Acacetin	$C_{16}H_{11}O_5$	284.641	-1.579
	Jaceosidin	$C_{17}H_{13}O_7$	330.557	-1.490
	Asterric acid	$C_{17}H_{15}O_8$	348.490	-1.412
	Centaureidin	$C_{18}H_{17}O_9$	378.489	-1.401
	Lecanoric acid	$C_{16}H_{13}O_7$	317	0.066
	trans-caffeic acid	$C_9H_7O_4$	180.430	-1.395

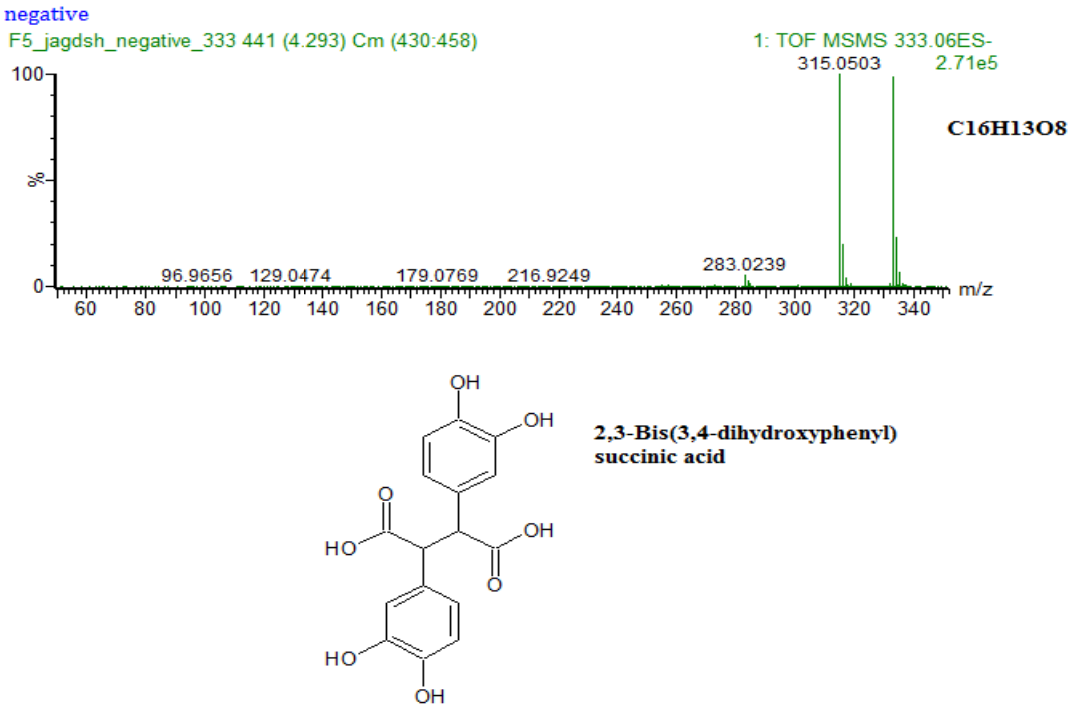


Figure IV.9. The MS/MS fragmentation pattern and structure of 2,3-Bis (3,4- dihydroxyphenyl) succinic acid (ions 333.441 m/z) by negative ionization mode.

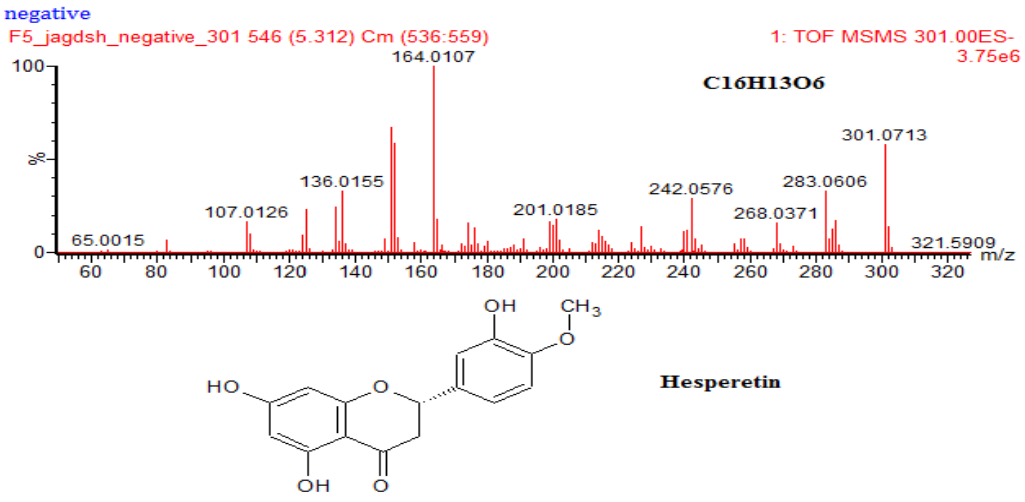


Figure IV.10. The MS/MS fragmentation pattern and structure of Hesperetin (ions 301.546 m/z) by negative ionization mode.

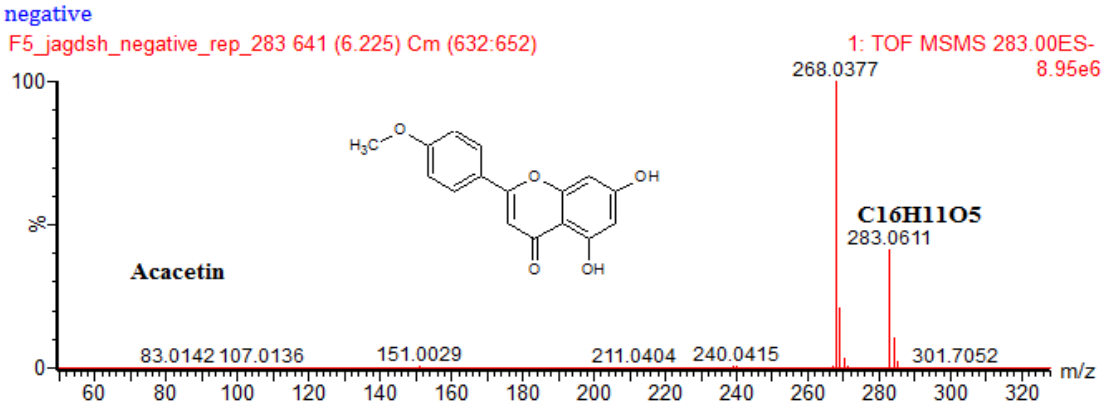


Figure IV.11. The MS/MS fragmentation pattern and structure of Acacetin (ions 283.641 m/z) by negative ionization mode.

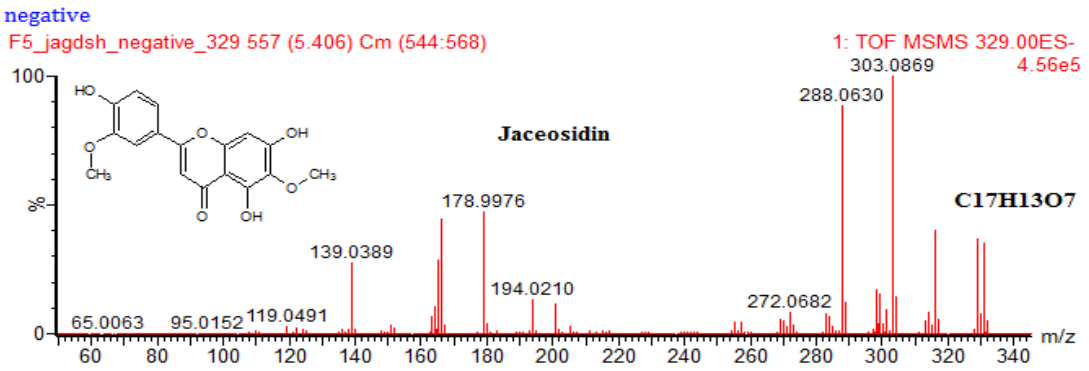


Figure IV.12. The MS/MS fragmentation pattern and structure of Jaceosidin (ions 329.557 m/z) by negative ionization mode.

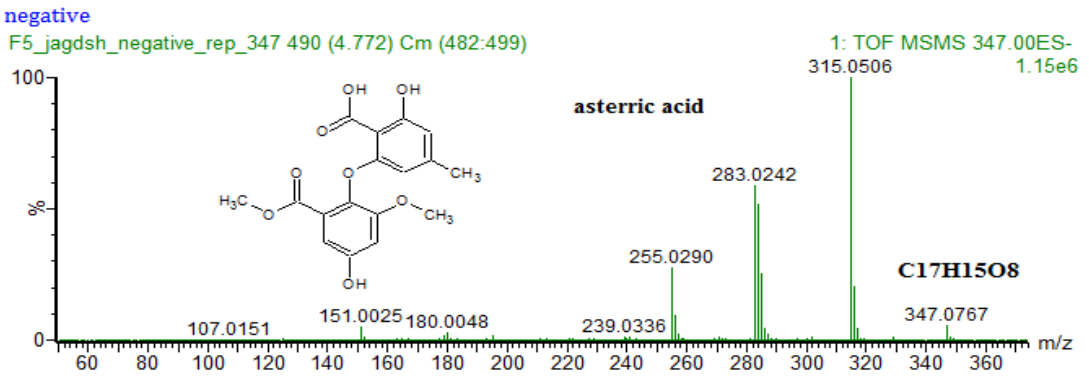


Figure IV.13. The MS/MS fragmentation pattern and structure of Asterric acid (ions 347.490 m/z) by negative ionization mode.

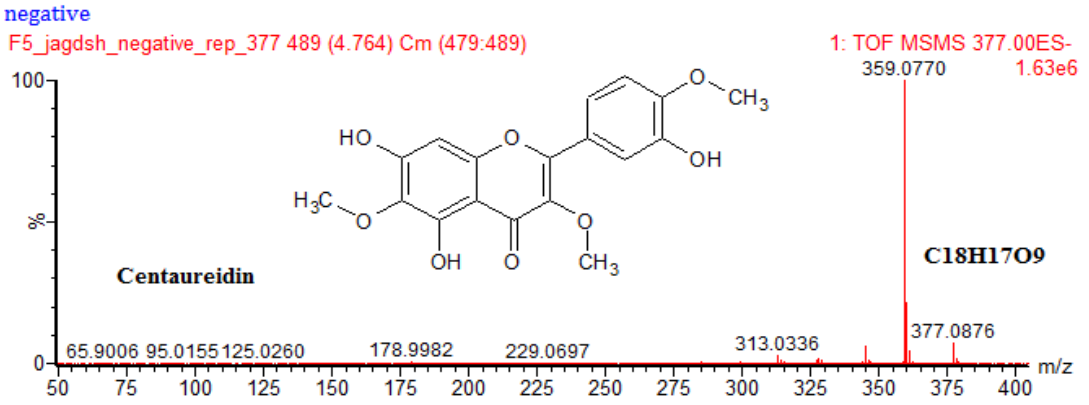


Figure IV.14. The MS/MS fragmentation pattern and structure of Centaureidin (ions 377.489 m/z) by negative ionization mode.

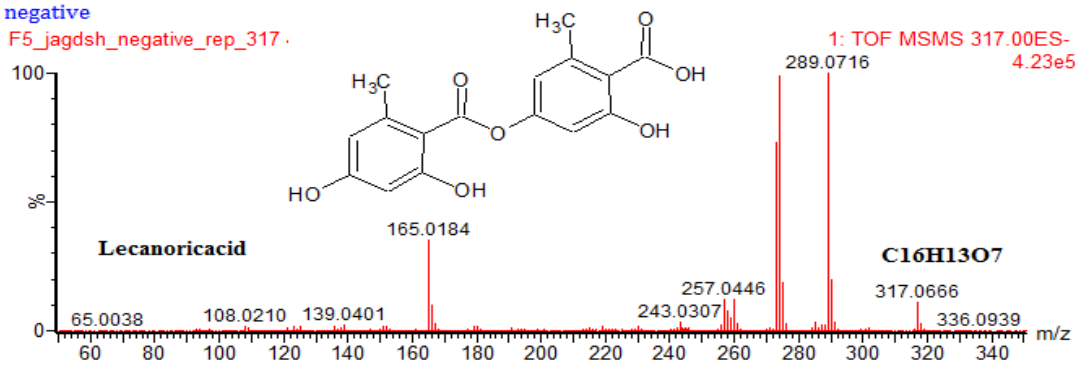


Figure IV.15. The MS/MS fragmentation pattern and structure of Lecanoric acid (ions 317.066 m/z) by negative ionization mode.

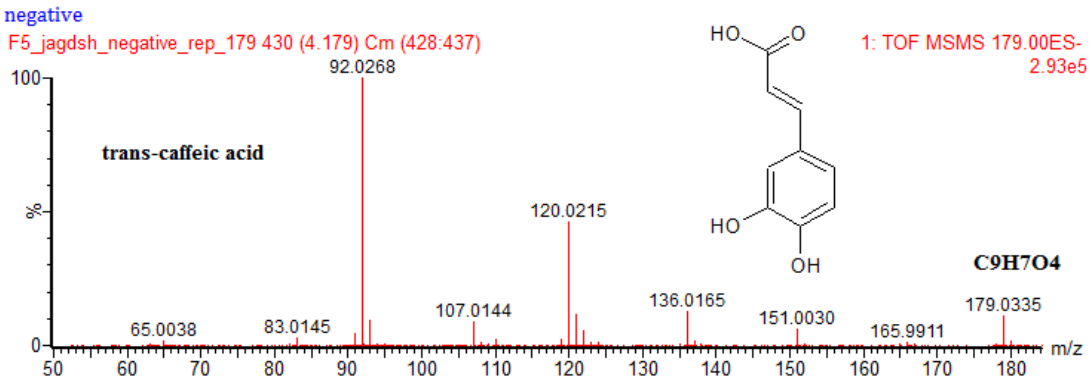


Figure IV.16. The MS/MS fragmentation pattern and structure of trans-caffeic acid (ions 179.430 m/z) by negative ionization mode.

4.4 Discussion

In the present investigation, the preliminary phytochemical studies revealed that methanol was the most suitable solvent system for the extraction processes. Selection of suitable solvent for extraction play a major role in phytochemical studies. It was reported that the solvent which was selected for the phytochemical extraction has an influence on the toxicity evaluation (Yankanchi, 2009). The preliminary toxicity screening test was carried out to select the solvent in which maximum compounds were extracted and also to ensure that the solvent did not have any direct toxicity to the organisms. Based on this criteria, the solvent methanol exhibited higher performance showing their maximum phytochemical separation in HPTLC profile and showed least toxicity towards larvae in the toxicity screening test.

The fractionation of both methanolic leaf extracts by column chromatographic technique has helped to club the phytochemicals on the basis of their chemical profile and molecular weight and it also helped to identify the compounds having greater toxicity that are present in botanicals. The results of preliminary toxicity screening test revealed 100% mortality of both fractions F2 of *C. infortunatum* and F5 of *C. odorata* and these were subjected for further evaluation of toxicity by LD_{50} calculation and it revealed that the mortality response was directly proportional to the applied dose and time. Similar, dose dependent toxicity studies with plant extracts and oils etc. have been reported by many authors (Fitzpatrick and Dowell, 1981; Pathak and Tiwari, 2010). The dose dependent mortality was reported in last instar nymph of *Dysdercus koenigii* treated with neem oil (Gujar and Mehrotra, 1990). When the LD_{50} values of fractions separated from *C. infortunatum* was compared with that of fractions of *C. odorata*, the fraction F5 from *C. odorata* showed greater toxicity than that of F2 from *C. infortunatum*.

Toxicity of separated fractions was directly related to their phytochemical composition. It was reported that insecticidal effects of plant essential oils are related to their chemical compositions (Isman *et al*., 2001; Tapondjou *et al*., 2002; Pascual-Villalobus and Ballesta-Acosta, 2003). Leaves and flowers of *C. infortunatum* contain clerodin, hentriacontane, fumaric acid, ethyl and methyl esters of caffeic acid, 2-sitosterol and its glucoside and flavones glycoside (Akihisa *et al*., 1988). The methanolic leaf extracts of *Chromolena odorata* contain steroids, triterpenes, flavanoids, alkaloids, tannins, diterpenes and saponins (Lovet and Zige, 2013). The activity of such compounds present in fractions of both *C. infortunatum* and *C. odorata* may be the reason for the mortality of *O. exvinacea* larvae.

The HPTLC profiling of both fractions showed the presence of phenols, flavonoids and terpenes and also it is suggested that the phytochemicals under these categories may be causing toxicity of fractions. Phenols and terpenes were commonly found in both fractions -F2 of *C. infortunatum* and F5 of *C. odorata*. Flavonoids were present only in F5 fraction of *C. odorata* and it may be the reason for the fraction F5 to have higher toxicity than F2. Plant flavonoids are a group of allelochemiclas with widely noted prooxidant effects (Halliwell and Gutteridge, 1985).

In the present study, the bioactive compounds identified from fraction F5 (*C. infortunatum*) by GCMS analysis were n-Hexadecanoic, squalene and digitoxin. Many other investigations also proved the toxicity of these compounds. The compound n-Hexadecanoic acid is generally known as palmitic acid, these are most common saturated fatty acids naturally produced by a wide range of animals, plants and microorganisms. The bioassay of the fraction separated from the acetone leaf extract of *Feronia limonia* showed larvicidal activity on mosquito larvae and revealed the presence *n*-hexadecanoic acid causing the larvicidal effect against fourth instar larvae

of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*, with the LC_{50} values of 129.24 ppm, 79.58 ppm and 57.23 ppm, respectively (Rahuman *et al*., 2000).

Similarly, the compound squalene is a kind of triterpene produced by all plants and also found in animals as a biochemical intermediate. The investigation carried by Chauhan *et al.* (2015) on the insecticidal activity of *Jatropha curcas* extracts against housefly *Musca domestica* and the GC-MS analysis of *J. curcas* leaf extract revealed the presence of squalene among the group of toxicity causing components present in it. Digitoxin is a phytosteroid and is similar in structure and effects to digoxin present in plants and animals. Toxicity of digitoxin was reported by Ahmed *et al.* (2013) in their investigation on the insecticidal and anti-oxidant activity of selected medicinal plants on stored grain pest *Tribolium castaneum* and noticed the digitoxin containing *Digitalis purpurea* extract showed highest mortality among the glycosides such as gitoxin, and digoxin.

In the case of *C. odorata*, major bioactive compounds in the fraction F5 possessing are cholestane, hexadecanoic acid-methyl esters, 1-Monolinoleoylglycerol trimethylsilyl ether and 1,2-Benzenedicarboxylic acid, diisooctyl ester. Bioactivities of these compounds were reported by many researchers. The compound cholestane is a saturated 27-carbon tetracyclic triterpene and its antioxidant activity was reported by Pandian and Nagarajan (2015) in their investigation carried out on the chemical composition and antioxidant potential of hydrodistilled oil and CO_2 extracts of *Valeriana wallichi*.

The insecticidal activities of Hexadecanoic acid-methyl esters were reported in the evaluation of active fractions from *Casimiroa edulis* leaf extract against the larvae of *Spodoptera litura* (Barakat, 2011). The antimicrobial activity of hexadecanoic acid- methyl esters was reported by

Abhubaker and Majinda, (2016) in their studies carried out on the preliminary antimicrobial effects of crude n-hexane and chloroform extracts of *Albizia adianthifolia* and *Pterocarpus angolensis* by GC-MS.

1-Monolinoleoylglycerol trimethylsilyl ether was also identified as most prevailing bioactive phyto constituent in ethanol extract of leaf and stem of *Sesuvium portulacastrum* (Sheela and Uthayakumar, 2013). The GC-MS analysis of bioactive components of ethanol extract of *Clerodendrum phlomidis* leaves revealed the presence of 1-Monolinoleoylglycerol trimethylsilyl and they were responsible for pharmacological activities (Kumaradevan *et al*., 2015). The bioactive constituent 10, 13-Eicosadienoic acid- methyl ester was also identified from the results of GCMS analysis of methanolic extract of *Nigella sativa* (Hadi *et al*., 2015).

The presence of compound 1,2-Benzenedicarboxylic acid- diisooctyl ester in carbon dioxide extraction from roots of *Stellera chamaejasme* revealed its potency as antifungal agent (Cheng *et al*., 2012). The GC-MS analysis of methanolic extracts of *Cassia italica* revealed the presence of bioactive component 1,2-benzenedicarboxylic diisooctyl ester along with other active compounds eicosane acid, n-hexadecanoic acid and ethyl ester (Sermakkani and Thangam, 2012).

The LC-Q-ToF and LC-MS/MS analysis of fraction F5 of *C. odorata* revealed the presence of bioactive compounds such as Hesperitin, Acacetin, Jaceosidin, Centaureidin and Caffeic acid. Hesperetin is naturally occurring flavanone present in plants and their bioactivity was reported in many investigations. Erlund (2004) investigated the bioactivities of the flavonoids especially quercetin, hesperetin, and naringenin and their protection towards cardiovascular disease. Acacetin is an oxygen methylated flavones present in many plants and the insecticidal properties of this component was reported by many workers. Auamcharoen *et al.* (2012) reported the toxicity of crude

methanolic extract of *Duabanga grandiflora* on *Sitophilus oryzae* and studied the toxicity causing components consisting of three flavones such as acacetin, apigenin and acacetin 7-O-glucoside along with triterpenes, phenolics and steroid.

Jaceosidin is a kind of flavone mainly found in *Artemisia* species and they have many pharmacological importance. Moscatelli *et al*. (2006) studied the anti inflammatory activities of flavanoid from *Artesemia copa* and noticed that jaceosidin was the most active flavonoid that inhibited cyclooxygenase-2 activity in mouse macrophages. Centaureidin is an O- methylated flavonol present in plants and it was isolated from the leaves of *Gynandropsis pentaphylla* growing in Egypt (Ali *et. al*., 1987) and the antioxidant and hypoglycemic activities of the flavonoid centaureidin, have been reported by Pe´rez-Gutie´rrez *et al*. (1998).

Caffeic acid is an organic compound found in all plants intermediate in the biosynthesis of lignin. Joshy *et al*. (2014) carried out a molecular investigation on the insecticidal action of caffeic acid against *Helicoverpa armigera* and found that caffeic acid have the potency to inhibit the gut protease activity and alteration of gene expression in caffeic acid fed larvae.

The present investigation suggested that the larvicidal potency of leaf extracts of *C. infortunatum* and *C. odorata* against *O. exvinacea* larvae was mainly due to the presence of active compounds present in the botanicals.

CHAPTER V

HISTOPATHOLOGICAL EFFECTS OF *CLERODENDRUM INFORTUNATUM* AND *CHROMOLAENA ODORATA* ON THE MIDGUT TISSUE OF *ORTHAGA EXVINACEA*

5.1. Introduction

In insect, digestion and absorption to various tissues are significant processes. Midgut is the important part of digestive tract in insect and plays a critical role in physiological regulations such as metabolism, homeostasis of electrolytes, immune response and circulation etc. The complexity of cellular organization in midgut provides a good digestive spectrum to their physiology. The digestive and absorptive cells are the most predominant epithelial cells in insect midgut and are responsible for processing of the diet by actively involving in enzyme production and secretion, as well as absorption of nutrients (Billingsley and Lehane, 1996; Van Zyl and Van Der Linde, 2000).

The alimentary canal of insects is a long narrow or coiled tube which extends from the mouth to the anus, with three main sections having different embryonic origin: the foregut or stomodaeum; the midgut or mesenteron, consisting of the ventriculus and gastric caeca; and the hindgut or proctodaeum (Uvarov, 1966; Borror and De Long, 1969; Belkin, 1976; Maranhao, 1976). Among the three regions, midgut is the main location for enzyme production, digestion and secretion (Anderson and Harvey, 1966; Humbert 1979; Chapman, 1998). Many studies carried out on the midgut region reveal that any kind of alteration on this region may directly affect the growth and development of insects as a result of changes occurred in their

different physiological processes (Mordue and Blackwell, 1993; Nisbet, 2000; De Sousa *et al*., 2009).

The digestive tract of insect is considered as an effective physical and chemical barrier against different invasive pathogens and toxicants. Intake of toxic food materials may disrupt the cellular architecture and it may lead to serious histological and functional disorder, thereby interfering the physiochemical barrier in tract. The cellular and tissue level changes can be used as direct indicator of the physiological state of insect under toxic conditions (Meyer and Hendricks, 1985). Cellular deformities indicate or are the solid evidence for the biochemical and metabolic changes occurred in insect under stress condition and these changes were reported by both light microscopic and ultrastructural studies. The light microscopic studies helped to reveal the structural deformities of cells and also the intensity of changes can be predicted through cellular dimensional variations by histomorphometry. The functional changes of different cells, secretary activities of cell organelles and their disorientation can be successfully established by the ultrastructural studies.

Generally, the cellular arrangements of insect gut consist of a single layer of epithelial cells resting on a continuous basal lamina or basement membrane and associated muscle layers. The epithelial layer is formed of four types of cells, especially columnar cells, which is concerned with the secretion of enzymes and absorption; goblet cells with function of active transportation of potassium and calcium ions; regenerative cells is concerned with renewal and replacement of injured cells and endocrine cells with endocrine function.

This chapter deals with the effect of *C. infortunatum* and *C. odorata* on the histopathology and histomorphometry of the midgut tissue of sixth instar larvae of *O. exvinacea*. The histomorphological and morphometric studies

were carried out using light microscopy and ultrastructural studies by Transmission Electron Microscopy.

5.2. Materials and methods

The methods used for the histopathological studies were described in section 3.2.5. The histological techniques for light microscopic studies and morphometric studies were mentioned in 3.2.5.1 and method used for the ultrastructural studies were detailed in section 3.2.5.2.

5.3. Results

5.3.1 Light microscopic observations

The five different concentrations (1%, 2%, 3%, 4% and 5%) of both *C. infortunatum* and *C. odorata* treated midgut tissue of sixth instar larvae of *O. exvinacea* were examined under light microscope and major histopathological changes were noted. These changes were showed in Plates V.1 to 6.

a. General histomorphology of the midgut tissue

Normal histomorphology of the midgut tissue of sixth instar larva of *O. exvinacea* is composed of a single epithelial layer on a basal lamina, surrounded by two muscle layers which are outer longitudinal muscle layer and inner circular muscle layer (Plate V.1 Figure. 1a, 1b and 1c). Basement membrane is associated with muscle layer which separate the epithelial cells from haemolymph. Generally the epithelial layer consist of four types of cells, columnar cells, regenerative cells, goblet cells and endocrine or secretory cells, which are not distinctly observed (Plate V.1 Figure. 1b and 1d). Columnar cells are the most commonly found epithelial cells and they constitute about 70% of total epithelial cells (Plate V.1 Figure. 1b and 1c) and they are engaged with secretion and absorption in the midgut. Regenerative

cells or undifferentiated cells are found in the basal region of epithelial layer (Plate V.1 Figure. 1b). They are engaged with renewal or replacement of injured cells. Goblet cells are flask shaped (Plate V.1 Figure 1b, 1c and 1d) and they are concerned with active transport of potassium and calcium ions.

b. Cytotoxic effects of *Clerodendrum infortunatum*

The cytotoxic effects of *C. infortunatum* leaf extract were studied by the treatment of five different concentrations (1%, 2%, 3%, 4% and 5%) on the sixth instar larvae of *O. exvinacea*. The results showed that at 1% concentration, there occurred elongation of epithelial cells, especially the columnar cells (Plate V.2 Figure. 2a). In columnar cells, vacuolization of cytoplasm, enlargement of columnar nucleus and some secretory activity were observed (Plate V.2 Figure. 2a and 2b). At 2% concentration, folding of the epithelium was noted (Plate V.2 Figure. 3a). In some regions alternative folds and infolds of epithelium tend to overlap (Plate V.2 Figure. 3b). And due to this overlapping, excessive vacuolization and congested nucleus were noticed (Plate V.2 Figure. 3a and 3b). The effect of 3% concentration showed that a portion of epithelial cell layer was bulged and alternative folds and infolds of epithelial layer got completely overlapped (Plate V.3 Figure. 4a). There exhibited the detachment of epithelium from basement membrane (Plate V.3 Figure. 4a and 4b). Due to this detachment of epithelium, columnar cytoplasm got vacuolated (Plate V.3 Figure. 4b). In higher concentration (4%), excessive elongation of epithelial cells was visible (Plate V.3 Figure. 5a). Numerous secretory vesicles were found in cytoplasm as a result of increased secretory activities of columnar cells (Plate V.3 Figure. 5a). Excessive cytoplasmic vacuolization were clearly seen (Plate V.3 Figure. 5a and 5b). At 5%, due to excessive elongation and vacuolization, the apical region of the columnar cells got sloughed off and moved into the lumen and the size of columnar nucleus was decreased (Plate V.4 Figure. 6a and 6b).

c. Cytotoxic effects of *Chromolaena odorata*

The effects of five different concentrations (1%, 2%, 3%, 4% and 5%) of *C. odorata* leaf extract on the midgut tissue epithelium of sixth instar larvae of *O. exvinacea* showed that in addition to increase in size of the goblet cells, there happened cytoplasmic vacuolization of the columnar cells and thinning of muscle layers. In the case of 1% and 2% concentrations, there observed increase in size of goblet cells and vacuolization of cytoplasm (Plate V.4 Figure. 7a, 7b and Plate V.5 Figure. 8a, 8b). There was increased secretory activity of the columnar cells in 1% concentration which was not observed in the case of 2%. In the case of 3% concentration, vacuolization of cytoplasm of columnar cells and pinching off apical tips of the cells were visible (Plate V.5 Figure.9a) and vacuolization and thinning of muscle layer was also noticed (Plate V.5 Figure.9b). Destruction of brush border of epithelial layer was found in 4% concentration (Plate V.6 Figure.10a). Swelling of the nucleus of columnar cells were also seen (Plate V.6 Figure.10b). In the case of 5% concentration, elongation of columnar cells, thinning of muscle layer, vacuolization of cytoplasm and sloughing off of apical region of epithelium into lumen were found (Plate V.6 Figure.11a and 11b).

5.3.2. Morphometric studies

The effects of both botanicals on the dimensions of cells were also observed. The 70% of epithelial cells comprising columnar cells were selected for the morphometric studies and the result shows significant morphometric changes in height and width of columnar cells and in their nucleus. The normal height and width of columnar cells of untreated tissue were 690.10 µm and 55.63 µm respectively and its nucleus having the height of 85.19 µm and width of 97.48 µm (Table V.1).

The effects of *C. infortunatum* at concentration of 1% showed that, height of columnar cells were increased to 732.70 µm and width decreased to 35.16 µm and the height and width of nucleus were reduced to 43.97µm and 38.47µm respectively. At 2% treatment, height and width of columnar cells and its nucleus were decreased (Table V.1). At 3% treatment as a result of cell elongation, increased height and decreased width were observed in columnar cells and decreased height in their nucleus as compared to normal cells but when compared to 2%, the width of columnar cells and their nucleus were increased. At 4%, the height and width of columnar cells were severely increased and its nucleus height was increased and width was decreased (Table V.1). When compared to 4%, excessive elongation caused sloughing off of apical parts of columnar cells and thereby reduced height was observed at 5%. In the case of nucleus the height was decreased in both 4% and 5% but when compared to control it increased (Table V.1).

The effects of *C. odorata* at 1% treatment showed that the height of columnar cells and its nucleus were increased, but the width of columnar cells and its nucleus were decreased (Table V.1). When compared to 1%, columnar cell height got decreased in 2% and the cell width and height of nucleus were increased at 2%. Disruption of apical region caused the rapid reduction in height of columnar cells treated with 3%. In higher concentration 4%, height and width of columnar cells were increased and enormous increase in height of nucleus was noticed but their width was decreased (Table V.1). When compared to 4%, destruction of brush border caused severe decrease in height of columnar cells at 5% and decreased height and increased width of nucleus was noticed (Table V.1).

Table V.1: Morphometric changes in columnar cells due to the effect botanical treatment

Treatment	Parameters	Measurements in different concentrations (in micron)						F value
		Control	1%	2%	3%	4%	5%	
C. infortunatum	Height of Columnar cells	690.10± 47.80	732.70± 34.87	508.6 ±37.49	1156.8± 47.20	2399.25± 144.23	1033.1± 54.84	455.11
	Width of columnar cells	55.63± 7.95	35.16± 1.43	32.16± 3.77	53.35± 6.07	84.21± 11.80	83.77± 6.11	52.55
	Height of Columnar nucleus	85.19± 10.73	43.97± 4.17	36.88± 4.52	150.38± 11.28	109.17± 5.67	130.66± 6.69	177.86
	Width of Columnar nucleus	97.48± 10.44	38.47± 2.74	28.28± 3.83	63.53± 4.09	68.65± 6.54	82.20± 10.32	68.531
C. odorata	Height of Columnar cells	690.10± 47.80	1305.47± 24.80	1102.22± 70.48	371.88± 20.84	1991.38± 53.88	918.88± 88.43	491.92
	Width of columnar cells	55.63± 7.95	40.10± 4.19	57.36± 7.74	52.72± 2.44	182.90± 6.37	82.85± 2.57	434.11
	Height of Columnar nucleus	85.19± 10.73	162.88± 13.09	183.0± 6.08	48.67± 2.89	363.28± 19.59	151.26± 4.07	489.71
	Width of Columnar nucleus	97.48± 10.44	68.07± 6.80	75.89± 7.09	75.57± 3.83	54.12± 5.58	76.80± 9.32	17.62

Each value represents Mean ± SD. Significance level: $p<0.01$ = highly significant

5.3.1 Ultrastructural studies

From the light microscopic observations, prominent changes found 5% of *C. infortunatum* and 4% of *C. odorata* were selected for ultrastructural studies. The selected concentrations of botanical treated midgut tissue of sixth instar larvae of *O. exvinacea* were examined under transmission electron microscope and the changes observed were recorded (Plates V.7 to 14).

Ultrastructural aspects of the midgut tissue of the sixth instar larvae of *O. exvinacea* generally comprised of a basement membrane with connective tissue and inner to it a layer of epithelial cells and outer to it a muscle layer (Plate V.7 Figure. 12a and 12b). This muscle layer consisted of inner circular and outer longitudinal muscles which were formed of actin and myosin filaments (Plate V.7 Figure. 12b and 12c). The epithelial layer was formed of columnar cells, regenerative cells, goblet cells and endocrine cells which were not distinctly noticed (Plate V.7 Figure.12f and Plate V.8 Figure. 13a).

a. Muscle layers

General structure of muscle layers consisted of discontinuous network of circular muscle and longitudinal muscle (Plate V.7 Figure. 12b and 12c). Circular muscle layer was seen on the inner side (Plate V.7 Figure.12e) and longitudinal layer was present on outer region (Plate V.7 Figure.12d). In untreated larva, thick arrangements of actin and myosin filaments were observed and they were rich in mitochondria (Plate V.7 Figure. 12d and 12e).

Effect of botanical treatment :- In circular muscle layer, the separation of myofibrils and vacuole like formation were noticed on treatment of 5% *C. infortunatum* (Plate V.11 Figure.16c) and 4% of *C. odorata* (Plate V. 13 Figure. 18c) extracts. Shrunken myofibrils were observed in longitudinal muscle layer with the treatment of *C. odorata* (Plate V.13 Figure. 18d).

b. Basement membrane

The basement membrane was found in basal region of epithelial cells, associated with circular muscle layer (Plate V.7 Figure.12a, 12b and 12c). Muscle layer and basement membrane together separated the epithelial cells from the haemolymph (Plate V.7 Figure. 12b and 12c). The basement membrane had structural specializations that facilitate elasticity exhibited by midgut that help to changes in volume.

Effect of botanical treatment: - Excessive vacuolization in the basal region of the epithelial cells and detachment of epithelial cell layer from the basement membrane were observed in both the botanical treatments (Plate. V.11 Figure.16a, 16b and Plate V.13 18a, 18b, 18c).

c. Columnar Epithelial cells

Ultrastructural studies on epithelial cells showed that columnar cells are the most commonly found cells in epithelium (Plate V.7 Figure. 12a, 12f and Plate V.8 Figure13a, 13b). The basal wall of columnar cells was folded along with invaginations of basement membrane (Plate V.7 Figure. 12f). The nucleus of columnar cells was located in mid-basal part of the cell with condensed chromatin granules (Plate V.8 Figure.13b and 13c). Abundant secretory vesicles and rough endoplasmic reticulum were concentrated around the nucleus (Plate V.8 Figure.13c, 13d and 13e). Secretory granules were present in variable density in the cytoplasm representing enzymes or their precursor proteins (Plate V.7 Figure. 13e). In control midgut tissue, columnar cells had numerous mitochondria and secretory vesicles in the cytoplasm (Plate V.8 Figure.13f and Plate V.9 14a). Apical region of columnar cells were rich in microvilli and presence of smooth endoplasmic reticulum were observed (Plate V.9 Figure.14b, 14c). Peritrophic membrane was noticed in

untreated larva which was situated in between lumen and brush border of epithelial cells (Plate V.9 Figure. 14d).

Effect of botanical treatment: - Enormous elongation and cytoplasmic vacuolization of columnar cells were observed in the midgut tissue on administration of both botanicals (Plate V.11 Figure.16d and Plate V.13 Figure.18f). Nucleus of the columnar cells became shrunken and some vacuoles appeared around the nucleus on treatment of 5% *C. infortunatum* (Plate V.12 Figure. 17a) and the rough endoplasmic reticulum was not distinctly seen along with a change in the shape of mitochondria (Plate V.12 Figure. 17b). Loss of microvilli in the apical region of columnar cells and absence of smooth endoplasmic reticulum were observed in both the cases (Plate V.12 Figure.17f and Plate V.14 Figure.19f). Numerous secretory vesicles were present beside columnar nucleus and the number of rough endoplasmic reticulum around the columnar nucleus was seen reduced on treating with *C. odorata* extract (Plate V.14 Figure. 19b) and also observed that the vacuoles present in the apical region of columnar cells were filled with amorphous materials (Plate V.14 Figure. 19e).

d. Goblet epithelial cells

Goblet cells were present in the basal region of the epithelium with flask shaped basal cavity. The cavity was formed from the invaginations of the apical border of the cell, called goblet chamber (Plate V.10 Figure. 15a). In control larvae, goblet cells had enormous microvilli inside the cells with rich mitochondria (Plate V.10 Figure. 15b, 15c). These microvilli were concentrated mainly at the basal region of the goblet cells and a valve like packing was observed (Plate V.10 Figure.15 a). Nucleus of the goblet cells was found in the basal part of the cell (Plate V.10 Figure. 15d).

Effect of botanical treatment: - In both case of botanical treatment, the whole microvilli present inside the goblet cells were lost or ruptured and became dense in colour with change in shape and reduction in the number of mitochondria (Plate V.12 Figure.17c,17d and Plate V.14 Figure.19c,19d). Excessive vacuoles and secretory granules were noticed in adjacent areas of the goblet cells with the treatment of *C. infortnatum* (Plate V.12 Figure. 17e).

e. Regenerative epithelial cells

Regenerative or undifferentiated cells were found in the basal region of epithelium in between columnar epithelial cells and goblet cells (Plate V.7 Figure. 12a and 12f). The cells were so called because of its undifferentiated state at the time of development. They are engaged with renewal or replacement of injured cells.

Effect of botanical treatment: The regenerative cells exhibited change in shape and their nucleus became shrunken in the case of 4% *C. odorata* treatment (Plate V.13 Figure.18b) while in the case of 5% *C. infortunatum* the cells became vacuolated (Plate V.11 Figure 16b)

5.4 Discussion

5.4.1 Light Microscopic studies

The histopathological changes that occurred in the larval midgut epithelium of *O. exvinacea* treated with both botanicals *C. infortunatum* and *C. odorata* mainly consisted of vacuolization and elongation of columnar epithelial cells, destruction of brush border of these cells and secretory activity and sloughing off of the apical region of the epithelial layer.

Sayed *et al*. (2011) reported that the treatment of *Azadirachta indica* and *Citrullus colocynthis* extracts on the larval midgut of *Spodoptera littoralis* showed vacuolization and necrosis of the epithelial cells and destruction of

epithelial cells and their boundaries. Jbiloua and Sayah (2008) noticed that the cytoplasmic vacuoles and occurrence of large intercellular space due to the effects of methanolic extract of *Peganum harmala* seeds on *Tribolium castaneum* midgut cells through diet incorporation. It was reported that cell elongations were the main reason for the appearance of vacuoles or as a result of excessive fat droplets which dissolved during fixation and dehydration process (Salkeld, 1951). Severe cytotoxic changes like extreme vacuolization, destroyed epithelial membrane and elongated columnar cells with destructed cell boundaries were observed in the larval gut of *S. littoralis* with the treatment of *Artemisia monosperma* (Adel *et al*., 2010).

Epithelial elongation, excessive vacuolization and enlargement of nucleus were observed as common effects of both botanicals in larval mid gut tissue and the degeneration of epithelial cells were increased as concentration of both botanicals increased. According to Humbert and Desportes (1977) the degeneration process allowed the removal of toxic elements from the alimentary canal. Plant flavonoids are a group of allelochemiclas with widely noted pro-oxidant effects (Halliwell and Gutteridge, 1985).The cytotoxic effect of tannins on different species of *Acridoidea* showed the histopathological changes on midgut epithelia such as lesions and necrotic nuclei in *Locusta migratoria* (Bernays *et al*., 1980).

The presence of active ingredients in both botanicals caused the histological and morphometric variations in epithelial layer which might lead to digestive and food absorptive disorders. The neem derivative azadirachtin caused influences on the midgut of *Schistocerca gregaria* and *Locusta migratoria* and exhibited histopathological changes such as swelling of cell organelles and rounding up of the cells, necrosis of midgut cells, enlargement of connective tissue with many invading cells and muscle layers became shrunken and swollen (Nasiruddin and Mordue,1994). Studies on the mid gut

tissue of *Hofmannophila pseudospretella* showed that there were differences in the size and activity of mid gut columnar cells and goblet cells depending on food quality (Gerard, 2002).

Significant differences were noticed in size of columnar and goblet cells in gypsy moth larvae fed with suitable *Quercus cerris* leaves compared with larvae fed on *Robinia pseudoacacia* leaves which were associated with higher digestive activity (Peric-Maturaga *et al*., 2006). According to Cottee (1984), the histological changes occurred such as vacuolization of cytoplasm, reduction in the size of the nuclei, regeneration and necrosis of cells in *S. gregaria* and *L. migratoria* was mainly due to the active compounds present in botanicals. The cell organelles that fill the apical region of the columnar cells were those involved in the elaboration of the digestive enzymes, so any morphometric changes or destruction of the apical region of the columnar cells might be the cause for digestive disorders (Terra *et al.,*1996).

Jing *et al*. (2005) reported the histopathological changes in midgut epithelial cells induced by ingestion of phorbol-type plant isolated compound Jatropherol-I, revealing that it caused disintegration of the epithelial cells and it led to severe turbulence in insect metabolism, especially in protein metabolism with alterations in activities of various midgut enzymes.

In the present study, the effect of active ingredients in the botanicals might be the reason for the histological and morphometric changes in the midgut tissue of *O. exvinacea* and might thereby affect digestion, absorption and various other physiological processes.

5.4.2 Ultrastructural studies

The ultrastructural studies on midgut tissue of control sixth instar larva of *O. exvinacea* revealed the normal structure and functions of both muscle and epithelial layer of the midgut. The midgut or mesenteron, is the second largest organ in insect body, formed mainly by columnar cells, goblet and regenerative cells and have the primary function of nutrient absorption (Hakim *et al*., 2010). The midgut of insects is the main part of the digestive tract, in which digestion and absorption occur; the wall comprises a single layer of digestive epithelium and two muscle layers (Rocha *et al.,* 2010) and also the midgut is considered as the best site of transporting epithelia, employed in intensive secretion of K+ and base equivalents (Dow and O'Donnell, 1990; Moffett and Koch, 1992). The peritrophic membrane in insect mid gut separates food from midgut tissue and plays an important role in the protection of the gut from food abrasion and various microbial challenges (Peters,1992; Lehane, 1997).

Results from the ultrastructural studies on the midgut of experimental sixth instar larvae of *O. exvinacea* treated with *Clerodendrum infortunatum* revealed that the active compounds present in *C. infortunatum* might be the cause for the ultrastructural variations in both epithelial and muscle layers of the midgut. It was reported that when *Spodoptera littoralis* was treated with the extract of both *Clerodendrum inerma* and *Conyzadios corids* there occurred slight and severe disintegration of the epithelium, fading of the boundaries of epithelial cells and detachment of epithelial cells (Emara and Assar, 2001). Marilza *et al*. (2014) reported the ultrastructural change in the shape of midgut digestive cells from columnar to cubic, vacuolization and changes in the brush border of the midgut digestive cells of *Aedes aegypti* larvae treated with squamocin. Similar result was reported in *Spodoptera frugiperda* treated with neem extract (Correia *et al*., 2009). In the

larval midgut of *S. littoralis* treated with *Azadiracta indica* and *Citrullus colocynthis* extracts, vacuolization and necrosis of the epithelial cells and destruction of epithelial cells and their boundaries were reported (Sayed *et al.,* 2011). Destruction of microvilli and reduction in number and changes in the shape of mitochondria might affect the function of goblet cells concerned with excretion of potassium. It was reported that squamocin from *Annona squamosa* on *A. aegypti* larvae prevented the production of ATP by the electrons in the mitochondrial complex I and caused the death of the insect by affecting cellular respiration (Lummen, 1998; Takada *et al*., 2000).

Active ingredients present in *C. odorata* might be the cause for the ultrastructural variations occurred in both epithelial and muscle layers of the larval mid gut tissue of *O. exvinacea* treated with the leaf extract of *C. odorata*. Effect of plant cyclotides on the larval midgut of *Helicoverpa armigera* showed that the cyclotides disrupted the plasma membrane of the epithelial cells forming holes or pores that led to cell swelling and lysis (Barbera *et al*., 2007).The cytotoxic and larvicidal effects of squamocin on *A. aegypti* larvae might be attributed to the structure of the molecule and it has been reported that the acetogenins with adjacent *bis*-THF rings have high toxicity (Chen *et al*., 2011; Cortes *et al*., 1992).

Hence it is suggested that the effect of the active ingredients present in both the leaf extracts of *C. infortunatum* and *C. odorata* might be the reason for the ultrastructural alterations in the epithelial cells, destruction of microvilli, large areas of cytoplasmic vacuolization, mitochondrial damage and shrunken nucleus. These deleterious changes might cause serious digestive and absorptive disorders and it might affect normal growth and reproduction of *O. exvinacea*.

CHAPTER VI

BIOEFFICACY OF *CLERODENDRUM INFORTUNATUM* AND *CHROMOLAENA ODORATA* ON TOTAL PROTEIN, PROTEIN PROFILE AND FREE AMINO ACIDS IN DIFFERENT TISSUES

6.1 Introduction

Protein, 'the body builders' are the most important biological unit or complex macromolecules of living cells. The performance of proteins was reported in vast areas of functions within organisms, including enzymatic catalyzing functions, DNA replication, transporting molecules from one location to another, responding to stimuli and metabolic reactions. In insects, proteins are very essential for the proper development and growth including much vital physiological processes like cell division, metabolism, transport of ions across membranes, cuticle melanization and sclerotization. Hence protein is also called as "stuff of life" due to their importance as a biological compound in every living cell constituents (Swaminathan, 1983). The biological significance of proteins can be proved by the fact that the animals can live for a long time without fat or carbohydrate, but not without protein.

Proteins are the key source of metabolic currency and also can be referred as an important analyte in evaluating the physiological standards of the cell (Young, 1970). The studies related with both quantitative and qualitative changes of protein can reveal the condition of the physiological state of an insect. Determination of total protein is an important factor to detect the intensity of toxic constituents that affect the physiology of an organism. In addition, the expression of proteins under different toxic conditions is also considered as an important indicator to predict the physiological condition of an insect. Polyacrylamide gel electrophoresis can

be used for assessing the different biological processes by detecting the changes in protein structure and molecular weight (Sharaawi *et al*., 2002).

Amino acids are the building blocks of protein and play an essential role in different phases of insect life, especially as a neural transmitter, morphogenesis, phospholipid synthesis and energy production and in some cases the detoxification of certain metabolites can be effected through their interaction with amino acid. The combination of amino acids to form protein occur in different ways and is species specific. The optimal growth of leaf-eating insects depends on their ability to acquire essential amino acids from dietary protein. So the amino acid can be an indicator to know the physiological state of an insect under toxic condition.

The alimentary canal of insect is compartmentalized into foregut, midgut and hindgut, among this midgut is the main site for food digestion, nutrient absorption and protection from pathogen invasion. Therefore, midgut is an inevitable area for the study of both quantitative and qualitative changes of protein and that helps to know the physiological condition of an organism because any kind of alteration may directly affect the growth and development of the animal (De Sousa *et al*., 2009). Studies on expression profiles of proteins in the midgut can facilitate identification of molecular targets that can influence on the stability of physiology of an insect under toxic condition.

The fat body is a loose meshwork of lobes composed of mesodermal cells (adipocytes or trophocytes) and distributed in insect haemocoel suspended in haemolymph. This distribution depends on the species and stage of development. Its suspension in haemolymph helps to provide a large area for the exchange of molecules with both haemolymph and other tissues. The fat body is a dynamic tissue involved in multiple metabolic functions. Proteins are synthesized in the fat body and released into the haemolymph,

the storage of protein and its release for energy are mainly based upon the energy demands by the insect (Chen, 1985; Ranjini, 2002).

The highly dynamic fluid haemolymph is usually colourless but certain pigments impart slight greenish or yellow colour and it circulates in the body cavity (haemocoel) of insect. It is composed of fluid plasma with suspended haemocytes which account for approximately 5%-40% of the total body weight of an insect, depending upon the species. The complexity of its functions such as lubricant action, hydraulic medium, transportation of materials (nutrients, metabolic wastes, foreign materials, hormones and gases), heat transfer and protection of body are supported by the dynamic characters of haemolymph which is similar to the blood of vertebrates. The haemolymph proteins are numerous and possess a variety of functions (Kanost *et al*., 1990). The protein concentration in insect haemolymph is generally higher than that of the internal fluids of other invertebrates and is almost similar to that of the blood of man (Florkin and Jeuniaux, 1974). The amino acids in the haemolymph of lepidopteran insects have greater concentration than that reported in other groups of arthropods. Insects have 100 to 300 times more free amino acids in their haemolymph than humans and these may be either dietary or synthesized by the insect. According to the physiological needs, the level of protein and amino acid may vary during the protein synthesis and under stress condition (Ranjini and Mohamed, 2004 a)

This chapter deals with studies on the efficacy of both botanicals on quantitative and qualitative estimation of protein and free amino acid content in the midgut, fat body and haemolymph of sixth instar larvae of *Orthaga exvinacea*.

6.2 Materials and methods

The materials and methods for the quantitative estimation of protein are described in section 3.2.6.1 and that for the protein profiling are discussed in section 3.2.6.2. The method followed for the quantitative studies on amino acid is mentioned in 3.2.6.3.

6.3 Results

The results obtained show that the treatment of *C. infortunatum* and *C. odorata* produced significant changes in both protein and amino acid concentration in different tissues of sixth instar larvae of *O. exvinacea*. The data obtained for the quantitative estimation of protein in both control and treated tissues were presented in Table VI.1 to VI.3 and Figure.VI.1 to VI.3 and also the percentage reduction of protein in different tissues were given in Table VI.4. The electrophoretic studies presented some effective changes on the protein profiling of treated larvae and these were presented in Plate VI.1 to VI.6. The studies on the amino acid concentrations of different tissues were presented in Table VI.5 to VI.7 and Figure VI.4 to VI.6 and the percentage reduction was shown in Table.VI.8.

6.3.1 Studies on the effect of botanicals on the total protein concentration in different tissues.

Significant variations were observed in protein content of midgut, fat body and haemolymph of sixth instar larvae of *O. exvinacea* with increasing botanical concentrations. The gradual decrease in protein content with increase in botanical concentrations from 1% to 5% of both *C. infortunatum* and *C. odorata* were observed. The maximum reduction of protein was noticed at 5% of both treatments (Table VI.1 to VI.3). From the comparison of results of both botanical treatments, it was revealed that *C. odorata* have the maximum potency to reduce the protein content than that of

C. infortunatum. The statistical analysis of data depicted the significant changes occurred in protein concentration with the treatment of both botanicals.

The impact of botanicals tested on the larvae was found to be effective in reducing protein content in the midgut tissue. The total protein concentration was significantly lower in treated larvae than in the control (1.9 mg/ml). The midgut protein concentration of larvae treated with five different concentrations (1%, 2%, 3%, 4%, and 5%) of *C. infortunatum* was found to be decreasing from 1.78 mg/ml to 0.53 mg/ml and in the case of similar five different concentrations of *C. odorata,* the protein concentration decreased from 0.91 mg/ml to 0.07 mg/ml (Table VI.1). The percentage reduction of protein content was progressed from 1% to 5% treatment of both leaf extracts. Initially 6.32% of reduction was noticed at 1% of *C. infortunatum* treatment and later it gradually decreased with increasing botanical concentration and reached to 72.10% at 5% of treatment. In the case of *C. odorata* treated midgut tissues, 52.10% reduction in protein content was noticed at 1% of treatment and it reached the maximum of 96.31% at 5% of treatment (Table VI.4).

The effect of botanicals on the total protein content in fat body of larvae was also found to be causing reduction in protein content with increased botanical concentration (Table VI.2). The total protein concentration of control tissue was 2.73 mg/ml and it significantly reduced from 2.69 mg/ml to 1.40 mg/ml with 1% to 5% treatment of *C. infortunatum*; that means in 1% treatment a reduction of 1.46% in protein content occurred that further progressively decreased and the maximum reduction of 48.72% was found at 5% of treatment. Similarly with *C. odorata* treatment decline in protein content was observed in the fat body from 2.38 mg/ml to 0.79 mg/ml (Table VI.2). The minimum reduction of 12.82% was found at 1% of

treatment and the maximum reduction of 71.06% noticed at 5% of treatment (Table VI.4).

The treatment of botanicals also caused a serious decline in protein content of haemolymph of larvae. The untreated larvae contained 23.84 mg/ml of protein in their haemolymph and progressively it decreased from 21.88 mg/ml to 11.80 mg/ml with the treatment from 1% to 5% of *C. infortunatum*. A steady increase in percentage of reduction in protein content was noticed from 8.22% at 1% treatment to 50.50% at 5% treatment. In the case of *C. odorata* treatment, a drastic reduction in protein content of haemolymph from 19.68 mg/ml to 3.74 mg/ml was occurred with increase in botanical concentration (Table VI.3). The treatment caused 17.45% of reduction in protein content at 1% of treatment and 84.31% of reduction in protein at 5% (Table VI.4).

In both botanical treatments, common tendency noticed in all tissues was that there was gradual decline in protein concentration in relation to increased botanical concentrations and *C. odoarata* had the maximum efficacy in decreasing the protein content in all tissues than that of *C. infortunatum*.

Table VI.1 Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on the protein concentration in midgut tissue of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of protein in midgut		
		mg/ml	mg/gm	mg/larva
Control		1.90±0.02	18.15±1.13	0.63±0.01
C. infortunatum	**1%**	1.78±0.07	18.09±0.82	0.59±0.02
	2%	1.40±0.17	14.63±2.30	0.46±0.06
	3%	0.95±0.04	9.81±0.42	0.32±0.01
	4%	0.67±0.06	7.17±0.47	0.22±0.02
	5%	0.53±0.05	5.91±0.48	0.17±0.02
	F value	236.63	111.69	245.55
C. odorata	**1%**	0.91±0.02	9.27±0.40	0.30±0.01
	2%	0.58±0.02	6.60±0.30	0.19±0.00
	3%	0.28±0.04	3.14±0.49	0.09±0.01
	4%	0.20±0.02	2.35±0.31	0.07±0.01
	5%	0.07±0.02	0.81±0.17	0.02±0.01
	F value	3662.00	643.27	2690.00

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Protein content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VI.2 Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on the protein concentration in fat body tissue of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of protein in fat body		
		mg/ml	mg/gm	mg/larva
Control		2.73±0.034	36.00±1.145	0.91±0.011
C. infortunatum	**1%**	2.69±0.012	35.38±1.474	0.89±0.004
	2%	2.54±0.010	33.29±0.546	0.84±0.005
	3%	2.00±0.067	30.49±0.301	0.67±0.022
	4%	1.63±0.112	28.65±0.230	0.54±0.037
	5%	1.40±0.055	24.15±0.229	0.47±0.018
	F value	457.318	153.257	452.081
C. odorata	**1%**	2.38±0.056	31.62±0.221	0.79±0.019
	2%	1.98±0.053	29.00±1.240	0.66±0.0176
	3%	1.48±0.070	26.60±0.424	0.49±0.023
	4%	1.19±0.043	21.13±0.878	0.39±0.015
	5%	0.79±0.075	14.04±0.704	0.26±0.025
	F value	838.636	423.641	837.897

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Protein content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VI.3 Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on the protein concentration in haemolymph of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of protein in haemolymph	
		mg/ml	mg/larva
Control		23.84±0.20	2.98±0.025
C. infortunatum	**1%**	21.88±0.267	2.73±0.033
	2%	20.82±0. 124	2.59±0.016
	3%	17.65±0.332	2.20±0.041
	4%	14.78±0.159	1.85±0.020
	5%	11.80±0.549	1.47±0.068
	F value	1113.975	1111.724
C. odorata	**1%**	19.68±0.611	2.46±0.076
	2%	16.17±0.226	2.02±0.029
	3%	13.33±0.354	1.66±0.044
	4%	6.52±0.255	0.81±0.032
	5%	3.74±0.181	0.47±0.023
	F value	2575.919	2577.615

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Protein content was expressed in the units of mg/ml and mg/larva.

Table VI.4 Percentage reduction of total protein concentration in different tissues of *Orthaga exvinacea*

Botanicals	Treatments	Percentage reduction in protein content in different tissues		
		Midgut (%)	Fat body (%)	Haemolymph (%)
C. infortunatum	**1%**	6.32	1.46	8.22
	2%	26.32	6.96	12.67
	3%	50.00	26.74	25.96
	4%	64.74	40.29	38.00
	5%	72.10	48.72	50.50
C. odorata	**1%**	52.10	12.82	17.45
	2%	69.47	27.47	32.17
	3%	85.26	45.79	44.09
	4%	89.47	56.41	72.65
	5%	96.31	71.06	84.31

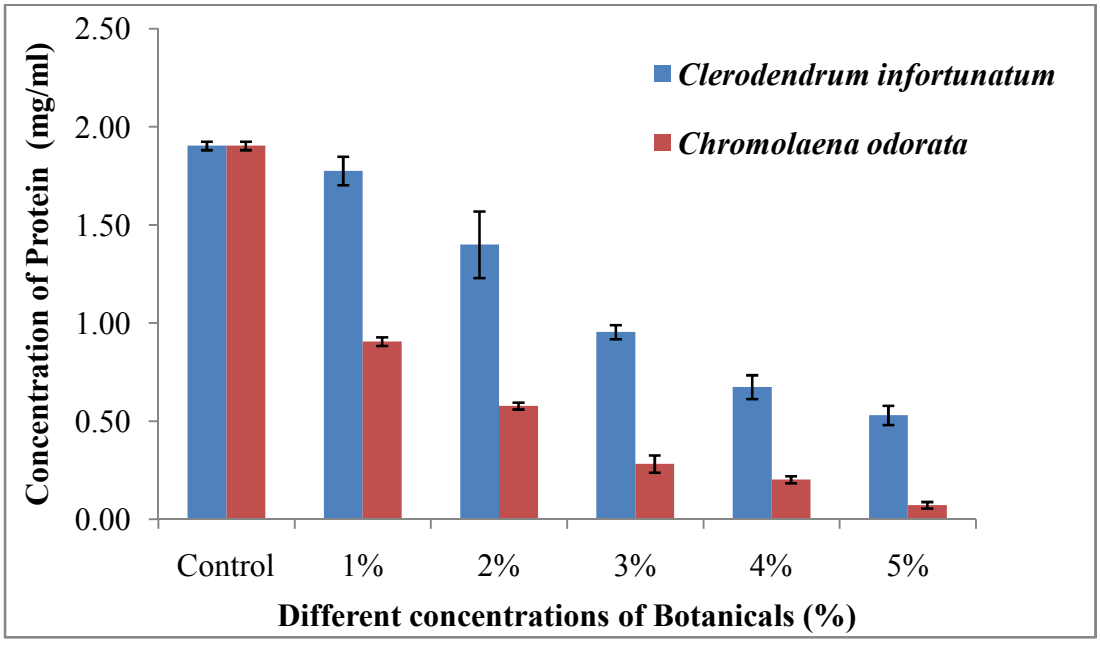


Figure VI.1 Graph showing the decrease in protein concentration in the midgut tissue with increase in concentrations of botanicals.

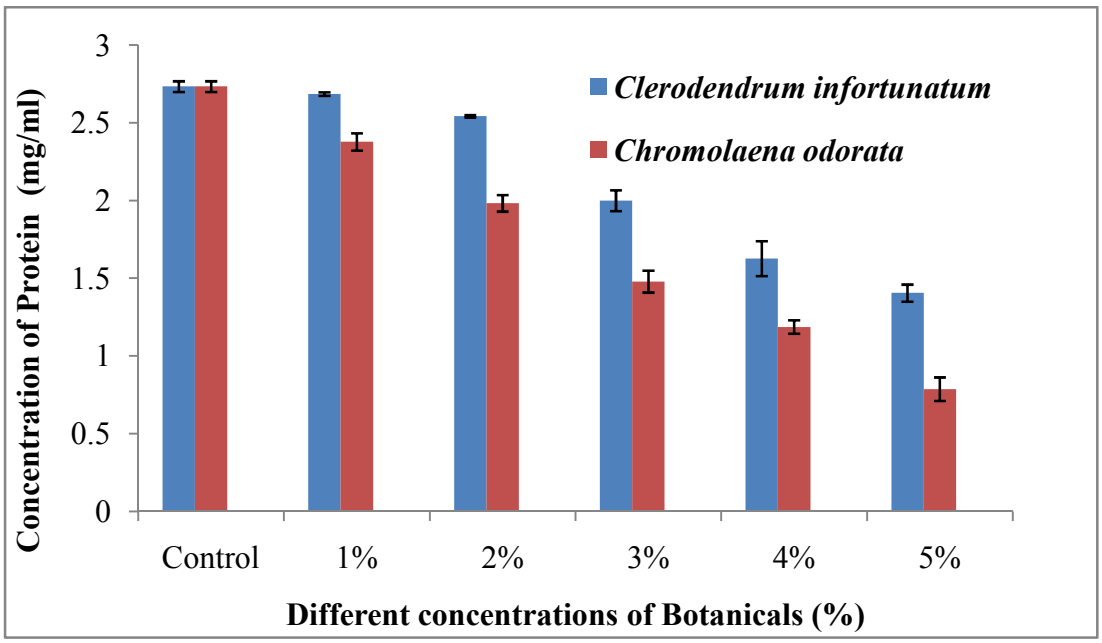


Figure VI.2 Graph showing the decrease in protein concentration in the fat body tissue with increase in concentrations of botanicals.

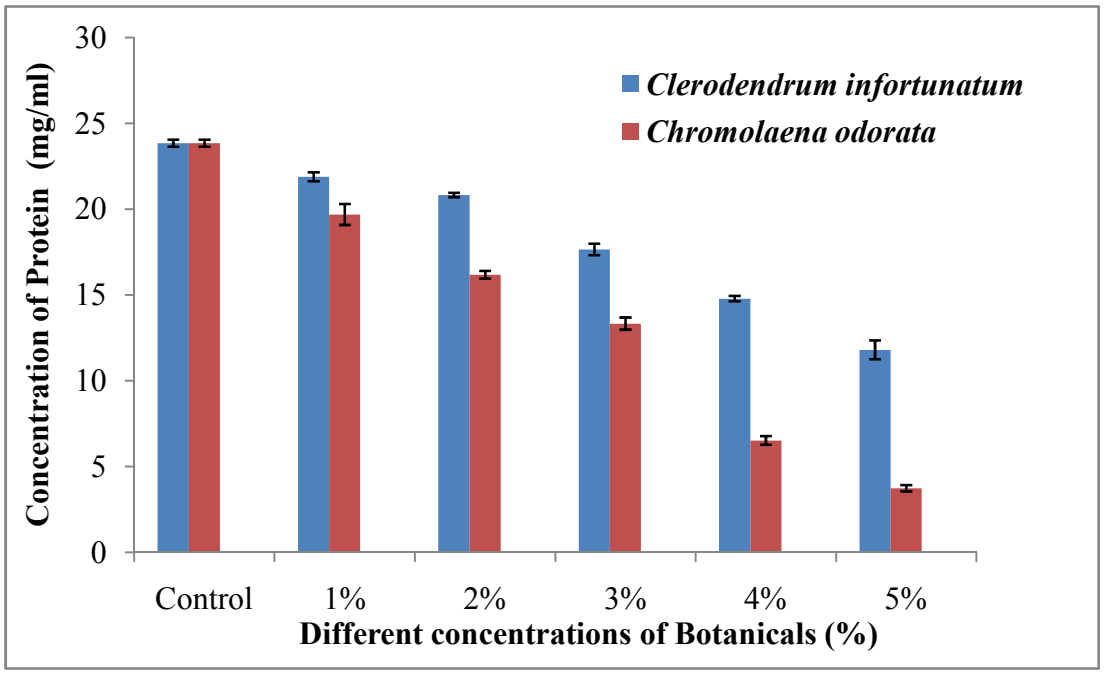


Figure VI.3 Graph showing the decrease in protein concentration in the haemolymph tissue with increase in concentrations of botanicals.

6.3.2 Electrophoretic studies of protein in different tissues

The influence of leaf extracts of *C. infortunatum* and *C. odorata* on protein profile of midgut, fat body and haemolymph of sixth instar larvae were recorded and it was presented in Plates VI.1 to Plate VI.6.

a. Effect of botanicals on the protein profile of midgut tissue

The protein profiles of the midgut tissue of both untreated (control) and treated larvae showed some considerable qualitative changes (Plate VI.1). A total of 12 protein bands were appeared in the protein profile of untreated larvae with molecular weights in between the range of 116 kDa to 14.4 kDa. At 1% of *C. infortunatum* treatment, two new bands with molecular weights of 30.19 kDa and 24.54 kDa were appeared, but these were disappeared in 2%, 3% and 4% concentrations and reappeared at 5% and also some disappearance of bands were noticed in both 3% and 4% treated larvae. The proteins having molecular weights 77.62 kDa, 58.55 kDa, 50.11 kDa, 44.77 kDa, 21.37 kDa, 14.12 kDa were commonly seen in 1% and 2% of treated tissues and the protein of 35.68 kDa present in control was absent in all treatments. The maximum disappearance of bands was noticed at 4% of treatment (Plate VI.1).

In the case of *C. odorata*, proteins appeared in control were mostly repeated in all the profiles from 1% to 3% treatments (Plate VI.4). Out of 15 bands appeared in the profile of 1% and 2% treated tissues, newly appeared 3 bands were noted other than that of control. They were 79.43 kDa, 22.90 kDa and 19.05 kDa for 1% treatment and 89.12 kDa, 23.44 kDa and 19.49 kDa for 2% of treatment. Some bands already present in control were disappeared at 3% and only one new protein band was appeared with molecular weight of 19.9 kDa. Disappearance of bands as well as appearance of 4 new bands having molecular weight 95.49 kDa, 36.30 kDa, 34.67 kDa

and 19.05 kDa were found at 4% of treatment. At higher concentration 5%, the treated profile exhibited appearance of 3 new proteins of 33.88 kDa, 28.84 kDa and 19.49 kDa and few bands were also disappeared (Plate VI.4).

b. Effect of botanicals on the protein profile of fat body tissue

The protein profile of fat body of control larvae exhibited 6 protein bands with molecular weights in between the range of 30 kDa to 100 kDa (Plate VI.2). The protein in the molecular range from 91.67 kDa to 66.86 kDa were common in all the treatments of *C. infortunatum* and protein having 40.40 kDa molecular weight was absent in all treated tissue protein profiles which was present in control. Two new protein bands with 51 kDa and 37 kDa were appeared at 1% of treatment and were absent in 2% and 3% treatments and again expressed at 4% treatment with slightly modified molecular weight. Similarly 36 kDa protein band was found in both 2% and 3% treated profiles with slight modification in weight but these were absent in all other concentrations. The 5% treated fat body profile showed 3 new protein bands with molecular weight ranging from 40 kDa to 90 kDa and they were absent in all other treatments (Plate VI.2).

In the case of *C. odorata* treatment, 5 protein bands were observed in control and after the treatment, 4 new bands were found in both 1% and 2% treated tissues with slightly varied molecular weight. The protein of 74.12 kDa and 66.69 kDa were absent in all the treatments which were present in control. The protein of 65 kDa was common in all treatments with slight changes in molecular weight except in the case of 4% treated tissue profile and also the protein 61 kDa was commonly noticed at 3% and 5% of treatments with slight molecular weight changes (Plate VI.5).

c. Effect of botanicals on the protein profile of haemolymph

The treatment of *C. infortunatum* made some changes in the protein profile of haemolymph of larvae (Plate VI.3). The control larvae exhibited 7 bands with molecular weights ranging from 30 kDa to 100 kDa. At 1%, 2% and 4% treatment, commonly found 4 bands with similar molecular weights ranging from 30 kDa to 60 kDa were noticed. The protein of 34.69 kDa was absent in all treated profile which was present in control and also 30 kDa protein was present in all the treatment with slight change in molecular weight except in the case of 3% treated tissue profile. The maximum appearance of protein bands (ranging between 30 kDa to 80 kDa) have been noticed at 5% of treatment than that of other treated profiles (Plate VI.3).

The treatment of *C. odorata* was also has profound effect on the protein profile of haemolymph of the larvae. Total number of 7 protein bands with molecular weights ranging from 20 kDa to 70 kDa were observed in the case of control larvae. The maximum number of protein expressions were noticed in both 1% and 5% of treated haemolymph profiles in which 57 kDa, 54 kDa and 47 kDa were commonly found with slight molecular weight changes. In all treatments protein having low molecular weights (below 20 kDa) were present in common but the protein of molecular weight 18 kDa present in control tissue profile was absent in all other treated profiles (Plate VI.6).

6.3.3 Effect of botanicals on the free amino acid concentration in different tissues

Efficacy of botanicals tested on the larvae was found be effective in reducing amino acid concentration in midgut, fat body and haemolymph. The results showed the progressive reduction of amino acid content in all tissues with respect to increasing botanical concentrations. In both botanical

treatments, the maximum amino acid reduction was noticed at higher concentration 5% and also the minimum was found at 1% treatment. Similar to protein results, the *C. odorata* have greater efficacy to decrease the amino acid content in all tissues than that of *C. infortunatum*. Statistical analysis revealed the significance of reduction in amino acid content in all 5 different concentrations (1%, 2%, 3%, 4%, and 5%) of both treatments with respect to control.

The amount of free amino acid concentration in the midgut tissue of untreated larvae was 7.37 mg/ml and it progressively decreased with increasing concentrations of botanicals. Due to the effect of *C. infortuntum* on the midgut tissue, the amino acid concentration was found to be decreased from 6.44 mg/ml to 2.79 mg/ml (Table VI.5). At 1% of treatment, 12.62% of reduction was noticed in the midgut amino acid content and later it increased to 62.14% of reduction at 5% treatment. In the case of *C. odorata*, similar five different concentrations reduced the amino acid concentration from 5.40 mg/ml to 1.31 mg/ml and there occurred 26.73% of amino acid reduction at 1% which progressively increased and the maximum percentage reduction of 82.22% was noticed in 5% treated midgut tissue (Table VI.8).

The effect of botanicals on the free amino acid content in fat body of larvae was found to be significantly reducing the amino acid content in the tissue. The total free amino acid concentration in fat body of control larvae was 8.91 mg/ml and due to the treatment with *C. infortunatum*, it was reduced significantly from 8.40 mg/ml to 2.84 mg/ml with corresponding increasing concentrations (Table VI.6). There occurred 5.72% of reduction at 1% treatment and it further showed 68.13% of reduction at 5% treatment. The treatment of *C. odorata* also reduced the amino acid content in fat body from 7.83 mg/ml to 1.90 mg/ml corresponding to different concentrations of treatment. The maximum percentage reduction of 78.68% was noticed at 5%

treatment and the minimum of 12.12% was found in 1% treated tissues. (Table VI.8)

In the case of haemolymph too, drastic decline in amino acid content was noticed. The amount of amino acid in control larvae was found to be 48.88 mg/ml and it decreased from 47.12 mg/ml to 27.33 mg/ml with increasing concentration of *C. infortunatum* (Table VI.7). At the lower concentration of 1% there observed a reduction of 3.60% and at the higher concentration of 5%, 44.09% of reduction occurred (Table VI.8). Similarly in the case of *C. odorata*, the increase in concentration of botanicals drastically reduced the amino acid from 42.48 mg/ml to 16.81mg/ml and the percentage of reduction was 13.09% at 1% treatment and 65.61% at 5% treatment (Table VI.8).

All the results prominently showed the efficacy of both botanicals to decrease the amino acid concentration in midgut, fat body and haemolymph of larvae. The percentage of reduction was gradually increased with increasing the botanical concentration from 1% to 5% in all tissues. The minimum effect of botanical was noticed at 1% of treatment and the maximum effect was caused at 5%. In all tissue treatments, *C. odorata* possessed greater potency in reducing the amino acid content than that of *C. infortunatum*.

Table VI.5. Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odarata* on free amino acid concentration in midgut tissue of sixth instar larvae of *Orthaga exvinacea.*

Botanicals	Treatments	Concentration of amino acid		
		mg/ml	mg/gm	mg/larva
Control		7.37±0.128	63.30±0.320	2.46±0.043
C. infortunatum	**1%**	6.44±0.151	60.23±0.477	2.15±0.051
	2%	6.06±0.127	56.72±0.496	2.02±0.042
	3%	4.99±0.117	46.43±0.409	1.66±0.039
	4%	3.40±0.119	36.32±1.604	1.13±0.040
	5%	2.79±0.083	29.08±0.565	0.93±0.028
	F value	1064.558	1573.230	1063.135
C. odorata	**1%**	5.40±0.205	49.37±0.340	1.80±0.068
	2%	4.36±0.091	45.70±0.435	1.45±0.030
	3%	2.95±0.036	32.70±0.558	0.98±0.012
	4%	1.79±0.021	19.69±0.334	0.59±0.007
	5%	1.31±0.030	15.03±0.45	0.44±0.010
	F value	2298.430	9978.586	2299.511

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Free amino acid content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VI.6 Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on free amino acid concentration in fat body of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of amino acid		
		mg/ml	mg/gm	mg/larva
Control		8.91±0.090	98.96±0.434	2.97±0.030
C. infortunatum	**1%**	8.40±0.122	97.19±0.620	2.80±0.041
	2%	7.51±0.256	90.92±0.670	2.50±0.086
	3%	5.41±0.152	71.25±1.79	1.80±0.051
	4%	4.42±0.144	65.53±0.657	1.47±0.048
	5%	2.84±0.090	42.98±0.690	0.94±0.030
	F value	1232.186	2807.171	1228.723
C. odorata	**1%**	7.83±0.140	92.35±0.373	2.61±0.047
	2%	6.86±0.162	81.15±0.824	2.29±0.054
	3%	6.14±0.056	77.32±0.543	2.05±0.019
	4%	5.10±0.395	70.18±1.386	1.70±0.131
	5%	1.90±0.082	29.09±1.304	0.63±0.027
	F value	822.543	3714.653	823.983

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Free amino acid content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VI.7 Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on free amino acid concentration in haemolymph of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of amino acid	
		mg/ ml	mg/ larva
Control		48.88±0.309	6.11±0.039
C. infortunatum	**1%**	47.12±0.524	5.83±0.138
	2%	41.26±0.340	5.16±0.042
	3%	37.15±0.297	4.64±0.037
	4%	31.72±0.341	3.96±0.043
	5%	27.33±0.703	3.42±0.088
	F value	1828.422	994.239
C. odorata	**1%**	42.48±0.229	5.31±0.029
	2%	36.29±0.246	4.54±0.031
	3%	32.23±0.497	4.10±0.204
	4%	24.62±0.689	3.08±0.085
	5%	16.81±0.627	2.10±0.078
	F value	3100.318	1093.764

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Free amino acid content was expressed in the units of mg/ml and mg/larva.

Table VI.8 Percentage reduction of free amino acid concentration in different tissues of *Orthaga exvinacea*.

Botanicals	Treatments	Percentage reduction in free amino acid content		
		Midgut (%)	Fat body (%)	Haemolymph (%)
C. infortunatum	**1%**	12.62	5.72	3.60
	2%	17.77	15.71	15.59
	3%	32.29	39.28	24.00
	4%	53.87	50.39	35.11
	5%	62.14	68.13	44.09
C. odorata	**1%**	26.73	12.12	13.09
	2%	40.84	23.01	25.76
	3%	59.97	31.09	34.06
	4%	75.71	42.76	49.63
	5%	82.22	78.68	65.61

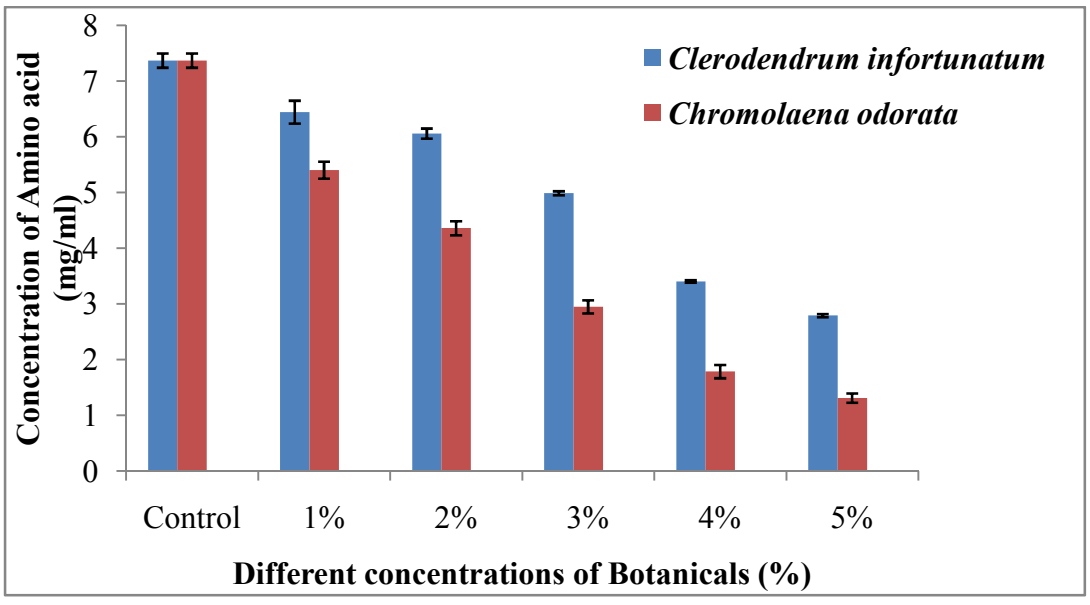


Figure VI.4 Graph showing the decrease in free amino acid content in the midgut tissue with increase in concentrations of botanicals.

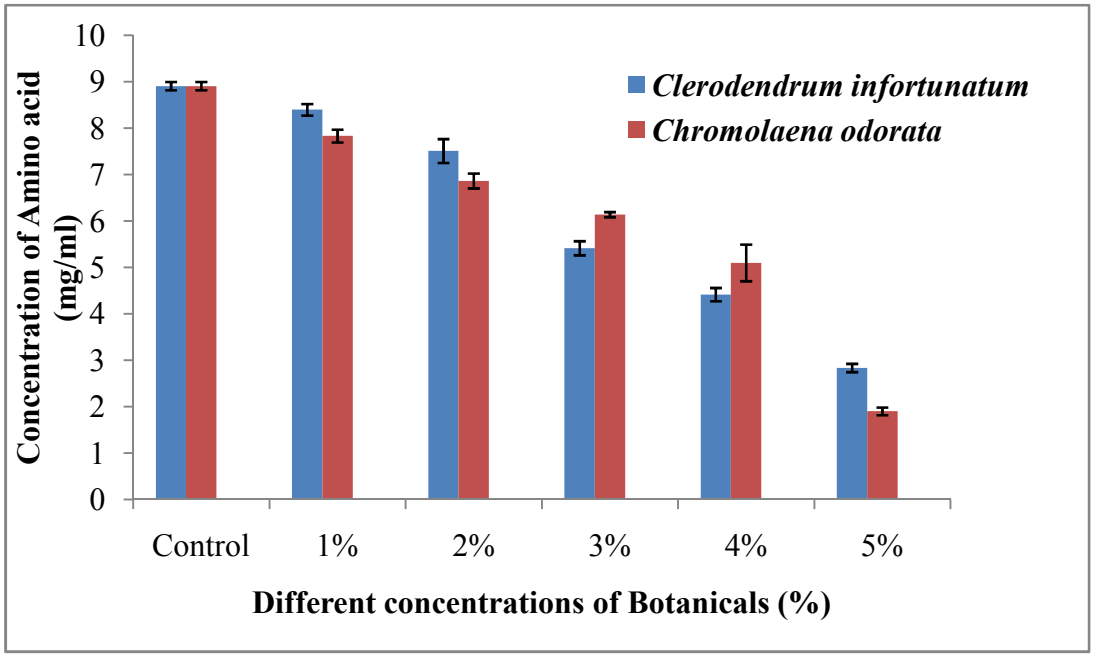


Figure VI.5 Graph showing the decrease in free amino acid content in the fat body with increase in concentrations of botanicals.

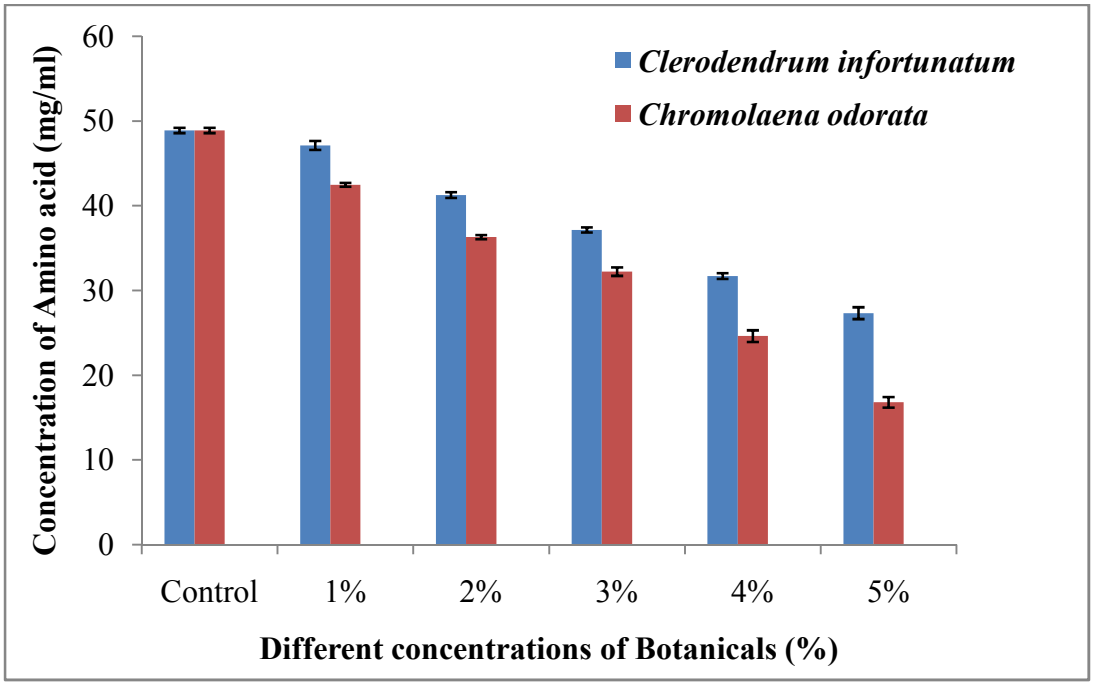


Figure VI.6 Graph showing the decrease in free amino acid content in the haemolymph with increase in concentrations of botanicals.

6.4 Discussion

The results of present investigation revealed the potency of both leaf extracts to reduce the total protein, free amino acids and to alter the protein profile of all tissues and also these results emphasize the toxicity of phytochemicals present in both botanicals to interfere the physiology of the larvae. Many comparable research works were reported with similar results and those findings can be correlated with this study.

Proteins are the important biological molecules that play an inevitable role in insect growth, development and various other physiological processes (Sahayaraj and Kombiah, 2010). The protein content in an insect is dependent upon its synthesis, breakdown, water movement between tissues and haemolymph, so the reduction of protein content in larvae might be due to the reduction in synthesis of protein or increase in breakdown to detoxify the active principles present in the plant extracts (Vijayaraghavan *et al*., 2010). Similar studies were carried out by Kiran Morya *et al.* (2010) and observed that the leaf extracts of *Lantana camara* reduced the protein content in *Corcyra cephalonica*.

Reduction of protein in *Crocidolomia binotalis* might be caused by the toxic principles present in the plant extracts (Vijayaraghavan *et al*., 2010). The volatile oils from *Cymbopogon citratus* and *Rosmarinus officinalis* decreased the protein content in *Musca stabulan* and also induced biochemical disturbances (Khalaf, 1998). Medhini *et al*. (2012) reported significant reduction of total protein content in midgut and haemolymph of *S. litura* larvae treated with leaf and flower extracts of *Calendula officinalis* in various solvents.

Many more investigations were reported on the reduction of protein in fat body and haemolymph of insects due to botanical effects. Schmidt *et al.*

(1998) reported the reduced amount of haemolymph protein in both *Spodoptera littoralis* and *Agrotis ipsilon* after the treatment of 6 days exposure to methanolic extract of *Melia azedarach* at 100 ppm and similar result was reported in the haemolymph protein concentration of *S. litura* treated with azadirachtin (Li *et al.*, 1995). Jadhav and Ghule (2003) evaluated the potency of azadirachtin on *C. cephalonica* through the diet incorporation and observed the decreased concentration of protein in the total body and haemolymph of the insect. The decrease in protein content might also be due to the mechanism of lipoprotein formation which is used to repair damaged cells and tissue organelles. Under stress condition an insect requires higher metabolic energy and this energy demand may have led to the protein catabolism to detoxify the toxic principles present in the *C. infortunatum* and *C. odorata*, but the lesser consumption of food for synthesis of protein might also be the reason for the reduction of protein.

The changes in protein profile showed in all results indicated that the botanicals influenced the different protein expressions of midgut, fat body and haemolymph of *O. exvinacea*. Comparable results were reported by many researchers regarding the toxic effects of phytochemical on the protein profiling of different insect tissues. The active principles present in both botanicals might have altered the protein profile and might have induced to appear new polypeptides or to disappear the existing ones. The impact of neem gold on *Cosmopolites sordidus* altered the normal physiological activity and induced the appearance of new polypeptides (Sahayaraj and Kombiah, 2010). The effect of *A. squamosa* seed extract on fourth instar of *S. litura* exhibited significant reduction in protein content and created conspicuous variations in electrophoretic banding pattern of larvae (Boreddy *et al*., 2000).

The electrophoretic studies on the effect of azadirachtin on *S. litura* showed an altered protein profile of larvae with affected 10 protein bands

(Huang *et al*., 2004). The structural deformities and diminution of protein profile were reported in *Anopheles* larvae after the methanolic treatment of *A. indica* (Sharma *et al.,* 2006 a).The effect of azadirachtin on protein profile of *Ostrinia furnacalis* showed significantly affected six proteins bands (Huang *et al*., 2007). The appearance and disappearance of protein bands were noticed in the midgut protein profile of *A. gemmatalis* after the treatment with the aqueous seed extracts of *Koelreuteria paniculata* (Martins *et al*., 2012). In *H. armigera,* the azadiractin treatment caused modified electrophoretic banding patterns of cuticular proteins (Rajkumar and Subrahmanyam, 2000).

The possible reason for the appearance and disappearance of polypeptides in protein profile may be due to the alteration occurred at molecular level of larvae. Al-Qahtani *et al.* (2012) reported that the treatment of some dried plants created transcriptional changes in *Oryzaphilus surinamensis* to develop resistance or detoxification mechanism. Hence any foreign particle interacting with the cellular metabolism created stress in the cell. It may either up-regulate or down-regulate gene expression. Changes that showed in the protein profile indicated that the treatment with both botanicals might have made changes at the genetic level. The disappearance of protein bands at higher concentration might be due to the phytochemiclas present in the botanicals which down- regulate some gene expressions. The protein patterns of different tissue of the treated larvae showed appearance of many new small proteins which are smaller in sizes. The small sized protein might be new peptides formed or it might be the peptides which are formed by the breakdown due to the action of protease. The present study suggest that the active components present in both botanicals might be the cause for the reduction of protein content as well as for the alterations in the protein profile of the mid gut tissue of the treated larvae.

The quantitative changes of amino acids in different tissues indicated the physiological status of organism. The amino acid content mainly depended upon the protein synthesis and osmotic stress caused by the larvae. Many research works illustrated the changes of amino acid concentration in different tissues in relation to different botanical treatment. Jadhav (2009) reported the reduced free amino acid content in *C. cephalonica* with the treatment of azadirachtin for 24 hrs, but increased after 48 hrs, 72 hrs and 96 hrs due to either protein breakdown or inhibition of amino acid incorporation into proteins. Similarly the treatment of botanicals caused the reduction of certain amino acid in *A. stephensi* larvae (Senthilkumar *et al*., 2009).

The decreased amount in amino acid and protein were observed in *Dysdercus koenigii* treated with the *Annona* extracts (Reddy *et al*., 1993) and similar effect was noticed in *An. Stephensi* larvae treated with *A. squamosa* extracts. The treatment of *H. suaveolens* and *V. negundo* created severe reduction in protein and amino acid content in the larvae of *O. exvinacea* (Ranjini and Ranjini, 2016).

Many scientific studies explained the comparable justification for the reduction of amino acid in insect due to botanical effect. Under toxic condition the increased rate of protein catabolism has been reported in *Spodoptera litura*, because insect degrades protein to resultant amino acids in order to let them enter into the TCA cycle as keto acid for compensation for the lower energy caused by stress and also to detoxify the active principles present in the plant extract (Medhini *et al*., 2012). According to Etebari *et al.* (2005) the serious protein reduction in tissues may constitute a physiological mechanism that could play a major role in compensatory process for insecticidal stress to provide intermediates to the Kreb cycle by retaining free amino acids in haemolymph.

Amino acids play a vital role in moulting process of an insect (Pandey *et al*., 1986) so the reduction may cause serious impacts in physiology as well as moulting process inducing morphological abnormalities in treated insects (Chen, 1966). Plant derived compounds have the efficiency to interfere the stability of various chemical process in the body of insects, this alteration may lead to serious metabolic disorders in insect and cause gradual reduction in their activity or death.

CHAPTER VII

EFFECT OF BOTANICALS ON THE CARBOHYDRATE CONCENTRATION IN DIFFERENT TISSUES

7.1 Introduction

Carbohydrates, the complex organic constituents are the major source of energy in living organisms and their breakdown facilitates the liberation of the energy required for many biological activities. The different forms of carbohydrates serve for the storage of energy and its usage is directly related to the physiological work of an organisms. The relationship between physiological activities and carbohydrate is particularly important one because the energy required for the physiological work is provided by energy reserves that are oxidized at rate prescribed by the amount of work being done. Hence the carbohydrate is inevitable biochemical parameter to study the physiological condition of an organism.

Generally, the glycogen and triglyceride are the major energy sources in animal and also serve as structural components like cellulose in plants and chitin in arthropods. In insects, carbohydrates are extremely important as a major energy source, act as 'identification tags' in cellular recognition, have role in protein translocation, in cold stress metabolism and also played as a major component in chitin synthesis. Normally, in the midgut of insect glucose contained in food is absorbed and converted to glycogen in the fat body and stored.

Fat body plays an important role in the carbohydrate metabolism because it is the major site of trehalose and glycogen synthesis. Only small amount of glucose absorbed from food is oxidized and utilized directly for

metabolic activity or flight while most of the remaining sugars are converted to glycogen and trehalose by the fat body. One of the functions of fat body is to maintain a relatively constant level of glucose in the haemolymph. The conversion of carbohydrates to fat was reported when there was too much reserved glycogen in the fat body and the conversion of fats into compounds suitable for oxidation during starvation in the fat body has been reported in insects (Downer, 1985; Steele, 1985; Wheeler, 1989).

Insect's haemolymph is characterized by the presence of the non-reducing disaccharide trehalose and also have the unique feature like very high concentration of trehalose compared with that in vertebrates and many other invertebrates. Especially the lepidopteran insects maintain relatively higher amounts of blood sugar when compared with other insects (Roeder, 1953; Ranjini and Mohamed, 2004 b). Insects have to spend energy for their biological needs, and if they are under starved condition, they can live on reserves accumulated in periods of food abundance but under stress condition, intensive utilization of reserve energy lead to mortality of insect. Hence the quantitative estimation of carbohydrate in different tissues may help to predict the physiological state of insect under stress condition.

This present investigation deals with the studies on the effect of both botanicals on quantitative estimation of carbohydrate content in the midgut, fat body and haemolymph of sixth instar larvae of *Orthaga exvinacea*.

7.2 Materials and methods

The materials and methods for the quantitative estimation of carbohydrate were described in section 3.2.6.4

7.3 Results

The results of quantitative analysis of carbohydrate content in midgut, fat body and haemolymph of *O. exvinacea* treated with *C. infortunatum* and *C. odorata* showed significant changes with increasing botanical concentrations. The data obtained from the quantitative estimation of carbohydrate in all tissues were statistically analyzed and the differences in the mean concentrations of carbohydrate between untreated and treated tissues were found to be highly significant. The significant changes in carbohydrate content in different tissues were presented in Table VII.1 to VII.3 and Figure VII.1 to VII.3 and the percentage reduction of carbohydrate was also given in Table VII.4.

7.3.1 The effect of botanicals on the carbohydrate concentration in different tissues.

The treatment of botanicals reduced the carbohydrate content in midgut tissue with increasing the botanical concentration from 1% to 5% of both leaf extracts (Table VII.1). The amount of carbohydrate content in untreated midgut tissue was 1.78 mg/ml and it progressively decreased with the treatment of *C. infortunatum* and found to be decreased from 1.69 mg/ml to 0.48 mg/ml (Table VII.1). The percentage reduction of carbohydrate content was increased with the treatment of *C. infortunatum* from 5.06% at 1% to 73.03% at 5% (Table VII.4). Similarly in the case of *C. odorata*, the carbohydrate concentration was decreased from 1.53 mg/ml to 0.23 mg/ml with the increasing botanical concentration from 1% to 5% (Table VII.1). The maximum percentage reduction of 87.08% in carbohydrate content was found at 5% treatment and the minimum reduction of 14.04% was noticed at 1% treatment (Table VII.4). Among the botanicals tested, *C. odorata* showed the maximum efficacy in reducing carbohydrate content in midgut tissue of sixth instar larvae of *O. exvinacea* (Figure VII.1).

In the case of fat body, the carbohydrate concentration was found to be significantly lower in treated larvae than in the control (1.97 mg/ml) (Table VII.2). 1% to 5% treatment of *C. infortunatum* was found to be decreasing the carbohydrate content from 1.60 mg/ml to 0.37 mg/ml in fat body (Table VII.2). The carbohydrate concentration at 1% treatment showed 18.78% of reduction which was gradually changed with increasing botanical concentration and reached 81.22% of reduction at 5% of *C. infortunatum* (Table VII.4). Whereas for *C. odorata* treatment, the carbohydrate concentration was gradually declined from 1.40 mg/ml to 0.18 mg/ml with the treatment of five different concentrations (1% to 5%) (Table VII.2). The treatment of 1% *C. odorata* reduced 28.93% of carbohydrate concentration in fat body and the percentage of reduction gradually increased and reached to 90.86% at 5% concentration (Table VII.4). In the case of fat body too, *C. odorata* possessed the maximum potency in reducing carbohydrate content than that of *C. infortunatum* (Figure VII.2).

The treatment with botanicals has also influenced the carbohydrate concentration in haemolymph of the larvae. The carbohydrate content in haemolymph of control larvae was found to be 14.62 mg/ml and it reduced from 12.75 mg/ml to 3.31 mg/ml with increasing concentrations of *C. infortunatum* from 1% to 5% (Table VII.3). Initially 12.79% of reduction in carbohydrate content was noticed at 1% treatment and it gradually increased with increasing the concentration of treatment and reached 77.36% at 5% of treatment (Table VII.4). A drastic decline in carbohydrate content was observed in the case of *C. odorata* treatment (Table VII.3). The carbohydrate concentration of haemolymph was reduced from 10.34 mg/ml to 1.18 mg/ml according to the increase in the treatment concentration from 1% to 5% and the maximum of 91.93% reduction in carbohydrate content was

noticed at 5% and the minimum of 29.27% reduction was observed at 1% treatment (Table VII.4). Among the botanicals tested, *C. odorata* showed the greater efficacy in reducing carbohydrate concentration in haemolymph than *C. infortunatum* (Figure VII.3).

In all the tissue treatment, the common effect of both botanicals observed was that the gradual reduction in carbohydrate content with increasing botanical concentrations and the minimum reduction was found at 1% treatment and the maximum was at 5% treatment. Comparison of results obtained from quantitative estimation of carbohydrate concentration revealed that *C. odorata* leaf extract possessed the maximum potency to reduce the carbohydrate concentration in all tissues of sixth instar larvae of *O. exvinacea* than that of *C. infortunatum* extract.

Table VII.1. Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on carbohydrate concentration in midgut tissue of sixth instar larvae of *Orthaga exvinacea.*

Botanicals	Treatments	Concentration of carbohydrate		
		mg/ml	mg/gm	mg/larva
Control		1.78±0.004	17.33±0.159	0.59±0.006
C. infortunatum	**1%**	1.69±0.008	16.70±0.156	0.56±0.002
	2%	1.59±0.034	16.09±0.095	0.53±0.012
	3%	1.44±0.017	14.72±0.153	0.48±0.006
	4%	0.87±.0131	9.32±0.340	0.29±0.004
	5%	0.48±0.012	5.54±0.192	0.16±0.004
	F value	4255.530	2999.695	4166.363
C. odorata	**1%**	1.53±0.010	15.63±0.670	0.51±0.003
	2%	1.27±0.011	13.77±0.123	0.42±.004
	3%	0.77±0.016	8.45±0.214	0.26±0.005
	4%	0.53±0.010	5.95±0.190	0.18±0.004
	5%	0.23±0.008	2.64±0.115	0.08±0.003
	F value	16268.57	1747.16	15728.11

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Carbohydrate content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VII.2. Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on carbohydrate concentration in fat body of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of carbohydrate		
		mg/ml	mg/gm	mg/larva
Control		1.97±0.00624	25.39±0.441	0.6578±0.002
C. infortunatum	**1%**	1.60±0.012	20.76±0.544	0.53±0.004
	2%	1.31±0.012	17.12±0.206	0.4354±0.004
	3%	0.88±0.011	13.24±0.272	0.30±0.004
	4%	0.57±0.004	10.26±0.361	0.20±0.001
	5%	0.37±0.010	6.35±0.305	0.12±0.004
	F value	20224.78	1767.63	19610.99
C. odorata	**1%**	1.40±0.012	18.59±0.623	0.46±0.004
	2%	1.04±0.009	15.24±0.333	0.35±0.003
	3%	0.78±0.007	13.98±0.513	0.26±0.003
	4%	0.50±0.018	8.88±0.331	0.17±0.006
	5%	0.18±0.014	3.20±0.370	0.06±0.004
	F value	14875.44	1472.13	14866.48

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Carbohydrate content was expressed in the units of mg/ml, mg/gm and mg/larva.

Table VII.3. Efficacy of leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on carbohydrate concentration in haemolymph of sixth instar larvae of *Orthaga exvinacea*.

Botanicals	Treatments	Concentration of carbohydrate	
		mg/ ml	mg/ larva
Control		14.62±0.105	1.83±0.013
C. infortunatum	**1%**	12.75±0.132	1.59±0.016
	2%	10.81±0.074	1.35±0.009
	3%	7.60±0.281	0.95±0.035
	4%	4.54±0.181	0.57±0.023
	5%	3.31±0.031	0.41±0.004
	F value	4225.16	4218.87
C. odorata	**1%**	10.34±0.076	1.29±0.010
	2%	6.74±0.156	1.03±0.022
	3%	8.28±0.175	0.84±0.020
	4%	3.88±0.140	0.48±0.017
	5%	1.18±0.073	0.15±0.010
	F value	7067.89	7003.41

The values are expressed as means of five replicates for each concentration with standard deviation (Mean ± standard deviation). All values were found to be highly significant ($P<0.01$). Carbohydrate content was expressed in the units of mg/ml and mg/larva.

Table VII.4. Percentage reduction of carbohydrate concentration in different tissues of *Orthaga exvinacea.*

Botanicals	Treatments	Percentage reduction in carbohydrate content		
		Midgut (%)	Fat body (%)	Haemolymph (%)
C. infortunatum	1%	5.06	18.78	12.79
	2%	10.67	33.50	26.06
	3%	19.10	55.33	48.02
	4%	51.12	71.07	68.95
	5%	73.03	81.22	77.36
C. odorata	1%	14.04	28.93	29.27
	2%	28.65	47.21	53.90
	3%	56.74	60.41	43.36
	4%	70.22	74.62	73.46
	5%	87.08	90.86	91.93

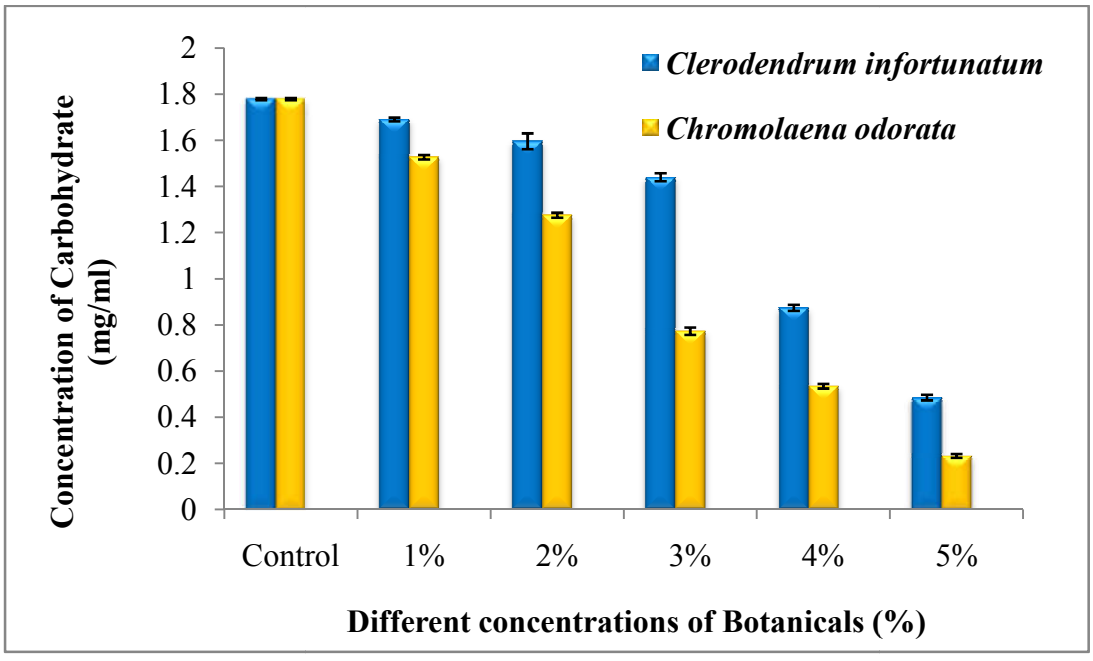


Figure VII.1.Graph showing the decrease in carbohydrate concentration in the midgut tissue with increase in concentrations of botanicals.

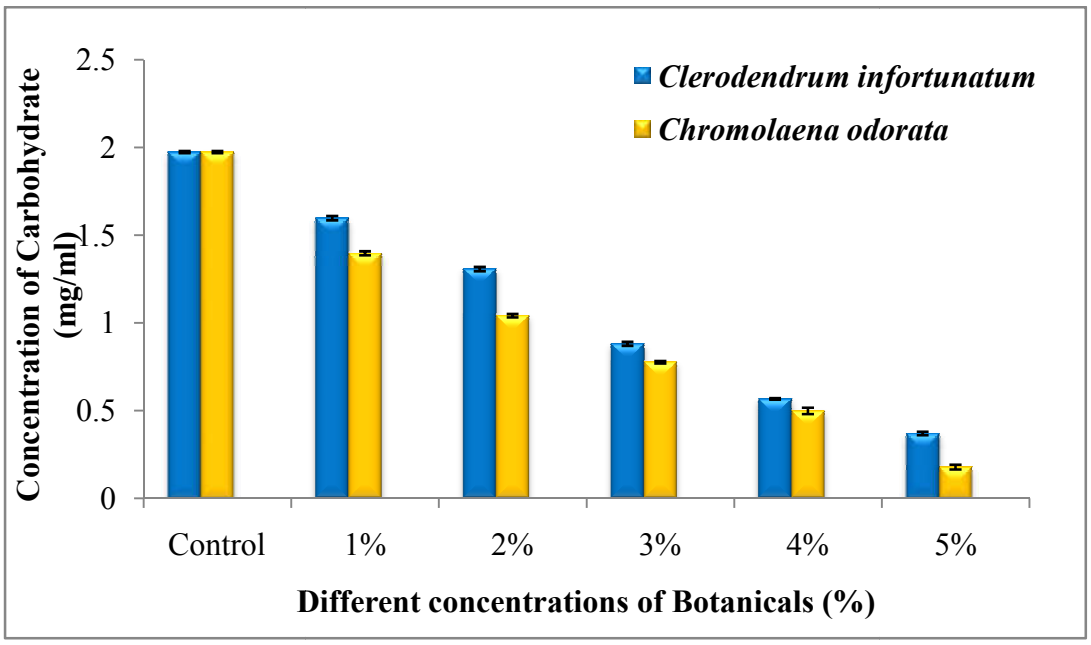


Figure VII.2. Graph showing the decrease in carbohydrate concentration in the fat body with increase in concentrations of botanicals.

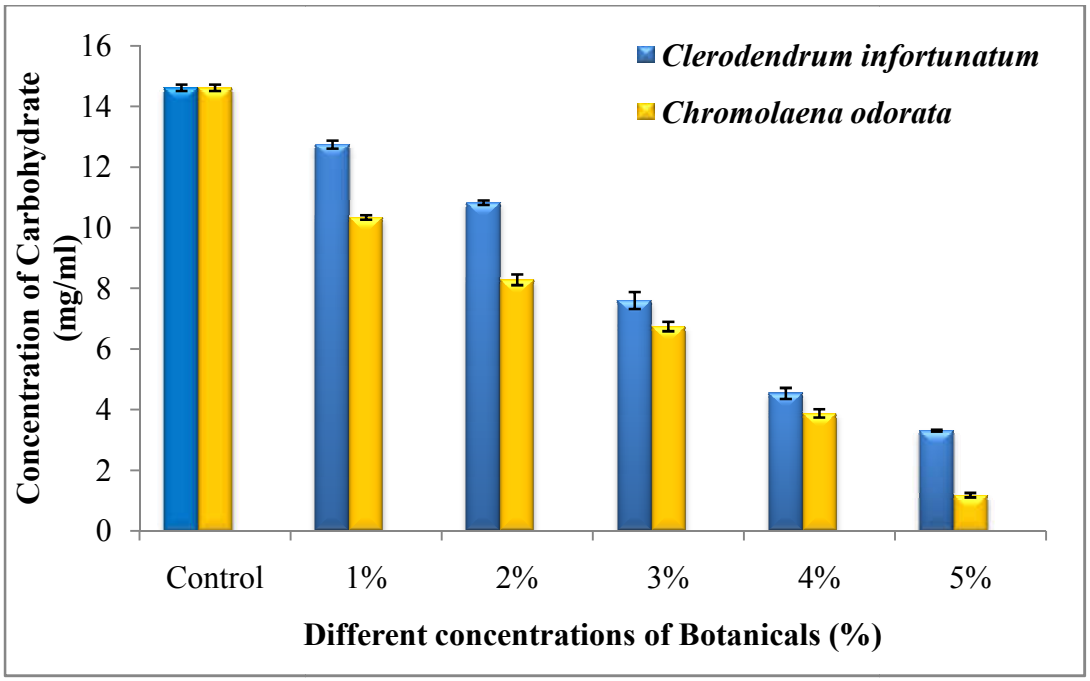


Figure VII.3.Graph showing the decrease in carbohydrate concentration in the haemolymph with increase in concentrations of botanicals.

7.4 Discussion

The results of quantitative studies of carbohydrate content in different tissues revealed the potency of both leaf extracts to reduce the carbohydrate concentration in all tissues. Several comparable research findings were reported with similar results. Sharma *et al*. (2011) investigated on the effect of petroleum ether extract of *Artemisia annua* and methanolic extract of *Azadirachta indica* on the *Culex quinquefasciatus* larvae and found reduced glucose level in larval tissue after 24 hrs of exposure. Severe decrease in carbohydrate content was observed in *Crysoperla carnea* female at higher concentration of neem oil treatment and it may be due to the antifeedant property expressed by the phytochemicals present in it (Abdul Razak and Sivasubramanian, 2007).

The secondary metabolites in leaf extracts were responsible for the antifeedant activity that may lead to serious digestive disorders and reduction in the carbohydrate content in different tissues of *O. exvinacea*. Acheuk and Mitiche (2013) proved the efficiency of plant alkaloids in *Pergularia tomentosa* in reducing both protein and carbohydrate contents of *Locusta migratoria* fifth instar nymph. According to Sak *et al*. (2006) the glycogen levels in juveniles and adults of *Pimpala turionellae* tend to decrease significantly when exposed to cypermethrin (Synthetic pyrethroid).

Insect midgut is the major site of digestion and absorption, so the intake of toxic food materials may disrupt the absorptive cells and adversely affect nutrient absorption system and it may be the cause for the severe reduction of carbohydrate content in tissues. Similar results were found in *Spodoptera litura* larvae treated with neem extract and it was reported that mode of action of phytoextracts can block the alimentary canal and it lead to the inhibition of feeding thereby lowering the glucose level in larvae (Koul, 1999). Similar reduction in total carbohydrate of the midgut tissue was

observed in *S. littoralis* upon treatment with extracts of *A. indica* and *Citrullus colocynthis* (Sayed *et al.,* 2011). The effect of neem kernel extract on the third instar larvae of *Anopheles stephensi* showed reduction of carbohydrate content and it was reported that reduction may be due to poor feeding and improper utilization of digested food by highly ruptured alimentary canal during the treatment (Sharma *et al*., 2006a). The significant reduction in carbohydrate content of *S. litura* larvae was noticed after the larvae were treated with *Calendula officinalis* extracts (Medhini *et al*., 2012).

Under toxic stress condition, the requirement of energy may increase that lead to catabolic activities of nutrients to meet the energy demand. To overcome the toxic condition through detoxification process, intensive utilization of carbohydrate reserve in fat body may occur and this may be the cause for decrease in carbohydrate content in fat body and haemolymph. According to Sharma *et al.* (2011) the significant reduction in glucose content was observed in mosquito larvae by the treatment of *A. indica* and suggested that it may be due to the utilization of stored glucose in tissues as a result of insecticidal stress. It was reported that under stress conditions, the absorbed nutrients get catabolized to overcome the high energy demand (Seyoum *et al.,* 2002) and finally, it may lead to the mortality of insect due to decreased energy metabolism (Etebari *et al*., 2006).

The reduction in carbohydrate content in haemolymph of *Plodia interpunctella* was reported due to the toxic impacts of two volatile oils from leaves and stems of *Piper cubeba* and *Salvia officinalis* and three fixed oils from *Rumex dentatus* and *Trigonella foenumgraecum* seeds and *Acacia nilotica* leaves (Shoukry *et al.*, 2003). Similar reduction in carbohydrate content in haemolymph was reported by Vijayaraghavan *et al.* (2010) in the larvae of *Crocidolomia binotalis* treated with extracts from *Lippia nodiflora, Vitex negundo* and *Strychnos nuxvomica*. The petroleum ether extract of

Ammi majus and *Apium graveolens* fed to 6th instar larvae of *Agrotis ipsilon* produced greatly reduced carbohydrate concentration in haemolymph of the larvae (Abo El-Ghar *et al.*, 1995). The effect of sub lethal concentrations of two volatile oils and three fixed oils on *Plodia interpunctella* larvae showed that all oil treatments declined the level of haemolymph carbohydrate content in larvae (Shoukry *et al.,* 2002 a, 2003).

Many researchers have reported comparable possibilities of carbohydrate reduction in insects due to the botanical effect. Fell *et al*. (1982), Rajendra (1990), and Shakoori and Saleem (1991) suggested that carbohydrates were converted to proteins in detoxification mechanism against toxicants that enter the animal body. Shoukry and Hussein (1998) observed similar findings with larvae of *Galleria mellonella* when they were treated with volatile oil of *Lantana camara* and *Vitex aganus-castus* plants. Hence the phytochemicals can directly or indirectly cause metabolic disorders in insects by creating biochemical changes that may lead to inactive or lethal state of insect.

CHAPTER VIII

INFLUENCE OF BOTANICALS ON THE ENZYME ACTIVITIES IN DIFFERENT TISSUES OF *ORTHAGA EXVINACEA*

8.1 Introduction

Enzymes are the globular proteins which act as catalysts for many biological reactions in living organisms. The biological processes that occur within all living organisms are chemical reactions and most of these are regulated by enzymes. Generally, enzymes catalyze all aspects of cell metabolism which includes the digestion of food, in which conversion of nutrient macromolecules (such as protein, carbohydrates and fats) are broken down into smaller molecules; conservation and transformation of energy and the construction of cellular macromolecules from smaller ones. In insects, a diverse group of enzymes are reported for fulfillment of many important chemical reactions. They are classified on the basis of their mode of catalyzing functions in different metabolic process. Many physio-chemical factors are influencing the activity of enzymes; they are mainly temperature and stress. Under imbalanced conditions, enzyme regulate biological processes by either changing their production concentration or increasing catalytic efficiency to overcome such situation. Hence the study on the activity of enzymes has more significance to understand the stress condition of an insect. The histopathological changes noticed in earlier chapter of the present research work also forced to think about the study on the activities of some selected digestive and stress related enzymes under botanical stress conditions.

The catalyzing activity of digestive enzymes for the successful absorption of food materials is a major process which is directly related to the

active growth and development of insect. The physiological conditions of insects are closely linked with the absorption in the alimentary canal. The pattern of digestive enzyme production in the alimentary canal show great variation among insects and depend mainly upon the food and feeding habits of the individuals. The character of the enzyme secretion in gut is dependent upon the nature of the meal that an insect can assimilate (Hubert *et al*., 1999; Agusti and Cohen, 2000; Torres and Boyd, 2009).

Generally, digestion in insects depends on activities of major enzymes including protease, amylases, glycosidases and lipases. Protease enzyme performs the proteolysis by protein catabolism or hydrolysis of peptide bonds and the enzyme amylase catalyses the hydrolysis of starch and other carbohydrates into sugars. According to the site of action, proteases are divided into exo- and endo-peptidases. Exo-peptidase removes amino acids from N- and C-terminals of peptide chain, known respectively as amino and carboxy peptidases (Terra and Ferreira, 2005). Endo-peptidases or proteinases are divided into several classes based on their active sites such as serine, cysteine and aspartic proteases (Terra and Ferreira, 2005). Serine proteases are also divided into three classes by their substrate specificity as trypsin, chymotrypsin and elastase.

Amylase is a major digestive enzyme and their activity is very essential for the active growth and development of insects and they improve insect digestive efficiency, help them to survive in diverse conditions and increase their fitness value (Kaur and Gupta, 2015). When the action of this enzyme is inhibited, insect nutrition is impaired, growth and development is retarded and eventually death occurs due to starvation. Hence the study on the activity of protease and amylase of *O. exviancea* is an inevitable factor that can indicate the state of stress caused by the botanical treatment.

In insect physiology, the cellular level of oxidative stress induces production of some detoxifying or antioxidant enzymes for its survival. Phosphatases are the major hydrolytic detoxifying enzymes and are involved in production of ATP. The phophatase enzyme includes acid phosphatase (ACP) and alkaline phosphatase (ALP) and both differ in their sub cellular distribution. ALP is a poly functional enzyme that found to be highly concentrated in plasma membrane and it hydrolyses a broad class of phosphomonoester substrates and acts as a transphosphorylase at alkaline condition whereas ACP is associated with lysosome and perform the hydrolysis of phosphate monoesters under acidic condition (Bai *et al*., 1993).

Generally, a normal level of Reactive Oxygen Species (ROS) is produced by all organisms as a result of metabolic processes under aerobic conditions. Oxygen is very essential for aerobic organisms for the production of efficient energy but paradoxically, produces chronic toxic stress in cells. Normally, the amount of ROS is counterbalanced by cellular antioxidant enzyme defense system, DNA repair and small molecular weight antioxidants (Tokarz *et al*., 2013). The antioxidant defense system protect insects by removing reactive oxygen species, which cause oxidative stress. As a result of oxidative stress, the production of reactive oxygen species exceeds the capacity of cellular antioxidant defenses to overcome these toxic species (Aging, 1956; Droge, 2002).

The antioxidant defense system of insect is primarily constituted by the enzymatic action of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), reduced glutathione (GSH) and glutathione reductase (GR) (Barbehenn, 2002; Meng *et al*., 2009). From this, superoxide dismutase is the major antioxidant enzyme that catalyzes the dismutation of the superoxide radical into oxygen (O_2) or hydrogen peroxide (H_2O_2). Hence the studies on the changes in the activity of ACP, ALP and SOD in the midgut

tissue, fat body and haemolymph of sixth instar larvae of *O. exvinacea* also help to analyze the toxic condition in the larvae under botanical stress.

8.2 Materials and methods

The materials and methods used for the enzyme assays were described in section 3.2.7. The methods for protease and amylase activity were explained in sections 3.2.7.1 and 3.2.7.2 and procedures for ACP, ALP and SOD were discussed in sections 3.2.7.3, 3.2.7.4 and 3.2.7.5 respectively.

8.3 Results

The higher concentration 5% of both botanicals was selected for the treatment and assayed the activities in different tissues at different hours of exposure (24 hrs, 48 hrs and 72 hrs). The effect of botanicals on the activities of both digestive and stress related enzymes were calculated and presented in tables VIII.1 and VIII.2 and the comparison of the effect of both botanicals were showed in figures VIII.1 to VIII.15.

8.3.1 Influence of botanicals on the activity of digestive enzymes (protease and amylase) in different tissues.

a. Digestive enzyme activity in midgut

Protease: - The influence of both botanicals on the activity of digestive enzymes in midgut showed significant variations in different hours of treatments (Table VIII.1). Reduced activity of protease and amylase was noticed in both botanical treatments. In control larvae, the specific activity of protease in midgut tissue was found to be slightly decreased in accordance with increased time of exposure and the activity noticed was 5.41 IU/mg at 24 hrs, 5.06 IU/mg at 48hrs and 4.99 IU/mg at 72hrs. The treatment with *C. infortunatum* decreased the specific activity at different time of exposure (24 hrs, 48 hrs and 72 hrs) and activity was found to be reduced to 3.72

IU/mg, 2.94 IU/mg and 2.27 IU/mg respectively. In the case of *C. odorata* treatment, sharp decline was observed in the activity of protease when compared to control and it was reduced into 2.93 IU/mg, 2.39 IU/mg and 1.95 IU/mg at 24 hrs, 48 hrs and 72 hrs respectively (Table VIII.1).

Amylase: - Normally, the specific activity of amylase in the midgut of untreated larvae showed slight changes with different exposure time. Reduced amylase activity was noticed in both botanical treatments. The specific amylase activity exhibited in the control larvae was 6.62 IU/mg at 24 hrs, 6.02IU/mg at 48hrs and 6.30 IU/mg at 72 hrs but after the treatment with *C. infortunatum*, activity was reduced to 5.52 IU/mg, 5.18 IU/mg and 4.62 IU/mg respectively (Table VIII.1). The treatment with *C. odorata* also reduced the amylase activity to 5.26 IU/mg, 4.84 IU/mg and 4.92 IU/mg with corresponding hours of treatment. Among the botanicals tested, *C. odorata* showed greater effect in reducing both protease and amylase activity in midgut tissue of *O. exvinacea* (Figure VIII.1 and VIII.4).

b. Digestive enzyme activity in fat body

Protease: - The influence of botanicals on the activities of protease and amylase in fat body was also found out. The specific activity of protease in fat body of control larvae was found to be`4.58 IU/mg, 4.52 IU/mg and 4.65 IU/mg at different hours of treatment (24 hrs, 48 hrs and 72 hrs) and this activity was further decreased to 3.18 IU/mg, 2.74 IU/mg and 2.78 IU/mg in accordance with the increasing time of exposure with the treatment of *C. infortunatum* (Table VIII.1). The treatment with *C. odorata* also decreased the protease activity in fat body of the larvae and the specific activity was found to be 2.70 IU/mg at 24 hrs, 2.50 IU/mg at 48 hrs and 2.26 IU/mg at 72 hrs (Table VIII.1).

Amylase: - Significant reduction was found in amylase activity of fat body with botanical treatments. The specific amylase activity in the fat body of untreated larvae was observed to be 5.47 IU/mg, 5.23 IU/mg and 5.33 IU/mg corresponding to the exposure time of 24 hrs, 48 hrs and 72 hrs respectively but after the treatment with *C. infortunatum* the activity was reduced to 4.62 IU/mg, 4.29 IU/mg and 3.83 IU/mg respectively (Table VIII.1). Similarly the treatment with *C. odorata* has also reduced the amylase activity to 3.54 IU/mg, 3.63 IU/mg and 3.42 IU/mg in accordance with increased time of exposure. Among the botanicals tested, *C. odorata* showed maximum influence in reducing both protease and amylase activities in fat body of *O. exvinacea* (Figure VIII.2 and VIII.5).

c. Digestive enzyme activity in haemolymph

Changes were also observed in the activities of digestive enzymes in haemolymph of the larvae with respect to different time of exposure. The specific protease activity exhibited in the haemolymph of the control larvae was 2.08 IU/ml at 24 hrs, 2.05 IU/ml at 48 hrs, 2.19 IU/ml at 72 hrs and this activity was gradually declined to 1.31IU/ml, 1.26 IU/ml and 1.06 IU/ml at different hours of treatment with *C. infortunatum*. In the case of *C. odorata*, the protease activity in the haemolymph of the larvae was also reduced to 1.15 IU/ml, 0.97 IU/ml and 0.81 IU/ml corresponding to the different hours of exposure (Table VIII.1).

Reduced amylase activity was noticed in both botanical treatments. In untreated haemolymph, the amylase activity was found to be 2.13 IU/ml at 24 hrs, 2.09 IU/ml at 48 hrs and 1.87 IU/ml at 72 hrs but after the treatment with *C. infortunatum*, activity was reduced to 1.57 IU/ml, 1.32 IU/ml and 1.08 IU/ml respectively. Due to treatment with *C. odorata*, the activity of amylase was declined to 1.42 IU/ml, 1.26 IU/ml and 0.65 IU/ml at different hours of exposure (24 hrs, 48 hrs and 72 hrs) (Table VIII.1). Among the botanicals tested, *C. odorata* showed greater effect in reducing both protease and amylase activity in haemolymph of the larvae (Figure VIII.3 and VIII.6).

TableVIII.1 Effect of botanicals on the digestive enzymes in different tissues exposed to different hours of treatment (activity expressed as unit of IU/mg protein for midgut and fat body and IU/ml protein for haemolymph).

Different tissues	Treatment groups	Protease			Amylase		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Midgut	Control	5.41±0.25	5.062±0.12	4.99±0.16	6.62±0.19	6.02±0.28	6.30±0.26
	C. infortunatum	3.72±0.16	2.94±0.13	2.27±0.22	5.52±0.13	5.18±0.12	4.62±0.092
	C. odoarata	2.93±0.05	2.39±0.36	1.95±0.11	5.26±0.09	4.84±0.41	4.92±0.09
	F value	260.82	183.06	485.25	129.60	20.87	139.65
Fat body	Control	4.58±0.21	4.52±0.073	4.65±0.05	5.47±0.05	5.23±0.06	5.33±0.07
	C. infortunatum	3.18±0.19	2.74±0.10	2.78±0.07	4.62±0.08	4.29±0.08	3.83±0.11
	C. odoarata	2.70±0.13	2.50±0.05	2.26±0.10	3.54±0.14	3.63±0.21	3.42±0.05
	F value	147.19	1046.35	1313.95	476.21	186.66	742.88
Haemolymph	Control	2.08±0.09	2.05±0.05	2.19±0.10	2.13±0.12	2.09±0.09	1.87±0.13
	C. infortunatum	1.31±0.06	1.26±0.14	1.06±0.09	1.57±0.15	1.32±0.02	1.08±0.11
	C. odoarata	1.15±0.14	0.97±0.08	0.81±0.07	1.42±0.06	1.26±0.05	0.65±0.06
	F value	117.70	155.81	349.16	53.58	310.63	181.64

Each value represent - Mean ± S.D. Significance level – $P<0.01$ = highly significant.

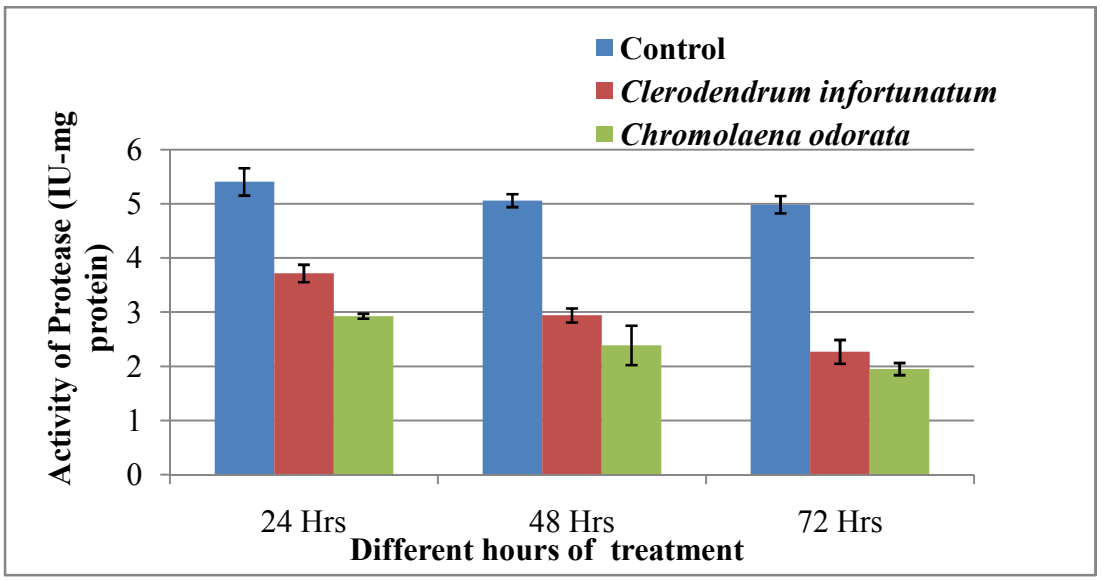


Figure VIII.1. Graph showing the reduced activity of protease in the midgut tissue with respect to different hours of treatment of botanicals.

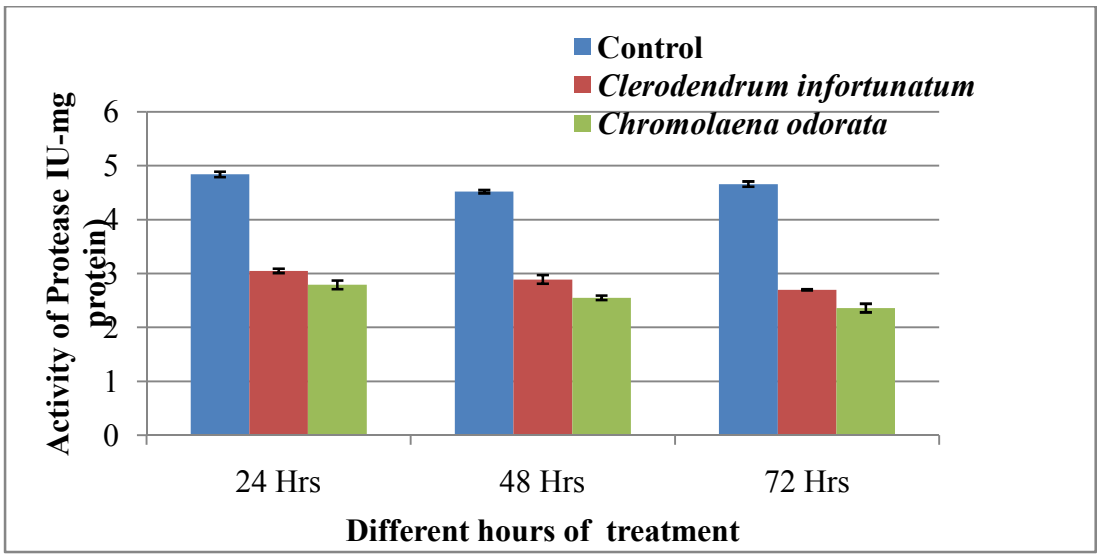


Figure VIII.2. Graph showing the reduced activity of protease in the fat body with respect to different hours of treatment of botanicals.

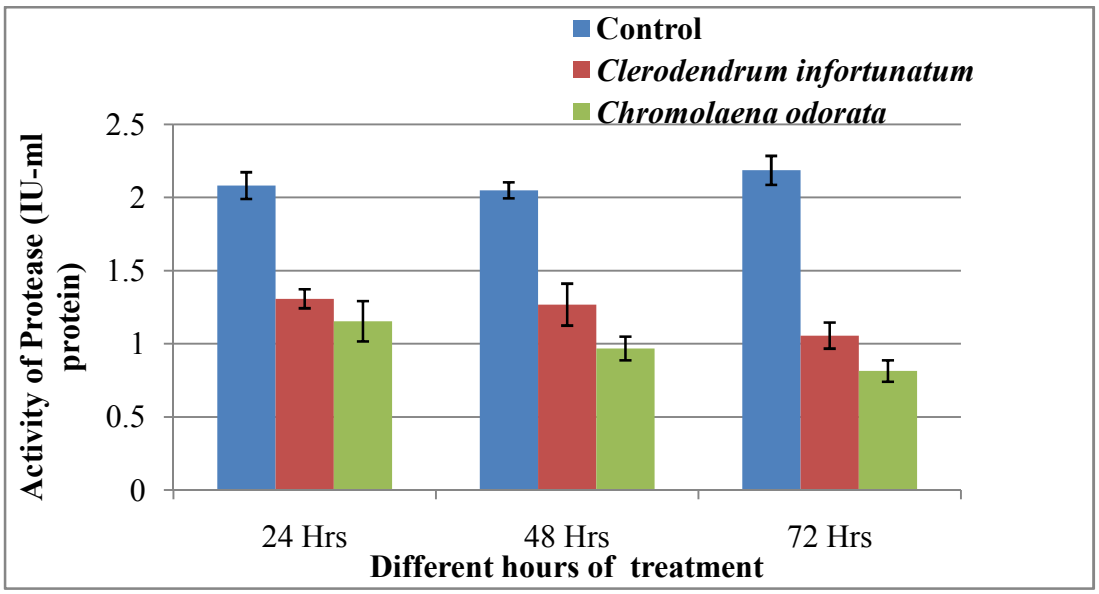


Figure VIII.3. Graph showing the reduced activity of protease in the haemolymph with respect to different hours of treatment of botanicals.

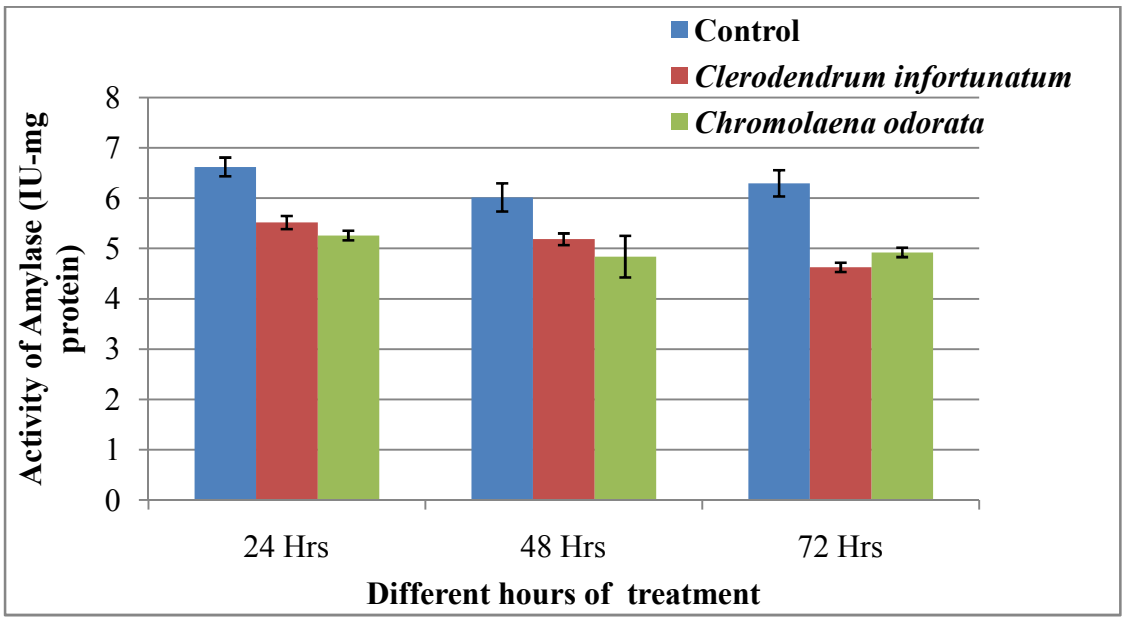


Figure VIII 4. Graph showing the reduced activity of amylase in the midgut with respect to different hours of treatment of botanicals.

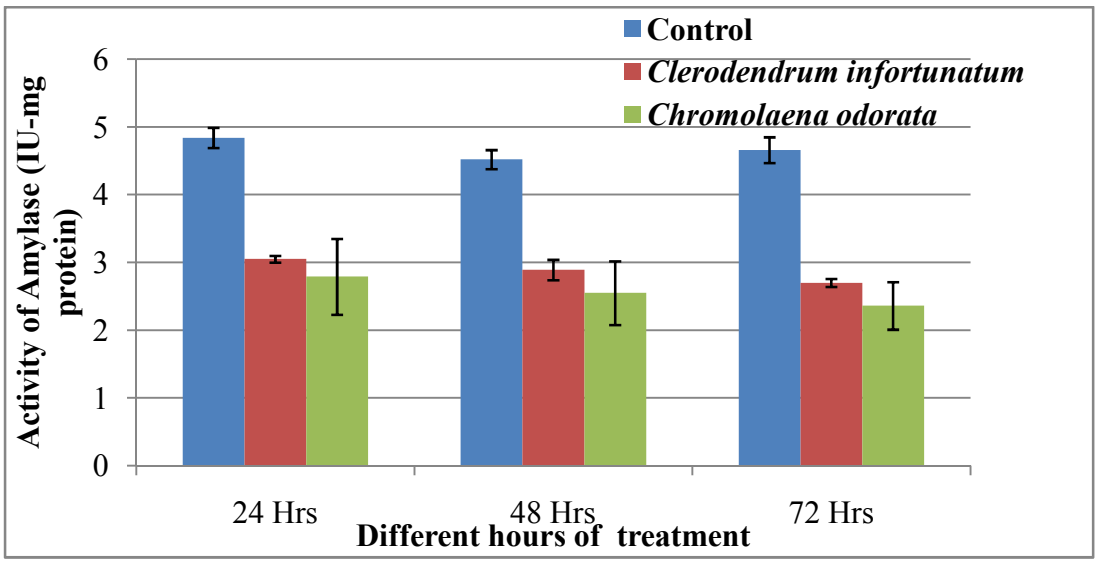


Figure VIII.5. Graph showing the reduced activity of amylase in the fat body with respect to different hours of treatment of botanicals.

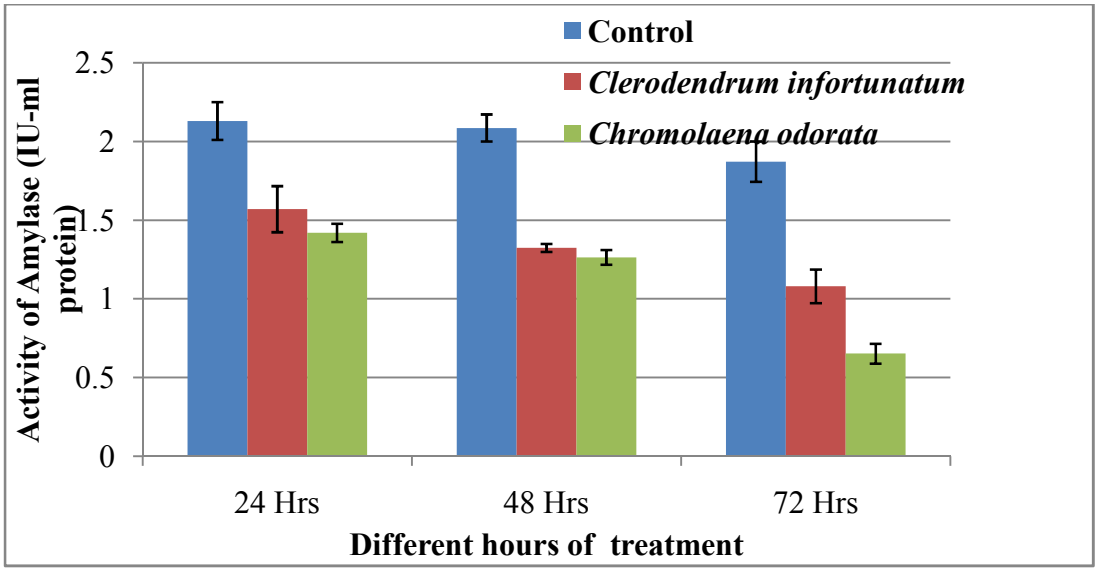


Figure VIII.6. Graph showing the reduced activity of amylase in the haemolymph with respect to different hours of treatment of botanicals.

8.3.2 Influence of botanicals on the activity of acid phosphatase, alkaline phosphatase and superoxide dismutase in different tissues of the larvae

a. Activity of stress related enzymes in midgut

ACP: - The botanical treatment has influenced the activities of stress related enzymes (ACP, ALP and SOD) present in larval midgut and exhibited significant changes with respect to different time of exposure (Table VIII.2). The specific activity of ACP in control larvae was found to be slightly decreased in its activity as 9.36 IU/mg, 9.27 IU/mg and 8.70 IU/mg in accordance with different exposure time of 24 hrs, 48 hrs and 72 hrs respectively. These values were increased to 9.67 IU/mg, 10.45 IU/mg and 11.35 IU/mg with *C. infortunatum* treatment related to different time of exposure (24 hrs, 48 hrs and 72 hrs) (Table VIII.2). But in the case of *C. odorata*, there occurred increased activity of ACP at 24 hrs and 48 hrs (13.20 IU/mg and 16.27 IU/mg) but later it decreased drastically at 72 hrs of exposure (3.48 IU/mg) (Table VIII.2).

ALP: - The specific ALP activity in the midgut of control larvae was found to be 15.04 IU/mg, 15.22 IU/mg and 14.83 IU/mg respectively with different time of exposure (24 hrs, 48 hrs and 72 hrs) (Table VIII.2). The ALP activity was increased to 17.53 IU/mg, 18.16 IU/mg and 20.20 IU/mg after different hours of treatment with *C. infortunatum*. A drastic increase in the activity of ALP was observed in the larval midgut of *C. odorata* treatment. The treatment increased the activity into19.60 IU/mg and 21.73 IU/mg at 24 hrs and 48 hrs respectively but later the activity gradually decreased at 72 hrs of treatment (10.81 IU/mg) (Table VIII.2).

SOD: - In untreated larvae, the specific activity of SOD was 42.19 IU/mg at 24 hrs, 41.59 IU/mg at 48 hrs and 41.19 IU/mg at 72 hrs but the treatment of

both botanicals caused severe increase in its activity in accordance with different hours of treatment (Table VIII.2). The *C. infortunatum* treated larval midgut showed the activity of 47.41 IU/mg, 51.95 IU/mg and 56.53 IU/mg corresponding to different time of exposure (24 hrs, 48 hrs and 72 hrs). Similarly, the treatment with *C. odorata* also caused severe increase in SOD activity and reached to 49.77 IU/mg, 67.76 IU/mg and 77.87 IU/mg with respect to increased exposure time (Table VIII.2).

Among the botanicals tested, *C. odorata* showed greater potency to influence the activities of ACP, ALP and SOD in the midgut tissue of *O. exvinacea* (Figure VIII.7, VIII.10 and VIII.13).

b. Activity of stress related enzymes in fat body

ACP: - Both leaf extracts also influenced the activity of stress related enzymes present in the fat body of larvae. The ACP activity in the fat body of control larvae was found to be 34.61 IU/mg, 34.89 IU/mg and 33.77 IU/mg and this activity was progressively increased to 36.44 IU/mg, 37.80 IU/mg and 41.45 IU/mg with increasing exposure time with *C. infortunatum* treatment (24 hrs, 48 hrs and 72 hrs)(Table VIII.2). In the case of *C. odorata* also a gradual increase in ACP activity was noticed at 24 hrs, 48 hrs and 72 hrs of exposure (43.58 IU/mg, 47.34 IU/mg and 49.22 IU/mg respectively)(Table VIII.2).

ALP: - The specific ALP activity in fat body of control larvae was found to be 12.54 IU/mg at 24 hrs, 12.09 IU/mg at 48 hrs and 12.04 IU/mg at 72 hrs and after treatment with *C. infortunatum* the activity increased to 13.82 IU/mg, 15.25 IU/mg and 15.99 IU/mg respectively (Table VIII.2). A drastic increase in the activity of ALP was observed in the case of *C. odorata* treatment and almost double of the value of control was noticed. The increased activity observed was 22.30 IU/mg, 23.54 IU/mg and 27.93 IU/mg

respectively at different time intervals (24 hrs, 48 hrs and 72 hrs) (Table VIII.2).

SOD: - The specific activity of SOD in fat body of control larvae was found to be 28.52 IU/mg, 28.38 IU/mg and 27.49 IU/mg with different time of exposure (24 hrs, 48 hrs and 72 hrs).The treatment of both botanicals caused severe increase in the activity of SOD in fat body. The larvae treated with *C. infortunatum* showed the activity of 31.12 IU/mg at 24 hrs, 33.18 IU/mg at 48 hrs and 36.86 IU/mg at 72 hrs. A gradual increase in the specific activity of SOD was noticed in the larvae treated with *C. odorata* and this increased activity was 34.58 IU/mg, 39.88 IU/mg and 42.08 IU/mg at different time of exposure (Table VIII.2).

The comparison of both botanical treatments showed that there was greater potency for *C. odorata* to influence the activities of ACP, ALP and SOD in the fat body of larvae than that of *C. infortunatum* (FigureVIII.8, VIII.11 and VIII.14).

c. Activity of stress related enzymes in haemolymph

ACP: - Effective changes were noticed in the activity of stress related enzymes (ACP, ALP and SOD) present in the haemolymph of larvae. The ACP activity in haemolymph of untreated larvae was observed as 5.12 IU/ml at 24 hrs, 5.06 IU/ml at 48 hrs and 5.45 IU/ml at 72 hrs and these values were gradually increased with increasing the exposure time of *C. infortunatum* (6.81 IU/ml at 24 hrs, 8.68 IU/ml at 48 hrs, and 9.62 IU/ml at 72hrs) (Table VIII.2). In the case of *C. odorata* treatment, a greater change in ACP activity was noticed with respect to different time of exposure. When compared to control, more than two folds of increase was observed at 24 hrs of exposure (13.29 IU/ml) and it further increased at 48 hrs (16.27 IU/ml) and almost five

folds of increase was noticed at 72 hrs (24.47 IU/ml) of treatment (Table VIII.2).

ALP: -The activity of ALP in haemolymph of the control larvae was found to be 8.35 IU/ml, 8.28 IU/ml and 7.70 IU/ml at the different hours of exposure (24 hrs, 48 hrs and 72 hrs). The activity of ALP was gradually increased with the treatment of *C. infortunatum* and showed 9.31 IU/ml, 11.74 IU/ml and 14.64 IU/ml activity in accordance with different exposure time (24 hrs, 48 hrs and 72 hrs)(Table VIII.2). The treatment of *C. odorata* also increased the activity of ALP in haemolymph of larvae and two folds of increase to that of control was observed in each different exposure time (15.24 IU/ml, 19.24 IU/ml and 21.66 IU/ml respectively) (Table VIII.2).

SOD: - SOD activity at different time intervals (24 hrs, 48 hrs and 72 hrs) in the haemolymph of control larvae was found to be 27.98 IU/ml, 28.27 IU/ml and 27.92 IU/ml respectively. The treatment of both botanicals caused increase in the activity of SOD in haemolymph (Table VIII.2). In the *C. infortunatum* treated larval haemolymph, there occurred slight increase in SOD activity at 24 hrs and 48 hrs (29.01 IU/ml and 29.96 IU/ml) but a little more increase was observed at 72 hrs of exposure (32.51 IU/ml). In the case of *C. odorata*, increase of SOD activity was progressed (31.67 IU/ml, 33.62 IU/ml and 36.20 IU/ml) with increasing exposure time (Table VIII.2).

Comparatively, *C. odorata* have greater potency to influence the activities of ACP, ALP and SOD in the haemolymph of larvae than that of *C. infortunatum* (Figure VIII.9, VIII.12 and VIII.15).

TableVIII.2 Effect of botanicals on the activity of stress related enzymes indifferent tissues of *Orthaga exvinacea* (activity expressed as unit of IU/mg protein for midgut and fat body and IU/ml protein for haemolymph).

Different tissues	Treatment groups	ACP			ALP			SOD		
		24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Midgut	Control	9.36±0.66	9.27±0.48	8.70±0.67	15.04±0.21	15.22±0.71	14.83±0.77	42.19±1.22	41.59±1.01	41.19±0.97
	C. infortunatum	9.67±0.73	10.45± 0.39	11.35±0.37	17.53±0.35	18.16±0.29	20.20±0.37	47.41±0.54	51.95±0.67	56.53±0.53
	C. odoarata	13.20±0.26	16.27±0.29	3.48±0.42	19.60±0.15	21.73±0.49	10.81±0.49	49.77±1.28	67.76±3.25	77.87±3.38
	F value	310.90	65.47	447.57	416.47	192.89	339.01	65.96	216.32	403.46
Fat body	Control	34.61±0.51	34.89±0.92	33.77±0.85	12.54±0.56	12.09±0.70	12.04±0.67	28.52±1.01	28.38±0.98	27.49±1.30
	C. infortunatum	36.44±0.87	37.80± 0.14	41.45±0.48	13.82±0.18	15.25±0.47	15.99±0.77	31.12±0.38	33.18±1.14	36.86±0.52
	C. odoarata	43.58±0.43	47.34±0.21	49.22±0.25	22.30±0.23	23.54±0.38	27.93±0.27	34.58±0.43	39.88±0.46	42.08±0.11
	F value	281.11	701.79	921.29	1052.46	608.96	916.88	103.73	200.70	412.68
Haemolymph	Control	5.12±0.87	5.06±0.71	5.45±0.84	8.35±0.89	8.28±0.79	7.70±1.074	27.98±0.18	28.27±0.48	27.92±0.54
	C. infortunatum	6.81±0.25	8.68±0.29	9.62±0.25	9.31±0.43	11.74±0.31	14.64±0.41	29.01±0.55	29.96±0.13	32.51±0.47
	C. odoarata	13.29±0.48	16.27±0.43	24.47±0.43	15.24±0.35	19.24±0.36	21.66±0.89	31.67±0.37	33.62±0.43	36.20±0.71
	F value	267.17	631.45	1565.67	186.67	546.47	345.45	116.04	257.71	252.94

Each value represent - Mean ± S.D. Significance level – $P<0.01$ = highly significant.

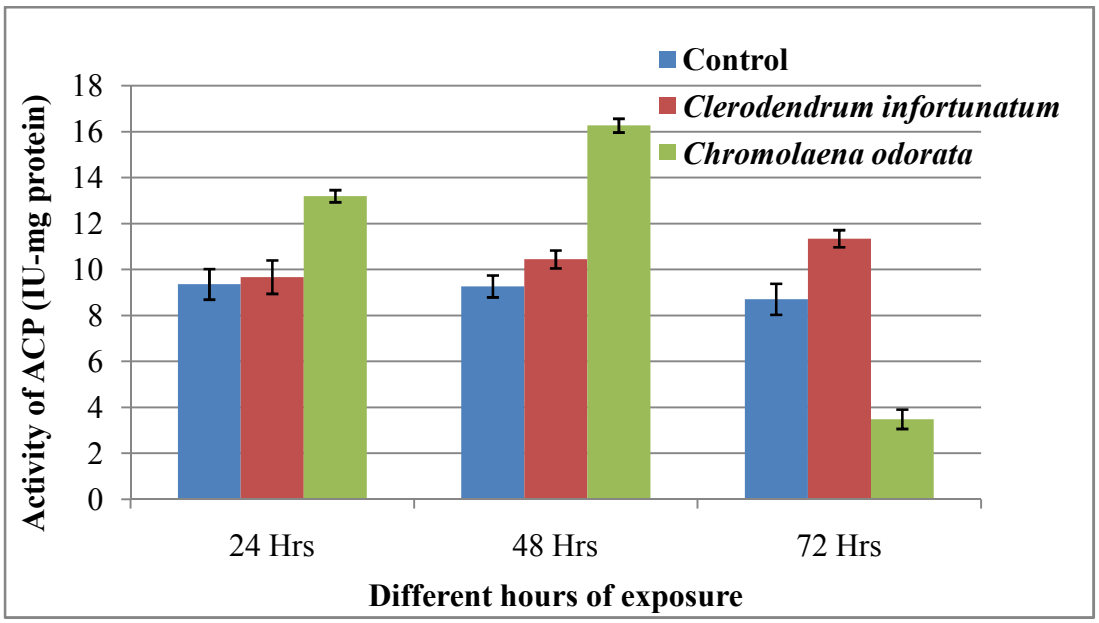


Figure VIII.7. Graph showing the activity of ACP in midgut with respect to different hours of treatment.

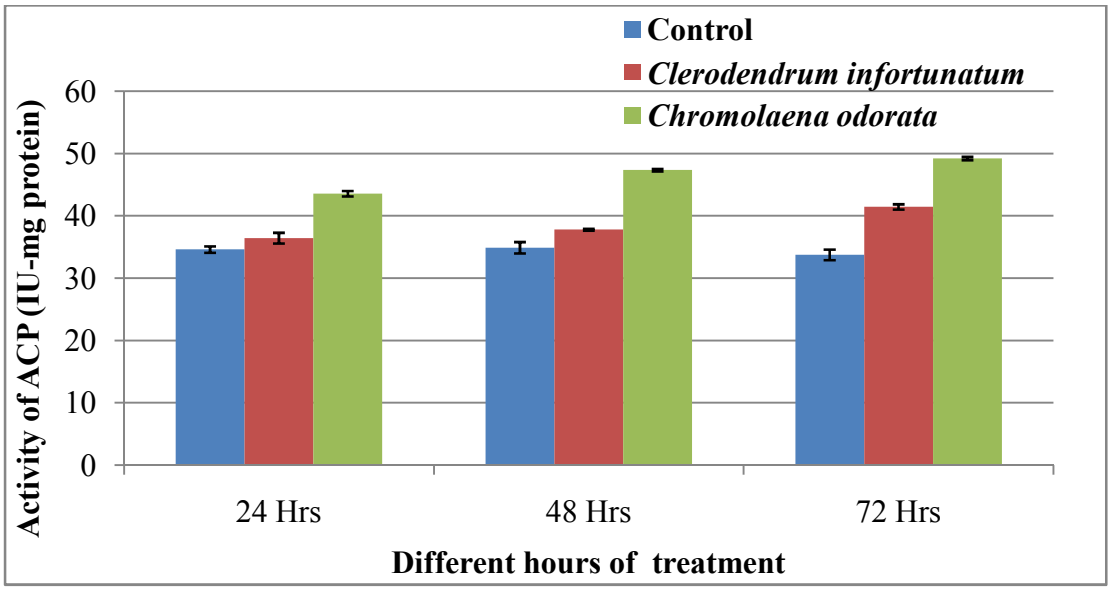


Figure VIII.8. Graph showing the activity of ACP in the fat body with respect to different hours of treatment.

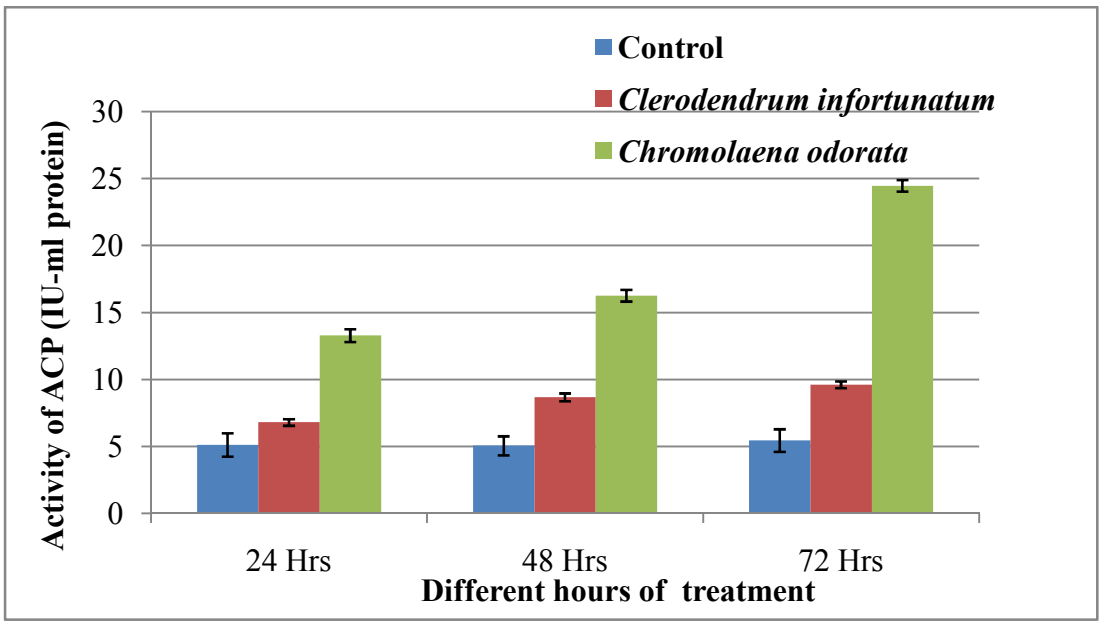


Figure VIII.9. Graph showing the activity of ACP in the haemolymph with respect to different hours of treatment.

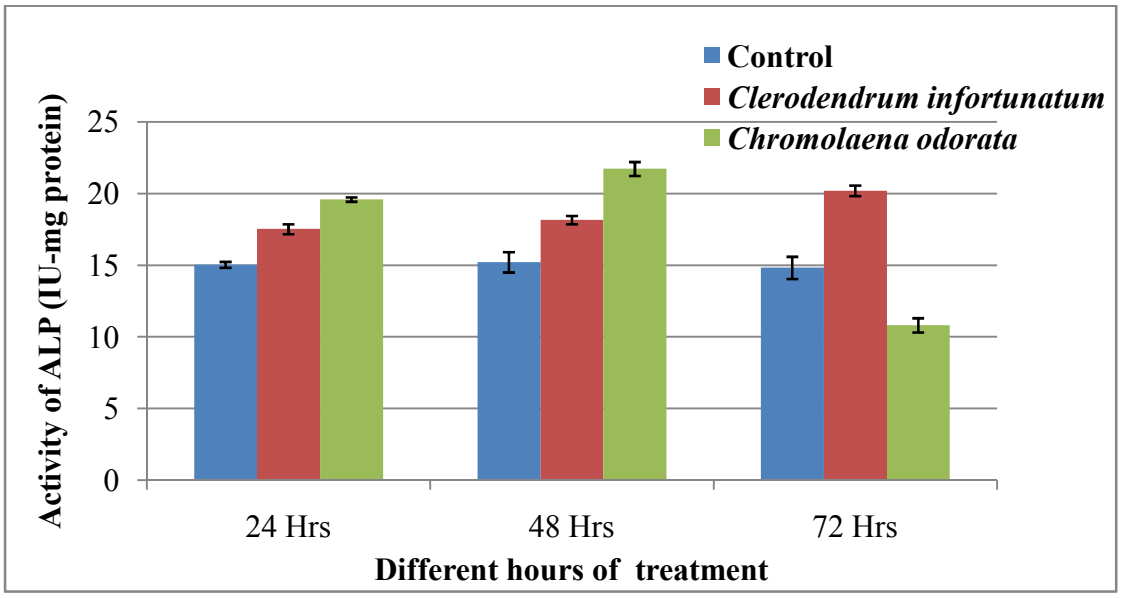


Figure VIII.10. Graph showing the activity of ALP in the midgut with respect to different hours of treatment.

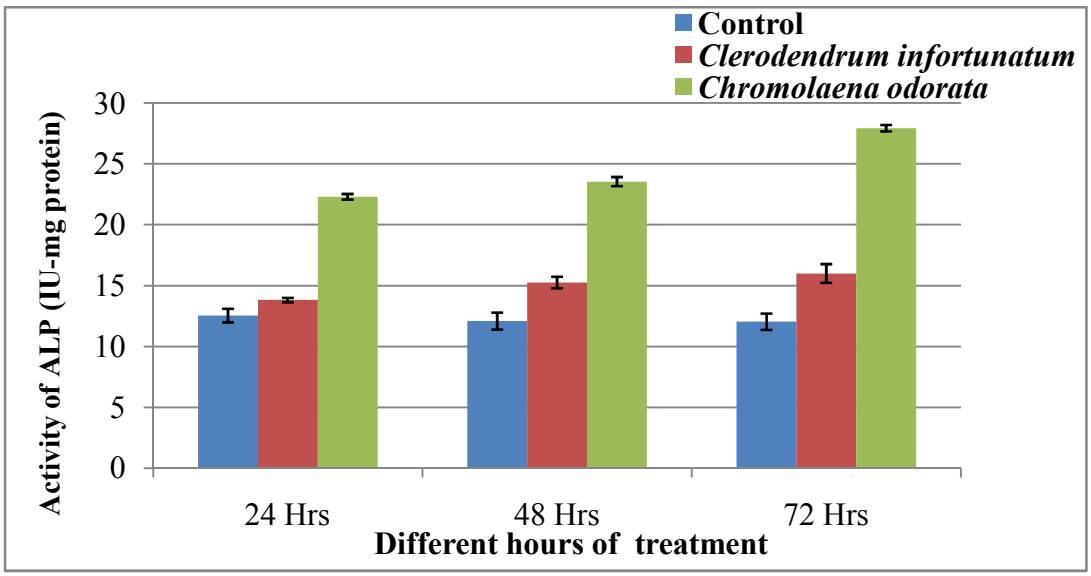


Figure VIII.11. Graph showing the activity of ALP in the fat body with respect to different hours of treatment.

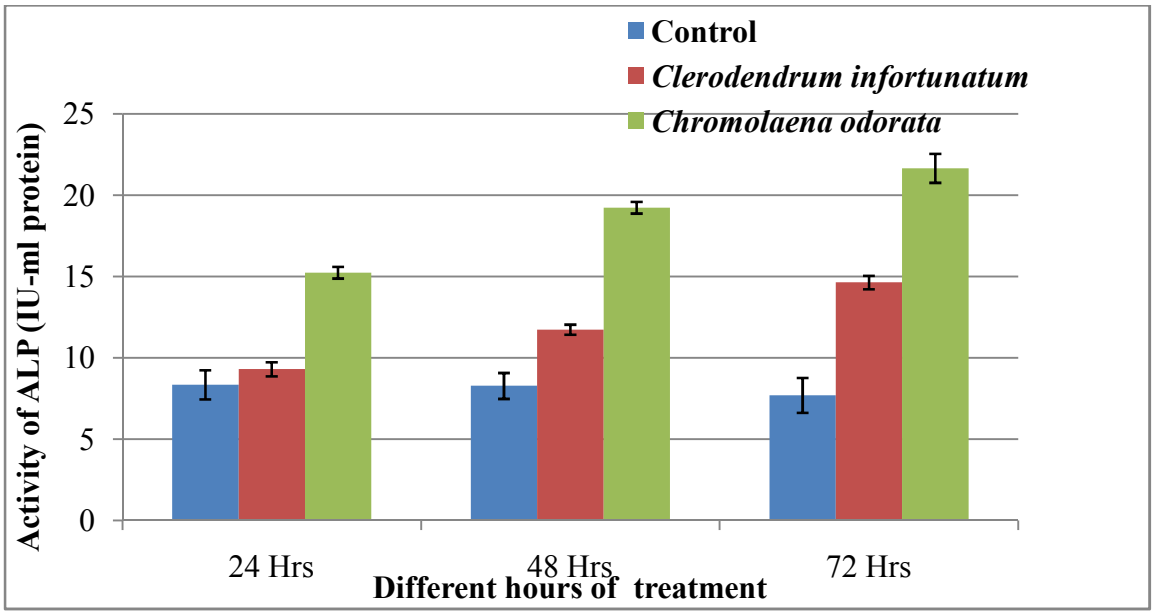


Figure VIII.12. Graph showing the activity of ALP in the haemolymph with respect to different hours of treatment.

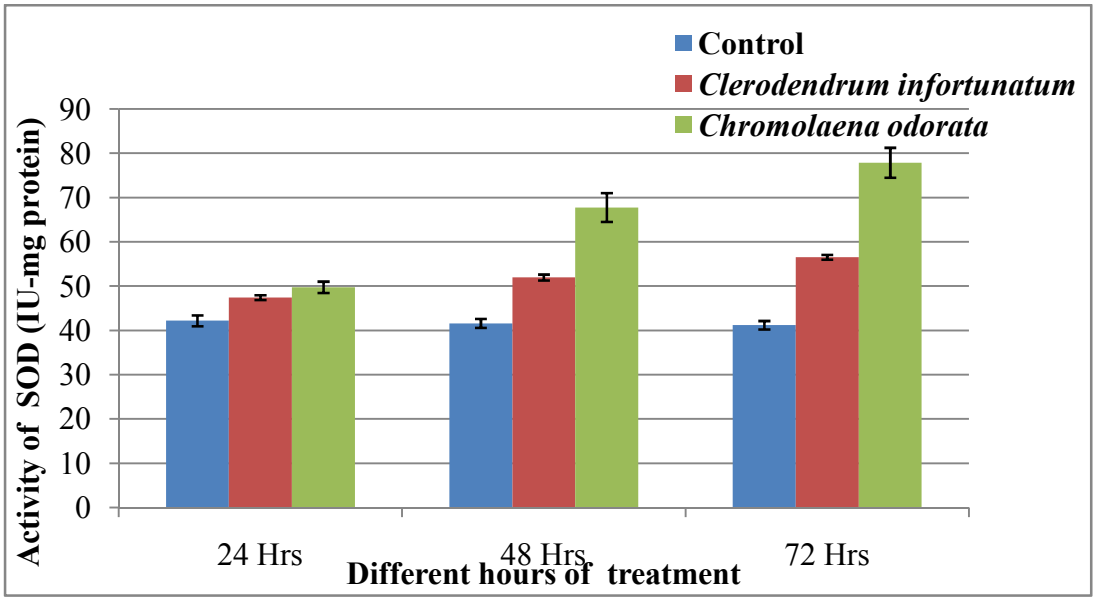


Figure VIII.13. Graph showing the activity of SOD in the midgut with respect to different hours of treatment.

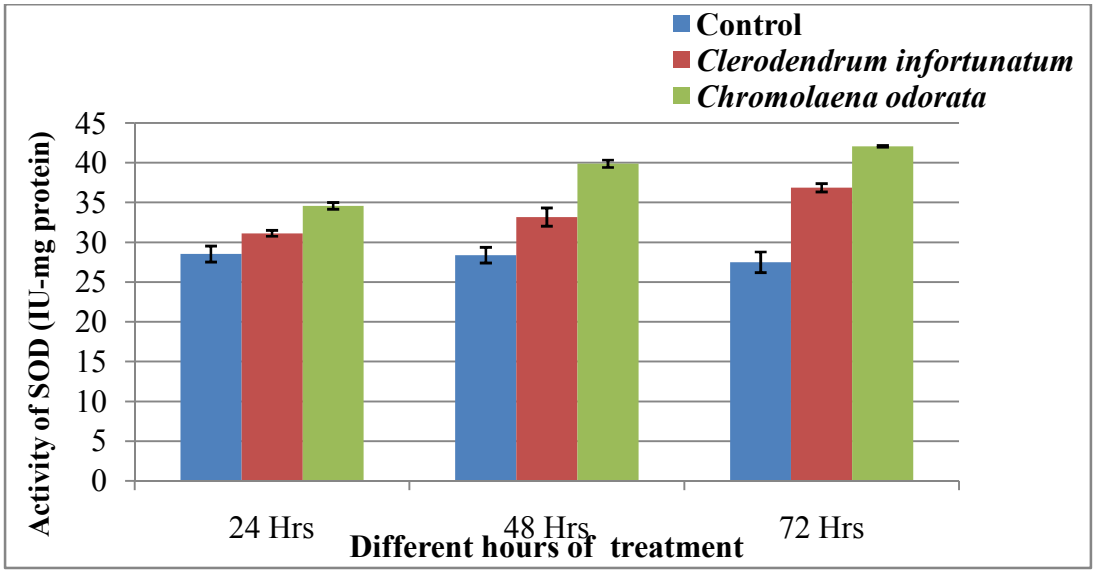


Figure VIII.14. Graph showing the activity of SOD in the fatbody with respect to different hours of treatment.

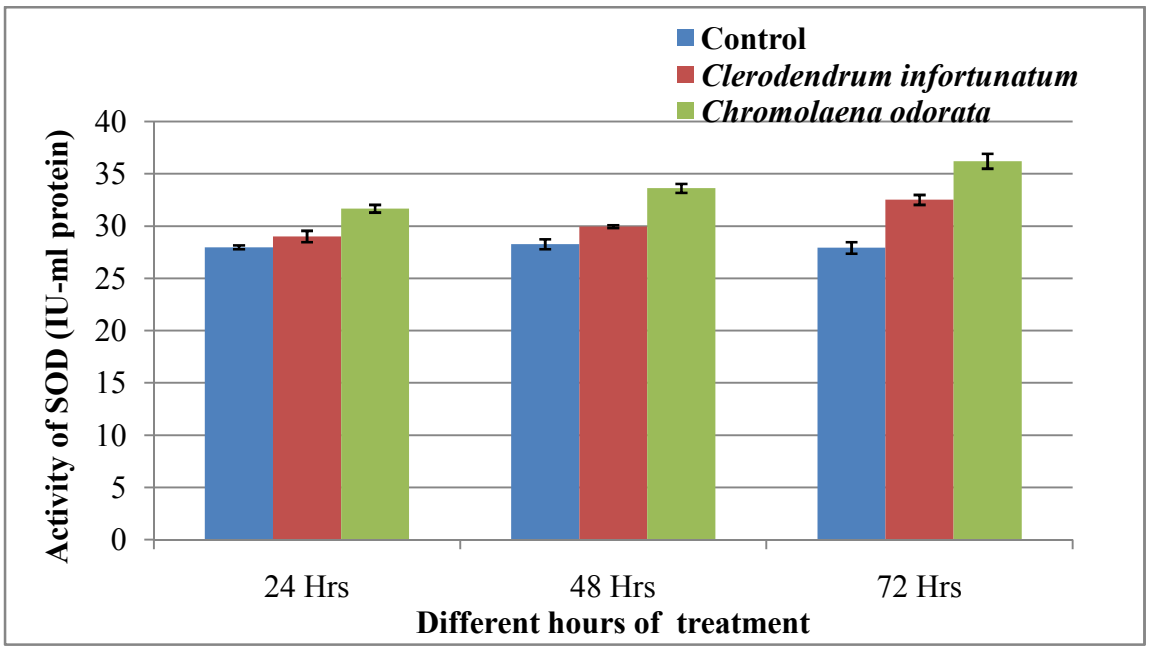


Figure VIII.15. Graph showing the activity of SOD in the haemolymph with respect to different hours of treatment.

8.4 Discussion

The results obtained from the quantitative assay of the activities of digestive enzymes and stress related enzymes showed the efficacy of both botanicals to influence the normal enzymatic activity in midgut, fat body and haemolymph of the sixth instar larvae of *O. exvinacea*.

Gradual decrease in the activities of protease and amylase are the common observation in all tissues with respect to increasing botanical concentration. The present results were in agreement with the findings of Senthil-Nathan *et al*. (2006 a, b) who noticed the impact of *Vtex negundo* extract and neem seed kernel extract on the gut of *Cnaphalocrocis medinalis* larva in combination with *Bacillus thuringiensis* through treated rice leaves and observed serious reduction in digestive enzyme activities such as protease, amylase and lipase and in the lactate dehydrogenase activity. Similarly, different concentrations of methanolic extract of *Artemisia annua*

at 48 hrs post-treatment in *Glyphodes pyloalis* larvae showed a serious decline in protease activity when compared to control (Khosravi *et al.,* 2011).

Many researchers reported the influence of botanicals on the digestive enzymes and found reduced enzyme activities with increasing the exposure duration. Salem *et al.* (2003) observed the effect of *Peganium harmala* and *Thymelae ahirsuta* extracts at 5% concentration against *Spodoptera littoralis* larvae for their efficiency on the activity of amylase and invertase and reported that the *P. harmala* water extract caused more inhibition than that of *T. ahirsuta* ethanolic extract on amylase activity. The suppressed activities of digestive enzymes like protease and lipase were noticed in the larvae of *C. medinalis* as a result of the treatment with *V. negundo* and *A. indica* (Senthil-Nathan *et al.,* 2006 c). Chockalingam *et al*. (1989) reported major reduction in the activities of amylase, invertase and protease of *S. litura* as a result of application of *Catharanthus roseus* extract. And also the treatment of azadiractin reduced the amylase and invertase activity in *S. litura* larvae and thereby lowers the digestibility (Senthil *et al*., 2005a). Mehrabadi *et al*. (2011) noticed the effect of some medicinal plant extracts on the digestive enzymes of some stored pests (*Callosobruchus maculatus*, *Rhyzopertha dominica*, *Sitophilus granaries* and *Trogoderma granarium*) and reported 4% to 95% of reduced amylase activity in their gut. Jbilou and Sayah *et al*. (2008) found that larvae of *Tribolium castaneum* fed on diet treated with methanol extracts from seven plant species had lower α-amylase activity than larvae fed on untreated diet.

Generally, digestive enzymes are synthesized in the rough endoplasmatic reticulum, processed in the golgi complex, packed into secretory vesicles and secreted by the gut via exocytosis, apocrine or microapocrine processes (Terra and Ferreira, 2012). As discussed earlier due to cytotoxicity of both botanicals, the total enzyme secretion in the midgut of

O. exvinacea was imbalanced. Rharrabe *et al*. (2007) noticed the inhibition of amylase activity in *Plodia interpunctella* when treated with Harmaline and also stated that the cytotoxic effect on epithelial cells of the midgut may cause the reduction in amylase activity. The histopathological changes that occurred in the midgut cells of *Dysdercus cingulatus* after the treatment with *Eupatorium odoratum* and *Vitex negundo* plant extracts caused dysfunctional digestive process due to the inhibition of digestive enzyme production (Premeela and Muraleedharan, 1995).

The presence of phytochemicals present in both leaf extracts may be the reason for the suppression of the enzyme secretion. Many scientists had reported the efficiency of plant secondary metabolite that can suppress the digestive enzymes secretion in insects. Khosravi and Sendi (2013) studied the effect of neem derived compound azadirachtin on *G. pyloalis* and suggested that this active component lowered the activities of both proteases and α-amylase in the midgut, due to its feeding deterrence ability. Khosravi and Sendi (2013) evaluated the capacity of azadirachtin to reduce the protease activity and also to reduce the level of α and β-glucosidases in *G. pyloalis* treated with neem pesticide (achook). The terpenoid present in the potato leaves interfere the activities of digestive enzymes and antioxidant enzymes of *Colorado potato* beetle, *Leptinotarsa decemlineata* larvae and thereby reduced the esterase activity (Ortego *et al*., 1999).The reduction in α-amylase activity by plant extracts could be due to the plant defense compounds that act on insect gut enzymes, α-amylases and proteinases (Ryan, 1990; Franco *et al*., 2002).

The phytochemicals can also act as digestive enzyme inhibitors, especially as proteinase and amylase inhibitors. Proteinase inhibitors are natural plant defensive mechanism against insect herbivores which were viewed as promising compounds for developing insect resistant transgenic

crops that over-express proteinase inhibitors (Gatehouse, 2011). Inactivation of digestive enzymes by proteinase inhibitors results in blocking the secretion of gut proteinases and that leads to poor nutrient utilization, stunted development and death because of starvation (Jongsma and Bolter, 1997; Gatehouse and Gatehouse, 1999). Several kinds of α-amylase and proteinase inhibitors present in plants act to regulate a number of phytophagous insects (Konarev, 1996; Gatehouse and Gatehouse, 1998). Sridhar and Sulochana (1989) also revealed the inhibitory effect of *Azadirachta indica* and *Pongamia glabra* leaf extract on the activity of digestive enzymes of *Euproctis fraterna.*

In the present study the leaf extracts of *C. infortunatum* and *C. odorata* significantly influenced the activities of phosphatase enzymes (ACP and ALP) in midgut, fat body and haemolymph of *O. exvinacea* with respect to different time of botanical exposure. These results could be correlated with many similar observations, such as the treatment with neem extracts in *C. medinalis* which showed drastic changes in activities of acid phosphatase (ACP), alkaline phosphatase (ALP) and adenosine triphosphatase (ATPase) in the larvae (Senthil-Nathan *et al*., 2004) treatment of azadiractin on *Helicoverpa armigera* (Babu *et al*., 1996) Neemzal on *S. gregaria* (Hamadah, 2009) and treatment of garlic oil on *T. castaneum* larvae (Beltagy and Omar, 2016).

Generally, the secretion of detoxification enzymes in insects is demonstrated as the enzymatic defense system against foreign compounds and play significant role in maintaining their normal physiological functions (Li and Liu, 2007). This can be either by increasing the activity of enzymes or by increasing their concentrated production but under extreme toxic conditions activity may be reduced due to cellular damage. Upadhyayi *et al*. (2011) noticed the toxic effect of synthetic termicides, fibronil, thiomthoxam and malathion on Indian white trermite, *Odontotermus obesus* which caused a

significant increase in phophatase activity up to 4 hrs of treatment and later on a decrease was noticed at 16 hrs of treatment with respect to control. The treatment of methanolic extract of *Artemisia annua* on the sunn pest, *Eurygaster integriceps* showed increased phosphatase activity at 12 hrs and 24 hrs of exposure but it later decreased at 48 hrs and 72 hrs of treatment.

In present study, the treatment of both botanicals induced the increased phosphatase activities (ACP and ALP) in all treated tissues except in the case of 72 hrs of midgut treatment. Many workers reported the induced effects of botanicals on the activities of both ACP and ALP. Koodalingam *et al*. (2014) observed a significant increased level of ACP activity in both larvae and pupae of *Aedes aegypti* upon exposure to NeemAzal (a neem formulation). The treatments of different extracts of *Ammi visnaga* fruits on penultimate instar nymphs of *S. gregaria* promoted or inhibited ACP activity in their haemolymph (Ghoneim *et al*., 2014).The increase in the activity of acid phosphatase may be due to the direct action of botanicals on the enzyme synthesis sites due to the lysis of the damaged tissue or have been released from the tissues due to the rupture of the cellular and lysosomal membranes (Bhardwaj *et al*., 2016).

In the present study, the treatment of *C. odorata* increased the ACP and ALP activity in the midgut at 48 hrs of exposure and later it declined at 72 hrs. These results were agreement with findings of Senthil-Nathan *et al*. (2004, 2006 d, e) who suggested that decreased level of ACP activity in *C. medinalis* at higher concentration of neem extracts was due to reduced phosphorus liberation for energy metabolism, decreased rate of metabolism as well as decreased rate of transport of metabolites, and may be due to the direct effect of neem seed extract.

The inducing effect on the ALP activity of the leaf extracts in different tissues can correlate with similar results of enhanced ALP activity in the

larvae of *Pieris rapae* exposed to them ethanolic extract of *Silybium marianum* (Hasheminia *et al*., 2013). The increased ALP activity in some tissues of nymphs of *S. gregaria* may indicate the involvement of this enzyme in detoxification process against the toxicants contained in the *Nigella sativa* seed extracts (Hasheminia *et al*., 2013). Sometimes the inhibited activities of both ACP and ALP enzymes might be due to the imbalance in enzyme substrate complex and inhibition of peristaltic movement of the guts in treated insects (Senthil-Nathan *et al*., 2005).

Generally, drastic changes in SOD activity of an organism indicate its stress condition under toxic environment. When an organism is exposed to toxic environment, it may encounter oxidative stress which lead to increased production of reactive oxygen species (ROS) (Knopowski *et al*., 2002). Its excessive production impairs cellular lipids, nucleic acid, protein and ultimately results in lipid peroxidation, genome instability or gene mutation; protein carbonyl formation and enzymatic lethargy, which in turn bring about various degenerative processes (Finkel and Halbrook, 2000). Normally, antioxidant defense system of an animal mainly consist of three major enzymes superoxide dismutase, catalase and glutathione peroxidase to defend against the enormously formed ROS to maintain the stable condition of ROS and other radicals in the cells (Meng *et al*., 2009). According to Curtis *et al.* (1972) superoxide dismutase (SOD) is one of the most important enzyme in the endogenous antioxidant defense system which scavenges the superoxide anion to form hydrogen peroxide and thus reduce the toxic effect through its radical.

The results of present investigation showed drastic increase in the activity of SOD in all tissues with respect to increase in exposure time. Similar findings were noticed in many investigations such as that of Krishnan and Kodrik (2006) who observed increased activity of superoxide dismutase

(SOD) in the midgut of *S. littoralis* larvae fed with potato leaves containing high content of allelochemicals. The larvae of *B. cucurbitae* treated with plant extracts showed induced activity of SOD after the treatment for 48 hrs and 72 hrs; increased catalase activity after 48 hrs and 72 hrs of treatment; decreased or increased esterase activity at different time periods were also noticed (Kaur *et al*., 2014). Rizwan-ul-haq *et al*. (2009) noticed the changes occurred in the activities of SOD and CAT in the larvae of *S. exigua* treated with harmaline and ricinine combined with *Bacillus thuringiensis*.

The present investigation which showed severe enzymatic changes in different tissues of *O. exvinacea* indicate the stress created by the influence of both botanicals. Reduced activities of protease and amylase indicate the imbalanced condition of digestive system and the drastic changes occurred in the activities of ACP, ALP and SOD may also indicate the condition of larvae in the state of recovering the energy loss due to botanical stress. The severe cellular damage occurred due to cytotoxicity can also be considered as a strong supporting evidence for the metabolic stress and it may lead to slow death of *O. exvinacea*.

CHAPTER IX
GENERAL DISCUSSION

Mango is the leading fruit crop of India and its production makes India become first among world's mango producing countries accounting for about 50% of the total world's production. So the mango cultivation has played an inevitable role in Indian agro-economy. This crop facing enormous losses due to ravages of wide range of pests and among them, 492 species of insects were reported as causing serious threat to the crop (Srivatsava, 1998). Among these, the leaf webber *Orthaga exvinacea* has gained a serious pest status due to their severe infestation causing tremendous losses of crop yield.

Ungovernable usage of synthetic insecticides to eradicate this pest has resulted in serious environmental pollution, lethal effects on non-target organisms, pest resistance to insecticides and pest resurgence. Because of serious public health concerns on the toxic effects of synthetic chemical pesticides, the pest management programme has paved away for an eco-friendly approach. On this current situation, insecticidal potency of *Clerodendrum infortunatum* and *Chromolaena odorata* has been identified as attractive alternatives to synthetic chemical insecticides. Plants, which have rich sources of natural substances, especially the secondary metabolites as defensive weapon, can be successfully exploited in eco-friendly pest management. Isolation of this wide range of phytochemicals from the plants and evaluation of their insecticidal efficiency and determination of structure would provide valuable possibilities for the development of novel eco-friendly method in insect pest management.

In the present investigation, the solvent selection for successful phytochemical extraction was a crucial step because the selected solvent

should have the ability to separate the maximum components from the plant and at the same time the solvent should not possess any direct toxicity to the organisms. On these conditions, the solvent methanol showed greater efficiency, with their maximum phytochemical separation in HPTLC profile and least toxicity towards larvae in the toxicity screening test.

The fractionation of both methanolic leaf extracts of *C. infortunatum* and *C. odorata* by column chromatography and toxicity evaluation of separated fraction has helped to club the phytochemicals on the basis of their toxicity and for the identification of the most toxic compounds present in leaf extracts. The preliminary toxicity screening test for both fractions, F2 of *C. infortunatum* and F5 of *C. odorata* showed 100% mortality and also the LD_{50} evaluation of both fractions confirmed the larvicidal activity of these fractions. The confirmed dose dependent mortality of fractions can be correlated with their phytochemical composition present in both leaf extracts. It was already reported that leaves and flowers of *C. infortunatum* contain bioactive compounds such as clerodin, hentriacontane, fumaric acid, ethyl and methyl esters of caffeic acid, 2-sitosterol and its glucoside and flavone glycoside (Akihisa *et al*., 1988). The methanolic leaf extracts of *Chromolena odorata* contained secondary metabolites like steroids, triterpenes, flavanoids, alkaloids, tannins, diterpenes and saponins (Lovet and Zige, 2003). One of the major attempts of present investigation was that the identification of toxicity causing compounds present in both fractions.

For this, primarily HPTLC profiling of both major fractions F2 of *C. infortunatum* and F5 of *C. odorata* was carried out and it revealed the presence of secondary metabolites such as phenols, flavonoids and terpenes. The phytochemicals under these categories were responsible for toxicity of fractions, especially phenols and terpenes were commonly found in both fractions but the presence of flavonoids were only observed in F5 fraction of

C. odorata and it may be the reason for the higher toxicity of this fraction. It was also reported that the secondary metabolites involving alkaloids, terpenoids, phenolics, essential oils and other organic chemicals provided a large spectrum of biological activities such as attractants, antifeedants, deterrents, phagostimulants or modify oviposition (Venkatachalam and Jebanesan, 2001).

Identification of compounds was carried out by GCMS analysis and revealed the presence of 8 compounds in fraction F2 and 13 compounds in fraction F5. Among the identified components in fraction F2, the bioactivities of n-hexadecanoic acid, squalene and digitoxin were already reported. The presence of *n*-hexadecanoic acid in the leaf extract of *Feronia limonia* showed larvicidal activity on mosquito larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Rahuman *et al*., 2000); similarly presence of squalene among the group of toxicity causing components in *Jatropha curcas* extracts possessed insecticidal activity against housefly, *Musca domestica* (Chauhan *et al.,* 2015) and also Ahmad *et al.* (2013) reported that the presence of digitoxin compound among the glycosides such as gitoxin, and digoxin in *Digitalis purpurea* extract showed highest toxicity against stored grain pest *Tribolium castaneum*.

Similarly, in the fraction F5 major bioactive compounds present are cholestane; hexadecanoic acid, methyl esters; 1-Monolinoleoylglycerol trimethylsilyl ether and 1, 2-Benzenedicarboxylic acid-diisooctyl ester. The antioxidant activity of cholestane was reported by Pandian and Nagarajan (2015) in their investigation carried out on the chemical composition and antioxidant potential of *Valeriana wallichi*. Presence of compound hexadecanoic acid- methyl esters in crude n-hexane and chloroform extracts of *Albizia adianthifolia* and *Pterocarpus angolensis* showed microbial activity (Abhubaker and Majinda, 2016) and also the presence of

1-Monolinoleoylglycerol trimethylsilyl in the ethanol extract of *Clerodendrum phlomidis* leaves showed pharmacological activities (Kumaradevan *et al*., 2015) . The LC-Q-ToF and LC-MS/MS analysis were also carried out for the identification of further compounds present in fraction F5 of *C. odorata* and revealed the presence of bioactive compounds such as Hesperitin, Acacetin, Jaceosidin, Centaureidin and Caffeic acid.

In the present investigation, the histopathological changes that occurred in the larval midgut epithelium of *O. exvinacea* treated with both botanicals *C. infortunatum* and *C. odorata* mainly consisted of vacuolization and elongation of columnar epithelial cells, destruction of brush border of these cells, secretory activity and sloughing off of the apical region of the epithelial layer. Similar comparable changes were observed in the case of the treatment of *Artemisia monosperma* on larval gut of *S. littoralis* which showed extensive cytotoxic changes like destroyed epithelial membrane, extreme vacuolization and elongated columnar cells with destructed cell boundaries (Adel *et al*., 2010). According to Sayed *et al*. (2011) the treatment of *Azadirachta indica* and *Citrullus colocynthis* extracts on the larval midgut of *Spodoptera littoralis* showed vacuolization and necrosis of the epithelial cells and destruction of epithelial cells and their boundaries.

Among different degenerative changes noticed in the midgut epithelium of *O. exvinacea* treated with *C. infortunatum* and *C. odorata,* cell elongation, excessive vacuolization and enlargement of nucleus were observed as common effects of both botanicals and the degeneration of epithelial cells was increased as concentration of both botanicals increased. Similar observations were recorded by Humbert and Desportes (1977) and they stated that the extreme degeneration process occurred may be for the removal of toxic elements from the alimentary canal. The excessive vacuoles seen in the treated tissues was due to the presence of fat droplets which

dissolved during fixation and dehydration process (Salkeld, 1951). Histomorphometric changes noticed in the larval midgut of *O. exvinacea* with the treatment of both botanicals consisted mainly changes in the size of columnar cells and their nuclei. It was reported that significant differences were observed in size of columnar and goblet cells in gypsy moth larvae fed with suitable *Quercus cerris* leaves compared with larvae fed on *Robinia pseudoacacia* leaves (Peric-Maturaga *et al*., 2006). The treatment of leaf extract of *Lantana camara* in fourth instar larvae of *Helicoverpa armigera* showed disrupted cell architecture and also reduced cell dimensions of midgut tissue (Prasad and Roy, 2011)

The ultrastructural aspects of midgut epithelium of control larva of *O. exvinacea* revealed the normal structure with single layer of epithelilal cells and outer to it two muscle layers. Normally, midgut is the major site of food digestion in insects and its comprises a single layer of digestive epithelium and two muscle layers (Rocha *et al.,* 2010) and it was also reported that the midgut is considered as the best site of transporting epithelia, employed in intensive secretion of K+ and base equivalents (Dow and O'Donnell, 1990; Moffett and Koch, 1992). The inner most covering peritrophic membrane play an important role in the protection of the epithelium from food abrasion and microbial attack (Peters, 1992; Lehane, 1997).

The treatment of both *C. infortunatum* and *C. odorata* caused drastic changes in the structure and functions of both muscle and epithelial layers of the larval midgut of *O. exvinacea*. In the case of muscle layer, the separation of myofibrils and vacuole like formation were noticed in *C. infortunatum* treated tissues and shrunken myofibrils in longitudinal muscle layer were observed with the treatment of *C. odorata.* Excessive vacuolization in the basal region of the epithelial cells and detachment of epithelial cell layer from

the basement membrane were observed in both the botanical treated tissues. The treatments of both botanical also caused ultrastructural changes such as cell elongation, cytoplasmic vacuolization and shrunken nucleus, reduced number of RER, change in shape of mitochondria, loss of microvilli in the apical region, absence of smooth endoplasmic reticulum, numerous secretory vesicles and absence of peritrophic membrane. It was reported that the ultrastructural changes occurred in the midgut digestive cells consisted of changes in shape from columnar to cubic, intensive vacuolization and changes in the brush border of the midgut digestive cells of *Aedes aegypti* larvae treated with squamocin from *A. mucosa* (Marilza *et al*., 2014). Similarly Sayed *et al.* (2011) reported that the treatment of *Azadiracta indica* and *Citrullus colocynthis* extracts caused enormous vacuolization, necrosis and destruction of epithelial cells and their boundaries in the larval midgut of *S. littoralis*. In present study, another important observation was the loss of microvilli inside the goblet cells. The destruction of microvilli and changes in the shape of mitochondria might affect the function of goblet cells concerned with excretion of potassium. It was reported that treatment of squamocin from *Annona squamosa* on *Aedes aegypti* larvae prevented the production of ATP by the electrons in the mitochondrial complex I and caused the death of the insect by affecting cellular respiration (Lummen, 1998; Takada *et al*., 2000).

The active ingredients present in both *C. infortunatum* and *C. odorata* extracts might have caused the histomorphological and ultrastructural variations occurred in both epithelial and muscle layers of the larval mid gut tissue of *O. exvinacea.* The neem derivative azadirachtin affected the midgut epithelium of *Spodoptera gregaria* and *Locusta migratoria* and exhibited histopathological changes such as swelling of cell organelles and rounding up of the cells, necrosis of midgut cells, enlargement of connective tissue with many invading cells and muscle layers became shrunken and swollen

(Nasiruddin and Mordue, 1994). Barbara *et al*. (2007) reported that the efficacy of plant cyclotides on the larval midgut of *Helicoverpa armigera* showed that the cyclotides disrupted the plasma membrane of the epithelial cells forming holes or pores that led to cell swelling and lysis. The cell organelles that located in apical region of columnar cells were actively employed with digestion of food and production of digestive enzymes, so any morphometric changes or destruction of the apical region of the columnar cells might be the cause for digestive disorders (Terra *et al*., 1996). Hence the presence of cytotoxic phytochemicals in both the leaf extracts of *C. infortunatum* and *C. odorata* might be the reason for the serious histopathological, histomorphometric and ultrastructural alterations in both epithelial and muscle layers and these deleterious changes may lead to serious digestive and absorptive disorders in *O. exvinacea*.

During present investigation, considerable quantitative changes in protein and amino acid content and changes occurred in protein profile of different tissues of treated *O. exvinacea* larvae revealed the efficacy of both leaf extracts to reduce the total protein, free amino acids and to alter the protein profile of all treated tissues, thereby interfering the physiological activities of the larvae. Many comparable research works were reported with similar results. Jadhav and Ghule (2003) observed a decreased concentration of protein in the total body and haemolymph of *C. cephalonica* after treated with azadirachtin through the diet. Schmidt *et al.* (1998) reported the reduced amount of haemolymph protein in both *Spodoptera littoralis* and *Agrotis ipsilon* after the treatment of 6 days exposure to methanolic extract of *Melia azedarach* and similar result was noticed by Li *et al.* (1995) in the haemolymph protein concentration of *S. litura* treated with azadirachtin. The requirement of higher metabolic energy under stress condition demands increased protein catabolism to detoxify the toxic principles present in the *C. infortunatum* and *C. odorata*, but the lesser consumption of food for

synthesis of protein might also be the reason for the reduction in protein content. According to Vijayaraghavan *et al*. (2010), the concentration of protein in an insect is mainly dependent upon its synthesis, breakdown and water movement between tissues and haemolymph, so the reduction of protein content in larvae might be due to the reduction in synthesis of protein or increase in breakdown to detoxify the active principles present in the plant extracts.

The treatment of *C. infortunatum* and *C. odorata* extracts also influenced the different protein expressions in midgut, fat body and haemolymph of *O. exvinacea*. Similar comparable results were reported by many researchers regarding the toxic effects of phytochemicals on the protein profiling of different insect tissues. Boreddy *et al*. (2000) reported that the treatment of *Annona squamosa* seed extract on fourth instar larvae of *S. litura* exhibited significant reduction in protein content and created noticeable variations in electrophoretic banding pattern of total protein profile of the larvae. The decline in protein content and structural deformities in protein profile were recorded in *Anopheles* larvae after the treatment with methanolic extract of *Azadirachta indica* (Sharma *et al.,* 2006 a). Appearance and disappearance of protein bands occurred in all protein profiles of different tissues of treated *O. exvinacea* larvae. Similar observations in appearances and disappearance of protein bands were noticed in the midgut protein profile of *A. gemmatalis* after the treatment with the aqueous seed extracts of *Koelreuteria paniculata* (Martins *et al*., 2012). The acceptable reason for the appearance and disappearance of polypeptides in protein profile may be due to the alteration occurred at molecular level of larvae. According to Al-Qahtani *et al.* (2012) the treatment of some dried plants created transcriptional changes in *Oryzaephilus surinamensis* to develop resistance or detoxification mechanism. It may be due to the effect of active principles present in both *C. infortunatum* and *C. odorata* acting at genetic level of

O. exvinacea larvae because any foreign particle interacting with the cellular metabolism created stress in the cell. It may either up-regulate or down-regulate gene expression and the disappearance of protein bands at higher concentration might be due to the activity of the phytochemiclas present in the botanicals which down- regulate some gene expressions. The protein patterns of different tissues of the treated larvae showed appearance of many new small proteins which are smaller in sizes. The small sized protein might be new peptides formed or it might be the peptides which are formed by the breakdown due to the action of protease.

The quantitative changes in amino acids of different tissues indicated the physiological state of organism. The reduction in free amino acid content of different tissues of *O. exvinacea* was noticed with increasing concentration of *C. infortunatum* and *C. odorata*. Similar findings were reported in the case of treatment with *H. suaveolens* and *V. negundo* which caused severe reduction in protein and amino acid content in the larvae of *O. exvinacea* (Ranjini and Ranjini, 2016) and similarly reduced amount of amino acid content was noticed in *Dysdercus koenigii* with the treatment of *Annona* extracts (Reddy *et al*., 1993). Many comparable justifications for the reduction of amino acid in insect due to the effects of botanicals were recorded. According to Medhini *et al.* (2012), botanical stress increased the rate of protein catabolism in *Spodoptera litura* due to the treatment with *Calendula officinalis* and insect degrades protein to resultant amino acids in order to let them enter into the TCA cycle as keto acid for compensating the lower energy caused by stress and also to detoxify the active principles present in the plant extract. Serious protein reduction in tissues may constitute a physiological mechanism that could play a major role in compensatory process for insecticidal stress to provide intermediates to the Kreb cycle by retaining free amino acids in haemolymph (Etebari *et al.,* 2005). Amino acids play a vital role in moulting process of an insect (Pandey

et al., 1986) so the reduction may cause serious impacts on physiology as well as moulting process inducing morphological abnormalities in treated insects (Chen, 1966).

During present investigation, quantitative studies on the carbohydrate content in different tissues revealed the potency of leaf extracts of *C. infortunatum* and *C. odorata* to reduce the carbohydrate content in all tissues of *O. exvinacea* larvae. Similar botanical effects were reported in many works. The treatments of petroleum ether extract of *A. annua* and methanolic extract of *A. indica* on the *Culex quinquefasciatus* larvae caused reduced glucose level in larval tissue after 24 hrs of exposure (Sharma *et al*., 2011). Severe decrease in carbohydrate content was observed in *Crysoperla carnea* female at higher concentration of neem oil treatment and it may be due to the antifeedant property of the phytochemicals present in it (Abdul Razak and Sivasubramanian, 2007). The secondary metabolites present in both leaf extracts has possessed antifeedant activity that may lead to serious digestive disorders and thereby reduced the carbohydrate content in different tissues of treated *O. exvinacea* larvae. It was also reported that the efficiency of plant alkaloids present in *Pergularia tomentosa* in reducing both protein and carbohydrate contents in *Locusta migratoria* (Acheuk and Mitiche, 2013). The intake of toxic food materials may disrupt majority of absorptive cells inside the midgut and the resultant imbalanced nutrient absorption system lead to severe reduction in carbohydrate content in all tissues. Similar supportive findings were found in *S. litura* larvae treated with neem extract and noticed that the phytoextracts can acts as antifeedant and thereby lower the glucose level in larvae (Koul, 1999). According to Sharma *et al*. (2006a) the treatment of neem kernel extract on the third instar larvae of *A. stephensi* showed poor feeding and improper utilization of digested food by highly ruptured alimentary canal and thereby serious carbohydrate decline was noticed in the larva.

Under stress condition, the increased catabolic activities of nutrients were noticed to compensate the high energy demand and it lead to intensive utilization of carbohydrate reserve in fat body and this may be the reason for the decrease in carbohydrate content in fat body and haemolymph. Sharma *et al.* (2011) also reported that the significant reduction in glucose content in mosquito larvae by the treatment of *A. indica* and suggested that it may be due to the utilization of stored glucose in tissues as a result of insecticidal stress. Similarly Vijayaraghavan *et al.* (2010) noticed the reduction of carbohydrate content in haemolymph of *Crocidolomia binotalis* larvae treated with extracts from *Lippia nodiflora, Vitex negundo* and *Strychnos nuxvomica*. Many researchers have reported comparable possibilities of carbohydrate reduction in insects due to the effect of botanicals. It was suggested that carbohydrates were converted to proteins in detoxification mechanism against toxicants that enter the animal body (Fell *et al*., 1982; Rajendra, 1990; Shakoori and Saleem, 1991). Hence the phytochemicals can directly or indirectly cause metabolic disorders in insects by creating quantitative changes in carbohydrate content of different tissues and that may lead to lethal state of insect.

The effect of leaf extracts of *C. infortunatum* and *C. odorata* also reflected on the activities of enzymes present in midgut, fat body and haemolymph of the sixth instar larvae of *O. exvinacea*. The quantitative assay of the activities of digestive enzymes and stress related enzymes showed considerable changes with respect to increased time of exposure to both botanicals. Gradual decrease in the activities of protease and amylase were commonly observed in all treated tissues with respect to increasing botanical exposure time. Similar observations were reported by Senthil-Nathan *et al*. (2006 a, b) in *C. medinalis* larva treated with *V. negundo* extract and neem seed kernel extract in association with *Bacillus thuringiensis* and found serious reduction in digestive enzyme activities such as protease and

amylase. Similarly, reduced activities of protease, amylase and invertase were found in *S. litura* larvae as a result of the treatment with *Catharanthus roseus* extract (Chockalingam *et al*., 1989).

As discussed earlier, the cytotoxicity of both botanicals lead to imbalanced enzyme secretion in the midgut of *O. exvinacea*. Similar findings were reported by Rharrabe *et al*. (2007) in *Plodia interpunctella* who noticed the inhibition of amylase activity after treatment with Harmaline and also stated that the cytotoxic effect on epithelial cells of the midgut may cause the reduction in amylase activity. Similary, the histopathological changes that occurred in the midgut cells of *Dysdecus cingulatus* due to the treatment with *Eupatorium odoratum* and *Vitex negundo* plant extracts caused dysfunctional digestive process by the inhibition of digestive enzyme production (Premeela and Muraleedharan, 1995). The phytochemicals present in both leaf extracts played an important role in the suppression of enzyme secretion. According to Ortego *et al*. (1999) terpenoid present in the potato leaves have the ability to suppress the activities of digestive enzymes and antioxidant enzymes of *Colorado potato* beetle, *Leptinotarsa decemlineata* larvae and thereby reduced the esterase activity. The active component azadirachtin lowered the activities of proteases and α-amylase in the midgut of *G. pyloalis* (Khosravi and Sendi, 2013).

In the present study, the leaf extracts of *C. infortunatum* and *C. odorata* significantly influenced the activities of ACP, ALP and SOD in midgut, fat body and haemolymph of *O. exvinacea* with respect to different period of botanical exposure. Generally, the secretion of detoxification or stress related enzymes in insects has demonstrated as the enzymatic defense system against foreign compounds and play significant role in maintaining their normal physiological functions (Li and Liu, 2007). This can be either by increasing the activity of enzymes or increasing in their concentrated

production but under extreme toxic conditions activity may be reduced due to cellular damage.

In present study, considerable changes were noticed in the activities of phosphatase enzymes with increasing time of exposure and it can correlated with many similar findings. The treatment with neem extracts in *Cnaphalocrocis medinalis* showed drastic changes in activities of acid phosphatase (ACP), alkaline phosphatase (ALP) and adenosine triphosphatase (ATPase) in the larvae (Senthil-Nathan *et al*., 2004). Generally, the treatment of both botanicals induced increased phosphatase activities (ACP and ALP) in all tissues of treated *O.exvinacea* larvae except in the case of 72 hrs of midgut treatment. Similar observations were noticed due to the effect of methanolic extract of *Artemisia annua* on the sunn pest, *Eurygaster integriceps* which showed increased phosphatase activity at 12 hrs and 24 hrs of exposure but it later decreased at 48 hrs and 72 hrs of treatment (Zibaee and Bandani, 2010). The reasonable explanation for this decreased activities of phosphatase at higher concentration was given by Senthil-Nathan *et al*. (2004, 2006 d, e) who stated that decreased level of ACP activity in *C. medinalis* at higher concentration of neem extracts was due to reduced phosphorus liberation for energy metabolism. It was also reported that decreased rate of metabolism as well as decreased rate of transport of metabolites may be due to the direct effect of neem seed extract.

The increased activity of SOD was found in all exposed tissues of *O. exvinacea* larvae which indicated the state of stress condition of the larvae due to botanical treatment. Similar findings were noticed in many investigations such as that of Krishnan and Kodrik (2006) who observed increased activity of superoxide dismutase (SOD) in the midgut of *S. littoralis* larvae fed with potato leaves containing high content of allelochemicals. The larvae of *B. cucurbitae* treated with plant extracts showed increased activity

of SOD and catalase after the treatment for 48 hrs and 72 hrs and decreased or increased esterase activity at different time periods (Kaur *et al*., 2014).

The discussions carried out on different criteria of present investigation established the bioefficacy of both botanicals on the management of larvae of *O. exvinacea*. The toxicity evaluation of separated fractions revealed the larvicidal potency of active compounds present in both leaf extracts of *C. infortunatum* and *C. odorata* and these identified cytotoxic compounds were responsible for the serious histopathological and histomorphometric alterations observed in the midgut tissue which imbalanced the total digestive and absorptive system of the larvae. This instability also reflects on various biochemical processes in the tissues of the larvae through the exhibition of severe enzymatic changes and metabolic stress which leads to gradual reduction in the larval activity or death of the organism. Hence this investigation suggest that these natural agents (botanicals) can be included as a novel method in the management of mango leaf webber *Orthaga exvinacea*.

CHAPTER X
SUMMARY

The present investigation was carried out to study the effect of methanolic leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* on the midgut, fat body and haemolymph of sixth instar larvae of *Orthaga exvinacea*. All the objectives were successfully achieved and finally concluded that the larvicidal potency of both botanicals can be executed as an eco friendly method for controlling mango leaf webber *O. exvinacea*.

The initial step of phytochemical studies included phytochemical separation, toxicity evaluation and identification of most toxic compounds present in both leaf extracts of *C. infortunatum* and *C. odorata*. From different polar solvent extraction, solvent methanol has been selected as best solvent for the phytochemical extraction on the basis of maximum phytochemical separation showed by HPTLC profile and minimum toxicity of the solvent to the larvae confirmed by toxicity screening of test. The fractionation of methanolic leaf extracts of both botanicals by column chromatographic method helped to group the phytochemicals present in leaf extracts on the basis of their similar chemical profile by TLC analysis and also preliminary toxicity screening test has been helped to select the most toxic fractions F2 from *C. infortunatum* and F5 from *C. odorata* for further toxicity evaluation of these fractions by determining LD_{50}value.

The HPTLC analysis of fractions F2 of *C. infortunatum* and F5 of *C. odorata* were carried out to realize the presence of secondary metabolites such as Phenols, Flavanoids and Terpenes by spraying appropriate visualizing reagents. It was confirmed that phenols and terpenes were commonly found in both fractions but the presence of flavonoids was found only in F5 fraction of *C. odorata.* Identification of active compounds in the fractions was

successfully carried out by GCMS, LC-Q-ToF and LC-MS/MS analysis. Out of 8 compounds identified in fraction F2 of *C. infortunatum*, n-Hexadecanoic acid, squalene and digitoxin were reported as bioactive compounds and similarly bioactivities of cholestane; hexadecanoic acid-methyl esters; 1-Monolinoleoylglycerol trimethylsilyl ether and1,2-Benzenedicarboxylic acid, diisooctyl ester were already reported out of 13 compounds identified from fraction F5 of *C. odorata*. For further identification of compounds, LC-Q-ToF analysis of both fractions was carried out and on the basis of selected ions from the elemental composition availed, the fraction F5 has been further analyzed by LC-MS/MS. But in the case of fraction F2 of *C. infortunatum*, there was no compound identified in selected ions from the elemental compositions obtained. The LC-MS/MS analysis of fraction F5 of *C. odorata* revealed the presence of bioactive compounds such as Hesperitin, Acacetin, Jaceosidin, Centaureidin and Caffeic acid.

To detect the cytotoxicity of both botanicals, the larvae were treated with five different concentrations of both leaf extracts of *C. infortunatum* and *C. odorata* and noticed the major histopathological changes in the midgut epithelium by using both light and ultra microscopic techniques. Histopathological changes that occurred in treated midgut epithelium of *O. exvinacea* mainly consisted of vacuolization and elongation of columnar epithelial cells, destruction of brush border of columnar epithelial cells and tremendous secretory activity and sloughing off of the apical region of the epithelial layer. In addition to these changes, folding of the epithelium and overlapped folds and infolds of epithelium were noticed in *C. infortunatum* treated larval midgut epithelium. And similarly in the case of *C. odorata*, addition to increase in size of the goblet cells, severe cytoplasmic vacuolization of the columnar cells and thinning of muscle layers were also observed. Both botanicals also affected the dimensions of cells. For evaluating this, 70 % of epithelial cells, comprising of columnar cells were

selected for the morphometric studies and noticed the significant morphometric changes occurred in height and width of columnar cells and in their nucleus also. As a result of excessive cell elongation, increased height and decreased width of columnar cells and nucleus were noticed and also the swelling of nucleus which caused increase in its height and width were recorded.

From the light microscopic observations on the prominent changes recorded, 5% of *C. infortunatum* and 4% of *C. odorata* were selected for ultrastructural studies by using Transmission electron microscopy. The major observations such as the separation of myofibrils and formation of vacuole were noticed in *C. infortunatum* treated tissues and shrunken myofibrils in longitudinal muscle layer was observed with the treatment of *C. odorata.* General observations of ultrastructural changes in the larval midgut with treatment of both botanicals were tremendous cytoplasmic vacuolization, shrunken cell nucleus with condensed chromatin granules, excessive vacuolization in the basal region leading to detachment of epithelial cell layer from the basement membrane, reduced number of RER, change in shape of mitochondria, loss of microvilli in the apical region, absence of smooth endoplasmic reticulum and presence of numerous secretory vesicles.

To explore the efficacy of both leaf extracts on different biochemical parameters, quantitative and qualitative estimation of protein and quantitative estimation of amino acid and carbohydrate were carried out in midgut, fat body and haemolymph of sixth instar larvae of *O. exvinacea* treated with 1% to 5% leaf extracts of *C. infortunatum* and *C. odorata*. Significant reduction in all biochemical parameters were noticed with increasing the botanical concentrations and severe polymorphic changes in protein such as appearances and disappearances of protein bands were noticed in the electrophoretic analysis of protein in different tissues. The minimum reduction in concentrations of total protein, total free amino acid and

carbohydrate were noticed at 1% of treatment and the maximum reduction was found at 5% treatment of both botanicals. When comparing the efficacy of both botanicals, *C. odorata* possessed the maximum potency to reduce protein, amino acid and carbohydrate and also to influence protein profile in all tissues than that of *C. infortunatum*.

The botanicals also influenced the activities of digestive enzymes (protease and amylase) and stress related enzymes (ACP, ALP and SOD) present in all tissues of *O. exvinacea* larvae. The treatments with both botanicals showed reduced activities of protease and amylase in all tissues of the larvae with increasing exposure time. But in the case of stress related enzymes, the treatment of both botanicals increased the activities of ACP, ALP and SOD in all treated tissues. But there was an exemption in the case of ACP and ALP activities in midgut tissue at 72 hrs on exposure of *C.odorata* treatment. It is assumed that the threshold level of activities of these enzymes may be at 48 hrs and after that it may show a decline in its activity. Comparatively, *C. odorata* had maximum influence on the activities of both digestive and stress related enzymes than that of *C. infortunatum*.

On the basis of all inferences, it is concluded that the identified compounds from the most toxic fractions F2 of *C. infortunatum* and F5 of *C. odorata* were responsible for the larvicidal activity (by topical application) of both botanicals and also confirmed that the phytochemicals present in the methanolic leaf extracts of *Clerodendrum infortunatum* and *Chromolaena odorata* have the efficacy to interfere with physiological processes in larvae. So these botanicals can be recommended for use as an effective control measure against *O. exvinacea.* As a future prospect of present investigation it is suggested that the active toxic compounds should be isolated from the leaves of both the plants and an effective biopesticide for controlling *Orthaga exvinacea* and related lepidopteran pests can be produced commercially. Also it is desirable to test the efficacy of these botanicals in field condition.

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PLATE III.1

Figure 1. a. Infestation of *Orthaga exvinacea* in field. **b.** Rearing of *Orthaga exvinacea* in lab.

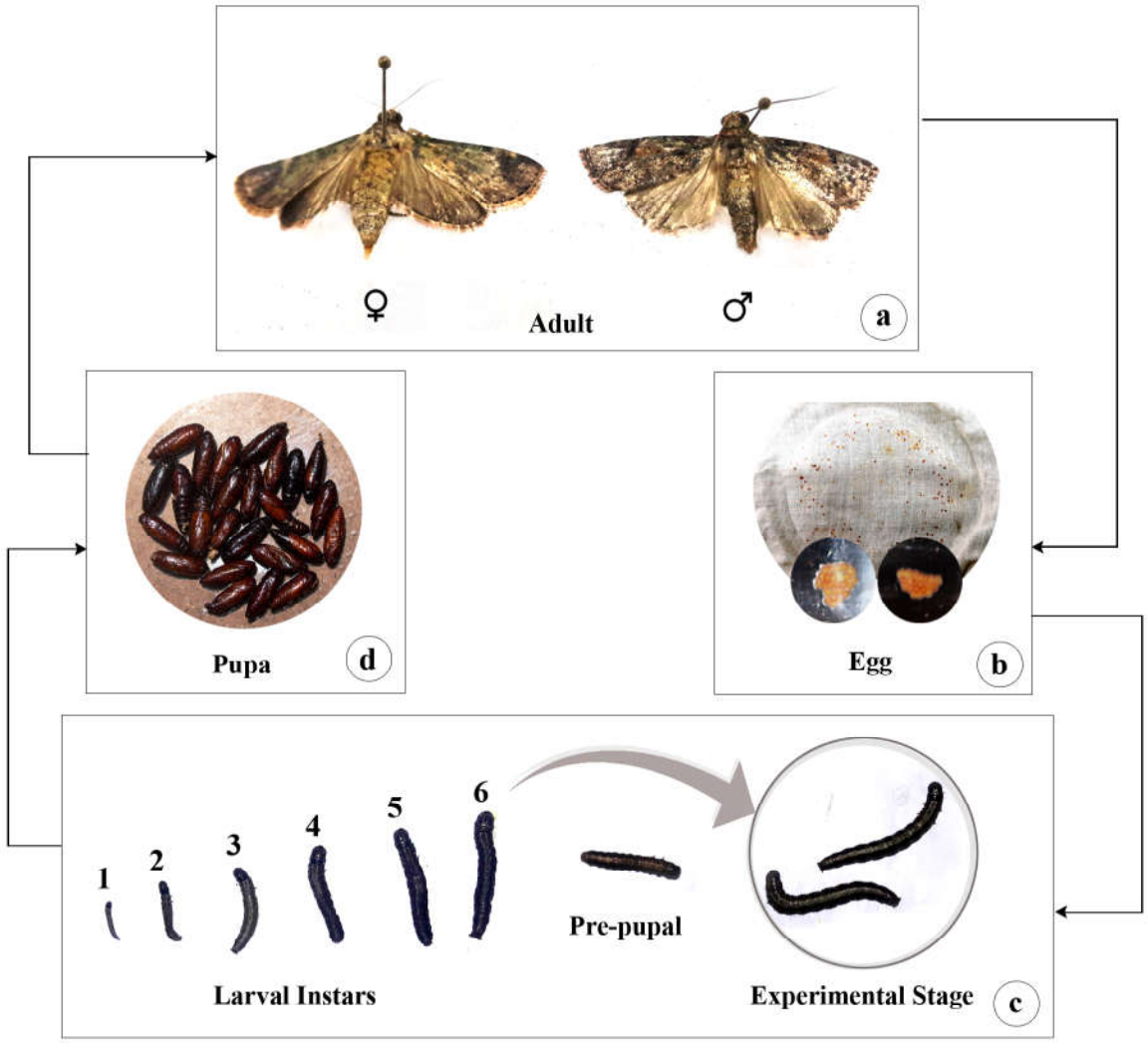


Figure 2. Life cycle of *Orthaga exvinacea* **a.** Adult; **b.** Egg; **c.** Different larval instars (1^{st} to 6^{th}), prepupal and experimental stage; **d.** Pupa.

PLATE V.8

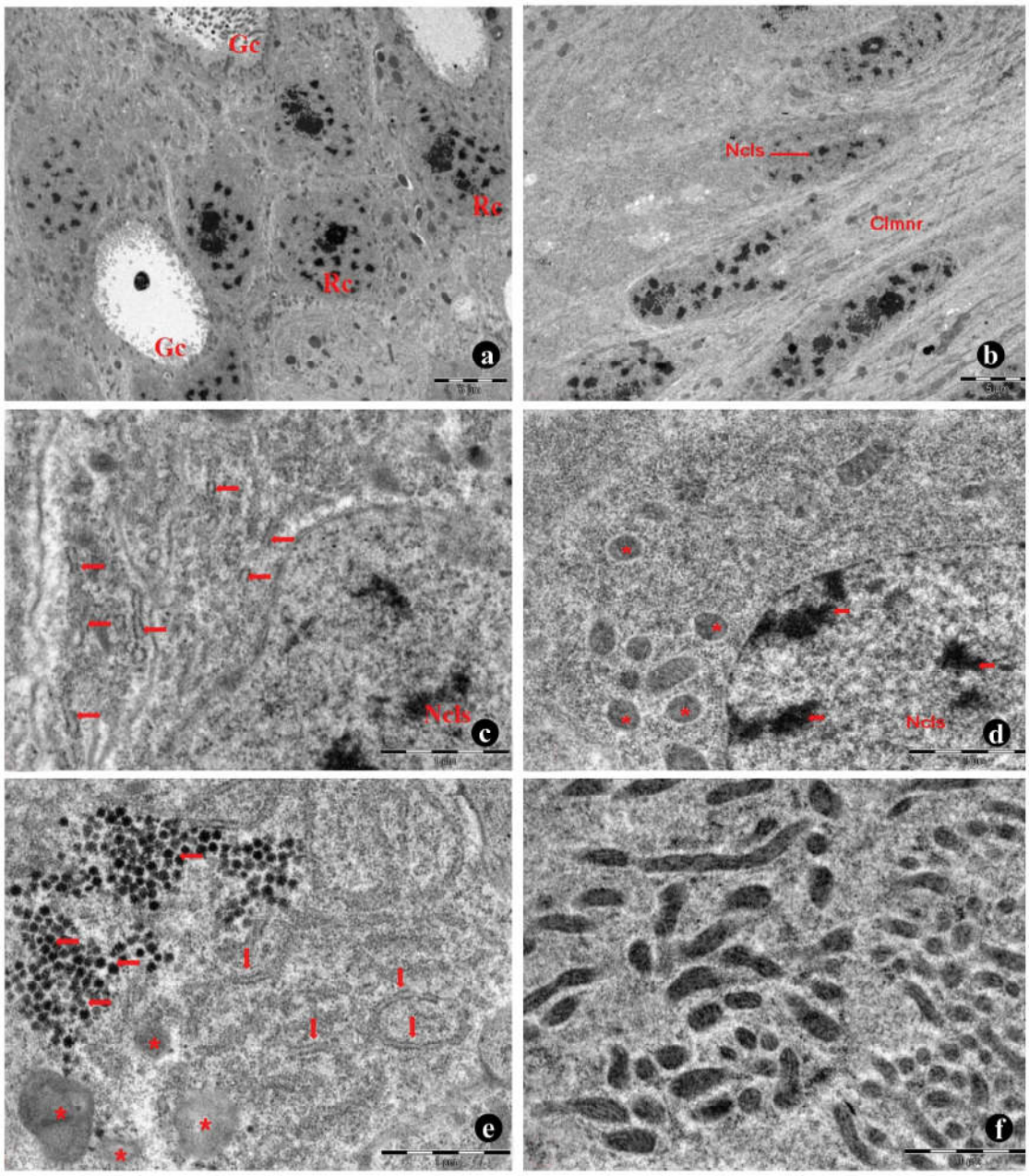


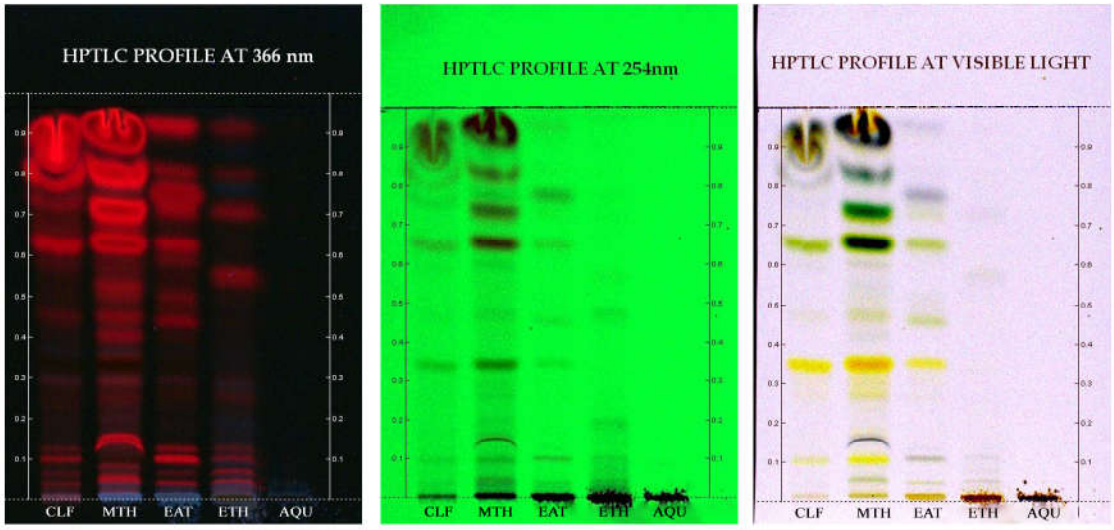
Figure 13. a. Ultrastructural view of columnar cells, regenerative cells and goblet cells of mid gut tissue of control larvae. (2900X).**b.** Columnar cells with distinct nucleus **c.** Nucleus of columnar cells and rich RER (arrows). (2900X).**d.** Secretory vessicles (asterisks) and nucleus having condensed chromatin grannules (arrows). (23000X). **e.** Showing numerous secretory grannules (arrows); secretory vessicles (asterisks); and RER (downward arrows) in columnar cytoplasm.(23000X). **f.** Rich in mitochondrial portion of columnar cell. (23000X).

Abbreviations:- Clmnr- Columnar Cell; Gc- Goblet cell; Ncls- Nucleus; Rc- Regenerative cell.

PLATE III.2

Experimental plants: - a. *Clerodendrum infortunatum* b. *Chromolaena odorata*

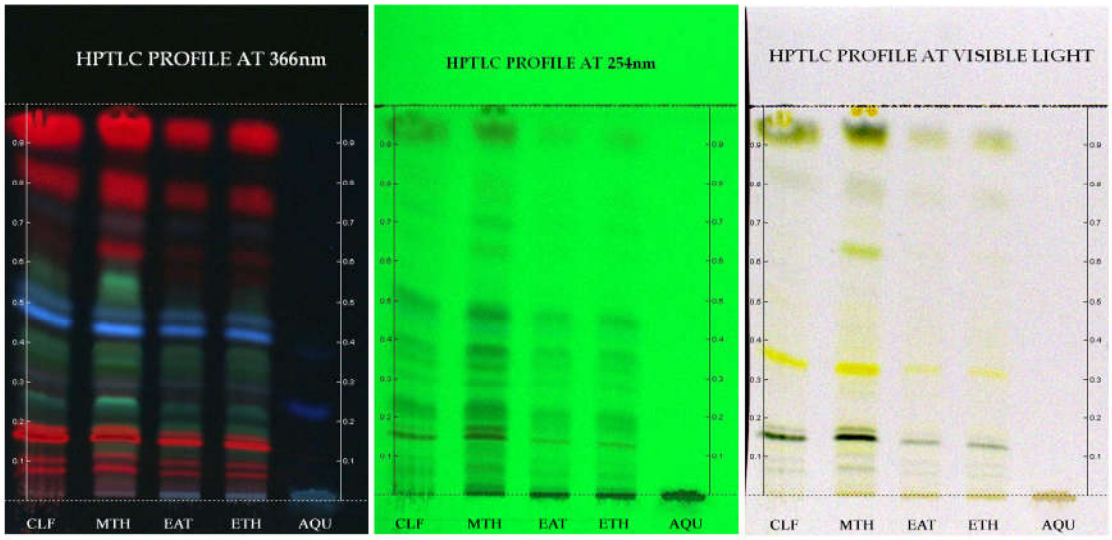
PLATE IV. 1



HPTLC profile of different polar solvent extracts of *Clerodendrum infortunatum*

Abbreviations:- CLF-Chloroform; MTH- Methanol; EAT- Ethyl acetate; ETH- Ethanol; AQU- Aqueous.

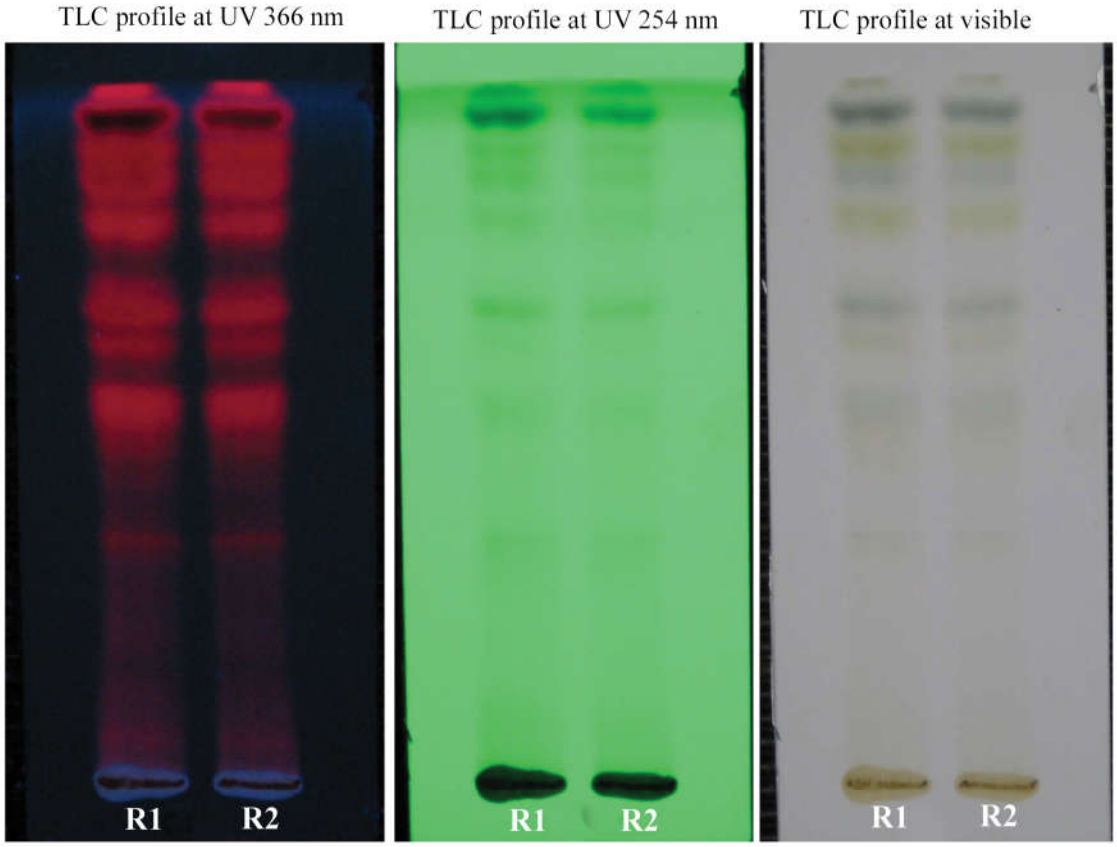
PLATE IV. 2



HPTLC profile of different polar solvent extracts of *Chromolaena odorata*

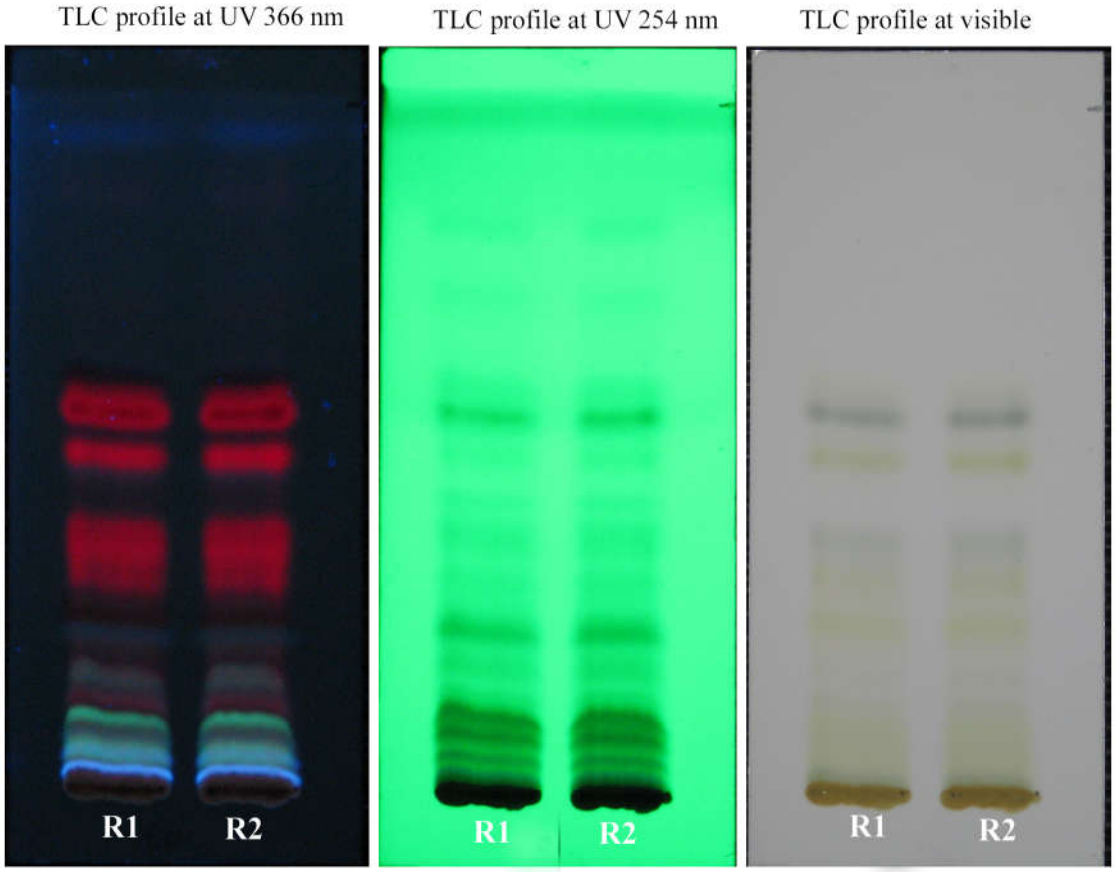
Abbreviations:- CLF-Chloroform; MTH- Methanol; EAT- Ethyl acetate; ETH- Ethanol; AQU- Aqueous.

PLATE IV. 3



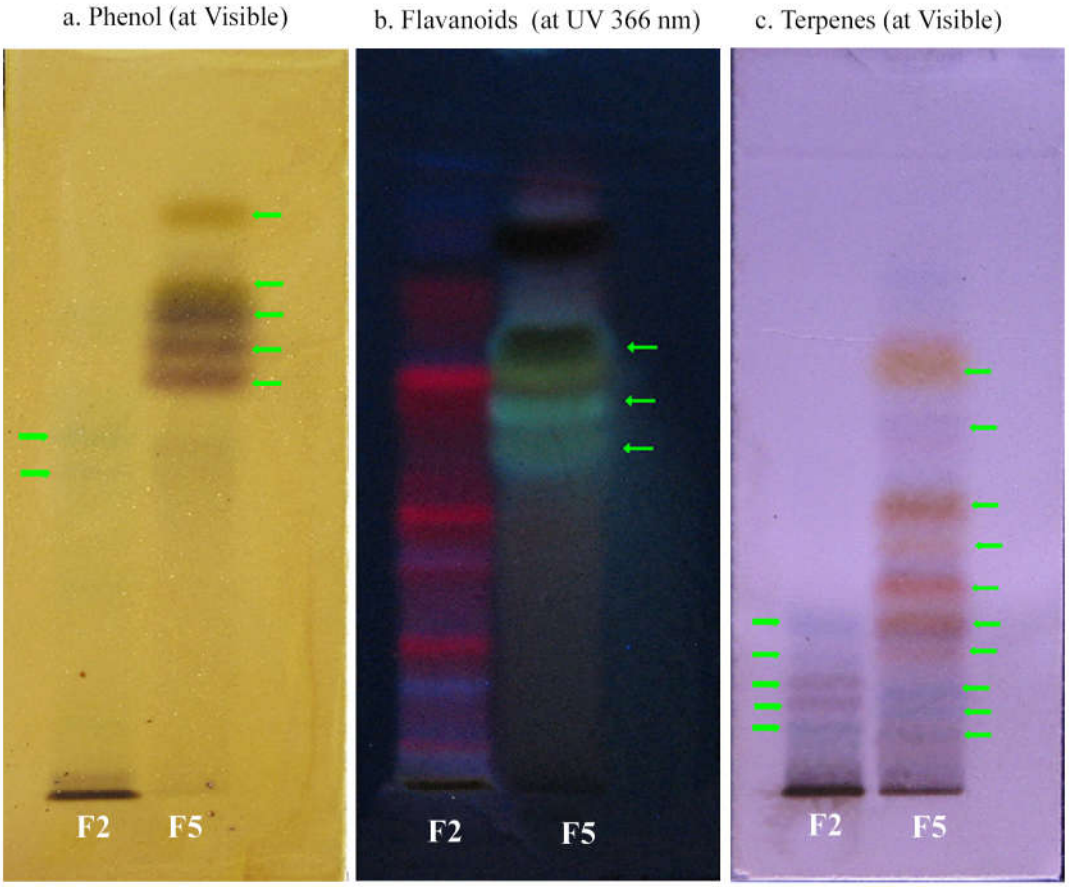
TLC profile of *Clerodendrum infortunatum* with different bands of separated fractions (two replicates R1 and R2).

PLATE IV. 4



TLC profile of *Chromolaena odorata* with different bands of separated fractions (two replicates R1 and R2).

PLATE IV. 5



The HPTLC profiles of fractions F2 and F5 showing the presence of Phenol, Flavonoids and Terpens.

PLATE V.1

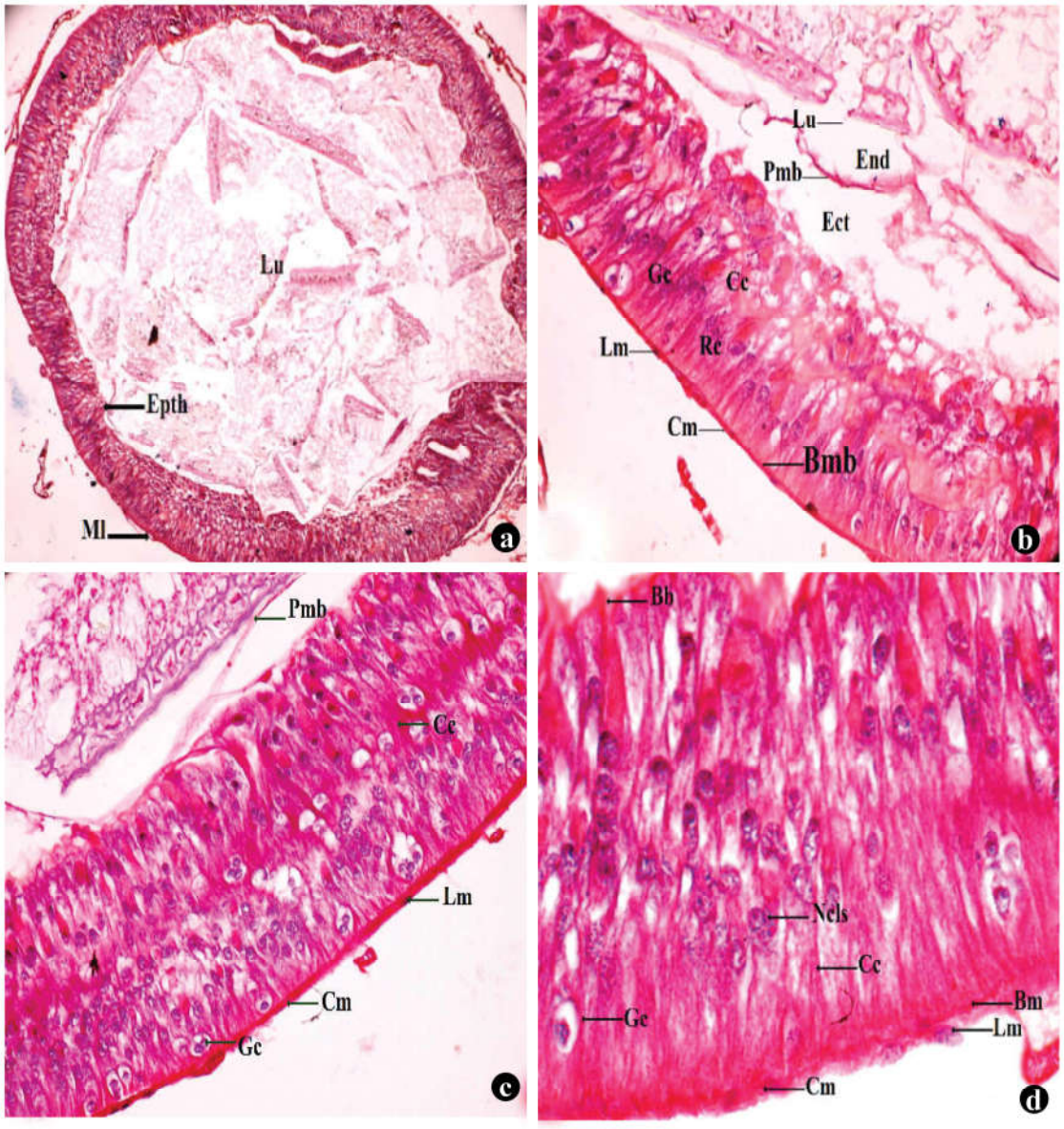


Figure 1.a. Cross section of the midgut tissue of untreated larva of *Orthaga exvinacea* (100X). **b** and **c.** General histomorphology of the midgut (400x); **d.** Showing an enlarged portion of different cells (1000X).

Abbreviations:- Bmb- Basement membrane; Cc- Columnar cells; Cm- Circular muscle layer; Ect- Ectoperitrophic spaces; End- Endoperitrophic spaces; Gc- Goblet cell; Lm- Longitudinal muscle layer; Lu- Lumen; Pmb- Peritrophic membrane; Rc- Regenerative cell.

PLATE V.2

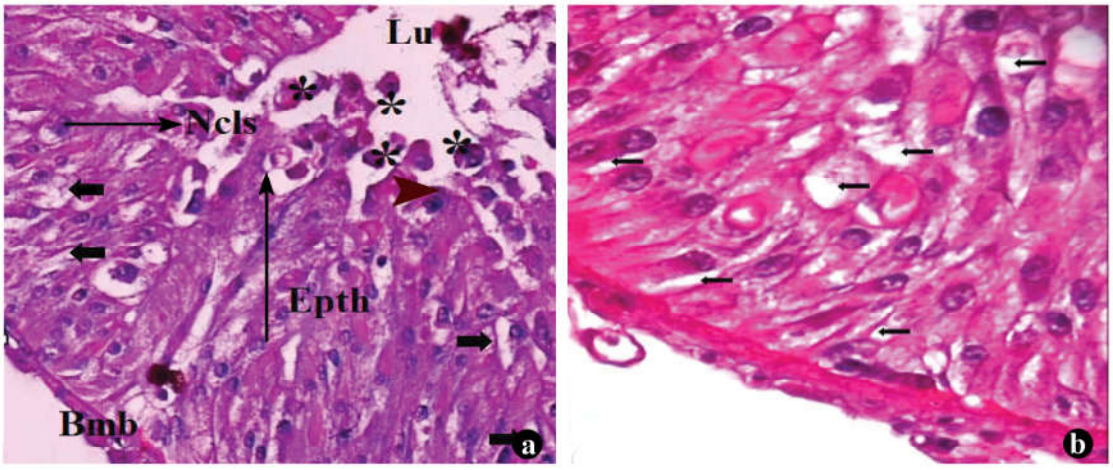


Figure 2.a. Transverse section of the midgut tissue of *Orthaga exvinacea* larva treated with 1% of *Clerodendrum infortunatum* showing elongation of epithelial cells (Long arrow); Vacuolization of cytoplasm (Thick arrows); enlargement of nucleus (Ncls); Secretory vesicles (asterisks) (400x). **b.** Enlarged portion showing cytoplasmic vacuolization (arrows) (1000X).

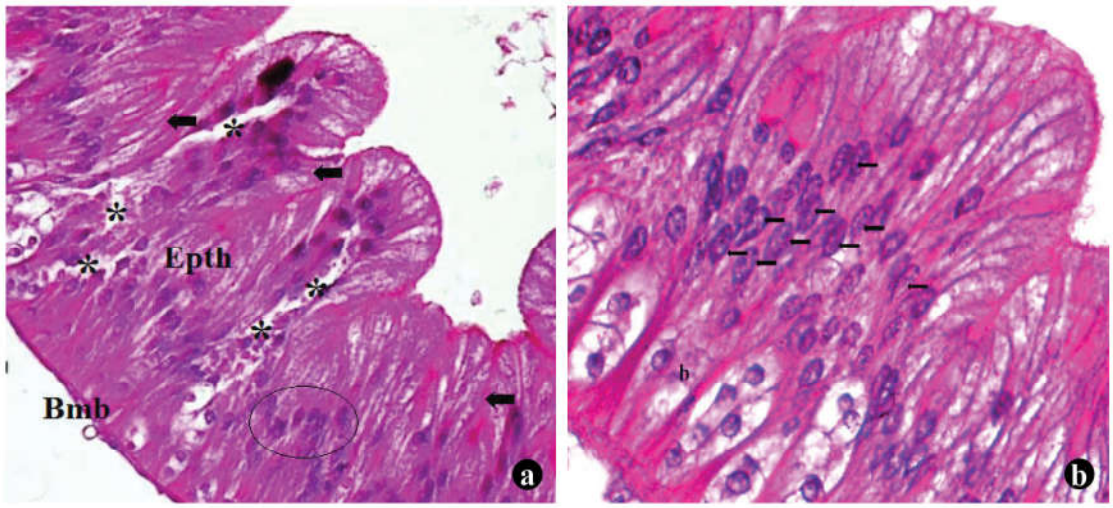


Figure 3.a. Cross section of the larval midgut treated with 2% *Clerodendrum infortunatum* showing the region of alternative folds and infolds in epithelial layer of migdut tissue overlapping (arrows); due to overlapping columnar nucleus became elongated and congested (in circle) and vacuolated (asterisks) (400x). **b.** Enlarged portion showing overlapping and congested nucleus (arrows). (1000X).

Abbreviations:- Bmb- Basement membrane; Epth- Epithelial cells; Lu- Lumen; Ncls- Nucleus.

PLATE V.3

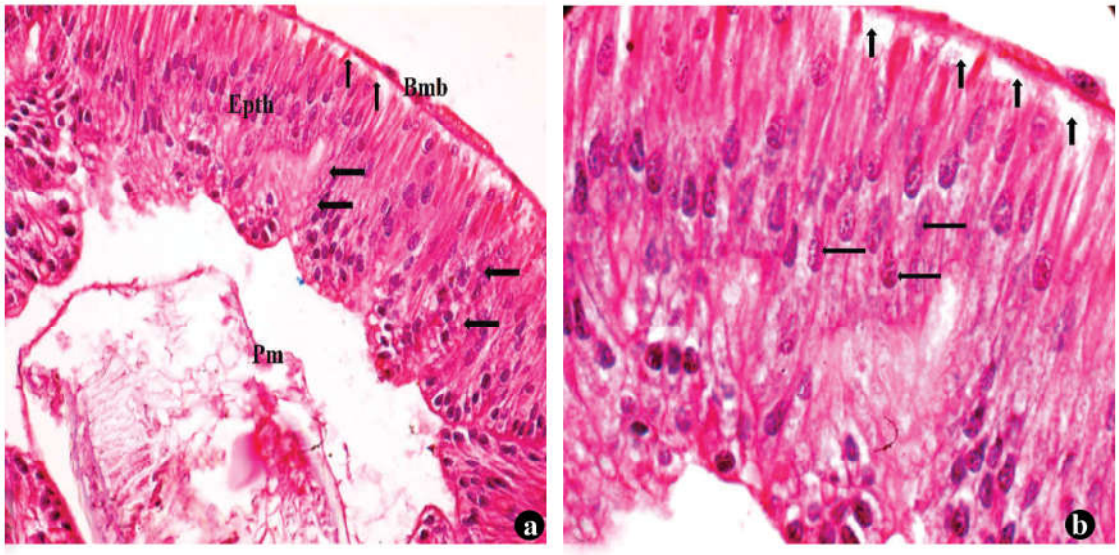


Figure 4.a. Cross section of the midgut tissue of larva treated with 3% *Clerodendrum infortunatum* showing the detachment of epithelial layer from the basement membrane (upward arrows); completely overlapped folded region (thick arrows) (400x).**b.** Enlarged portion showing detachment of epithelial cells (upward arrows) and elongated columnar nucleus (arrows) (1000X).

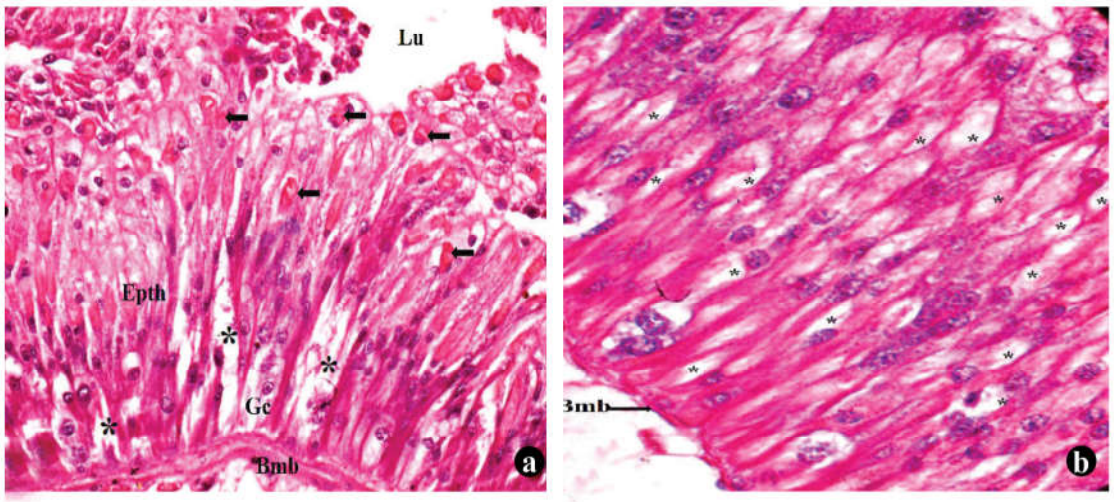


Figure 5.a. Midgut epithelium of larva treated with 4% showing the secretory vesicles (arrows); vacuoles due to excessive elongation (asterisks) (400x). **b.** Enlarged view showing vacuolated portion (asterisks) (1000X).

Abbreviations:- Bmb- Basement membrane; Epth- Epithelial cells; Gc- Goblet cells; Lu- Lumen.

PLATE V.4

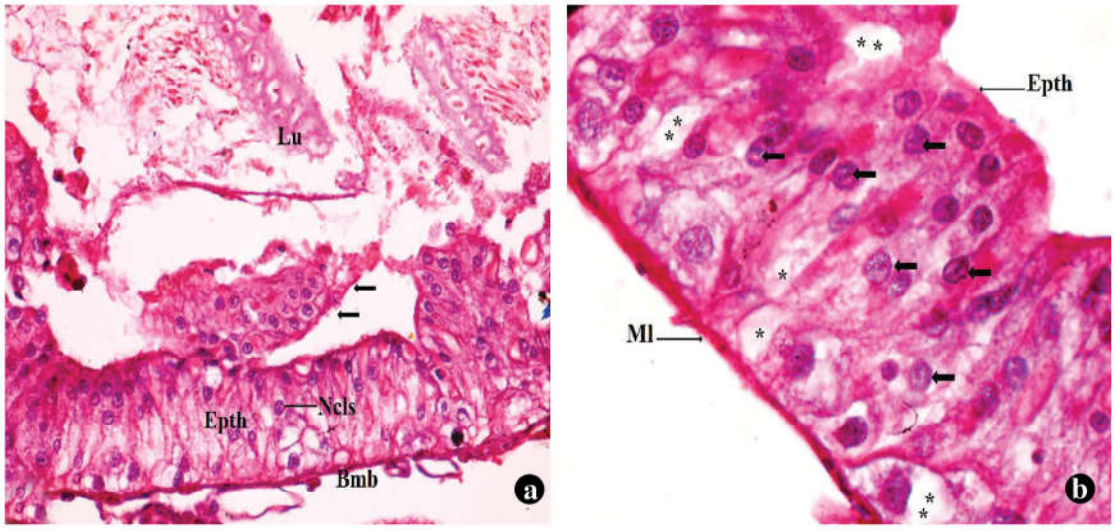


Figure 6. a. The larval midgut treated with 5% *Clerodendrum infortunatum* showing that due to excessive elongation, the apical region of the columnar cells get sloughed off and move into the lumen (arrows); size of nucleus decreased (Ncls)(400x). **b.** Enlarged portion showing vacuoles (asterisks) and size decrease in nucleus (arrows) (1000X).

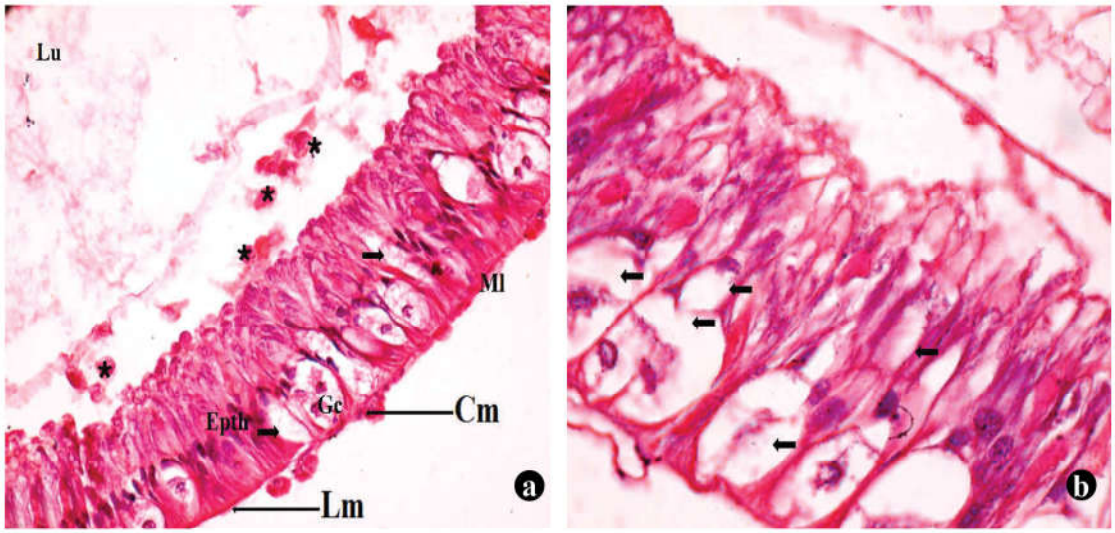


Figure 7.a. Cross section of the midgut tissue of *Orthaga exvinacea* larva treated with 1% *Chromolaena odorata* showing the vacuolization (arrows); Secretory vesicles (asterisks); Size of goblet cell get increased (Gc) (400x). **b.** Enlarged portion showing vacuolization (arrows). (1000X).

Abbreviations:- Bmb- Basement membrane; Cm- Circular muscle layer; Epth- Epithelial cells; Gc- Goblet cells; Lm- Longitudinal muscle layer; Lu- Lumen; Ml- Muscle layer; Ncls- Nucleus.

PLATE V.5

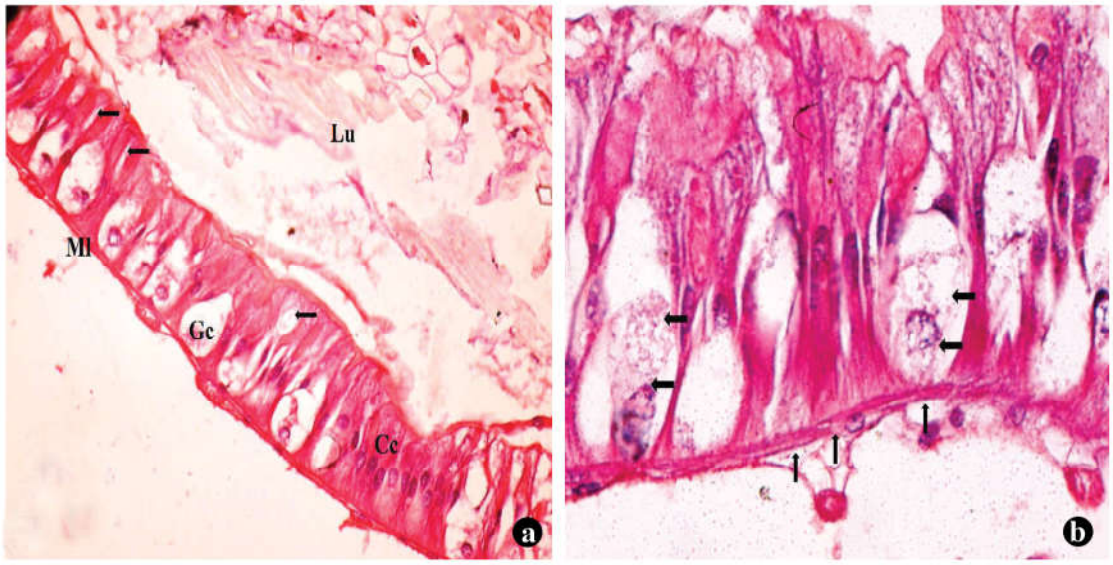


Figure 8.a. Larval midgut tissue treated with 2% of *Chromolaena odorata* showing vacuolization (arrows); Size of goblet cell increased (Gc) as in 1% (400x). **b.** Showing the swelling of goblet cells (arrows) and thinning of muscle layer (upward arrows) (1000X).

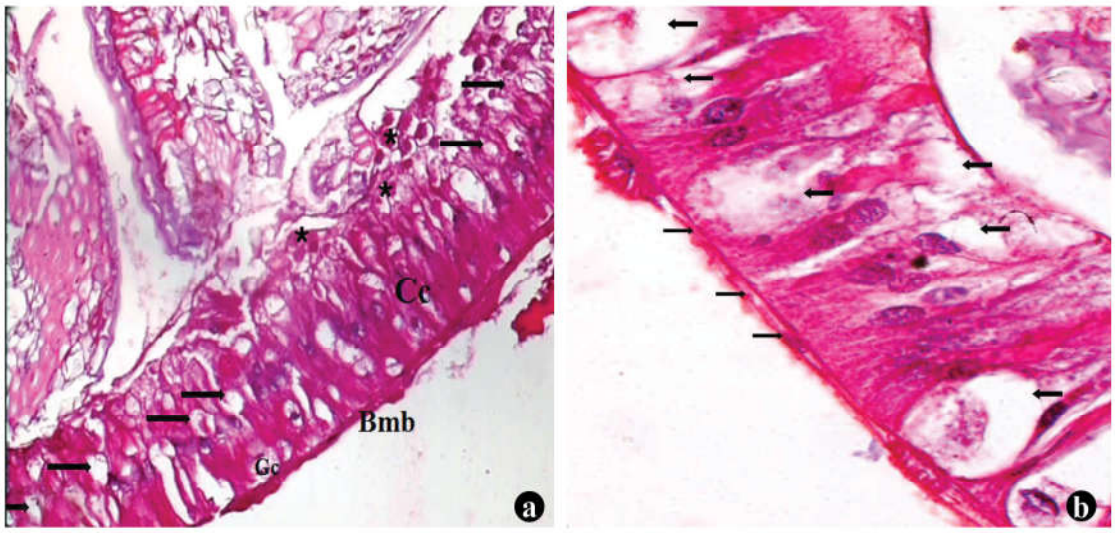


Figure 9.a. Cross section of the midgut of *Orthaga exvinacea* larva treated with 3% of *Chromolaena odorata* showing the excessive vacuolization and destruction of the apical region of epithelial cells (arrows) and secretory vesicles (asterisks) (400x). **b.** Showing vacuolization and thinning of muscle layer (arrows) (1000X).

Abbreviations:- Bmb- Basement membrane; Cc- Columnar cells; Gc- Goblet cells; Lu- Lumen; Ml- Muscle layer.

PLATE V. 7

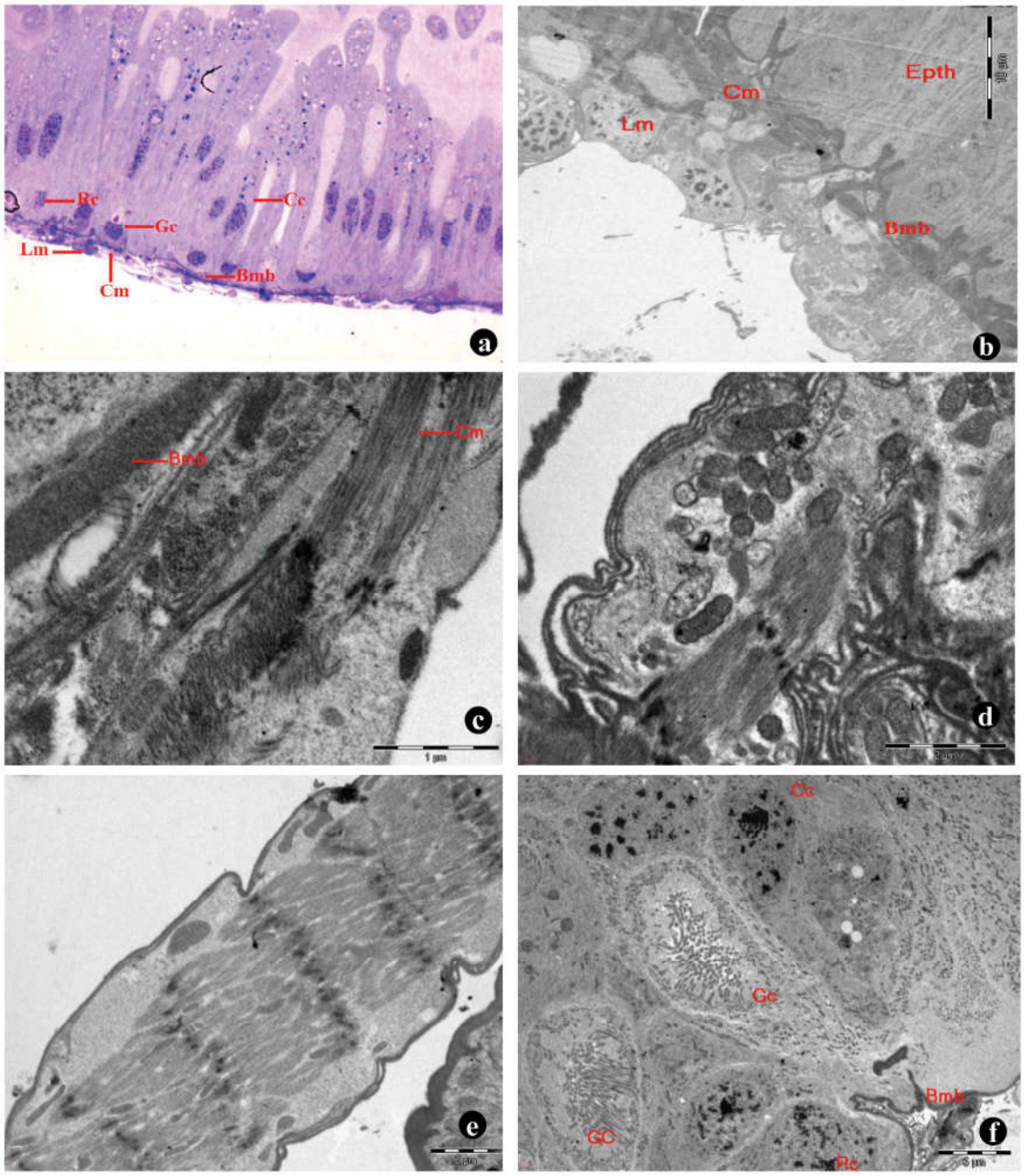


Figure 12. a. General aspects of the mid gut tissue of the sixth instar larvae of *Orthaga exvinacea* showing both muscle and epithelial layer (Semi thin section)(1000X). **b.** Ultrastructural aspects of longitudinal muscle layer (Lm); Circular muscle layer (Cm); basement membrane (Bmb). (1900X). **c.** Magnified portion of muscle layer and basement membrane (Bmb) (23000X). **d.** Longitudinal muscle layer (11000X). **e.** Circular muscle layer. (6800X).**f.** Ultrastructural aspects of different epithelial cells. (2900X).

Abbreviations:- Bmb- Basement membrane; Cc- Columnar cells; Cm- Circular muscle layer; Epth- Epithelial cells; Gc- Goblet cell; Lm- Longitudinal; Rc- Regenerative cell.

PLATE V. 9

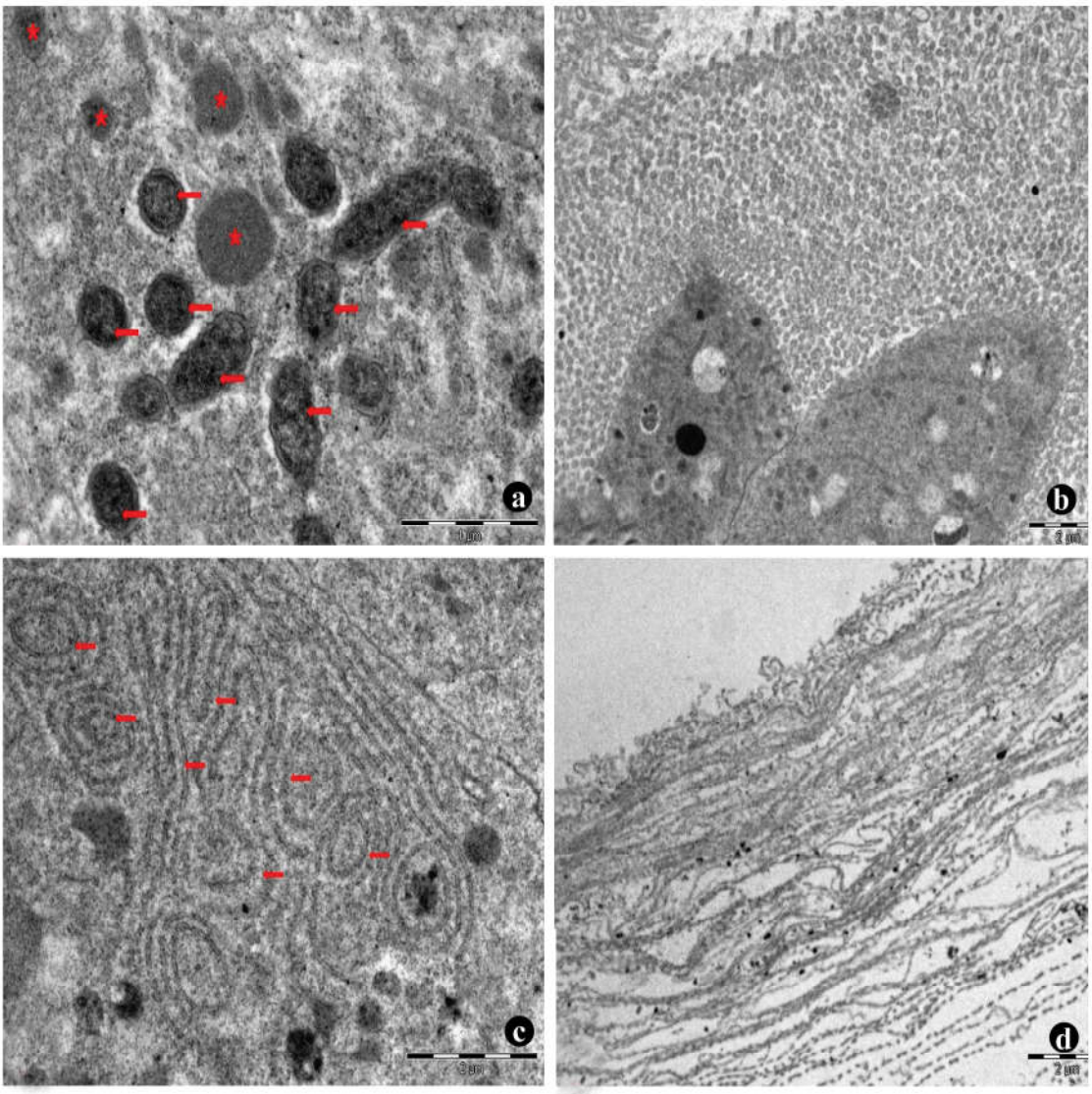


Figure 14 a. Enlarged portion of secretory vessicles (asterisk) and mitochondria (arrows) present in columnar apical portion. (23000X). **b.** Showing apical region of columnar cells with numerous microvilli. (6800X). **c.** Abundant SER in apical region of columnar cells.(11000X). **d.** A magnified portion of peritrophic membrane.(6800X).

PLATE V. 10

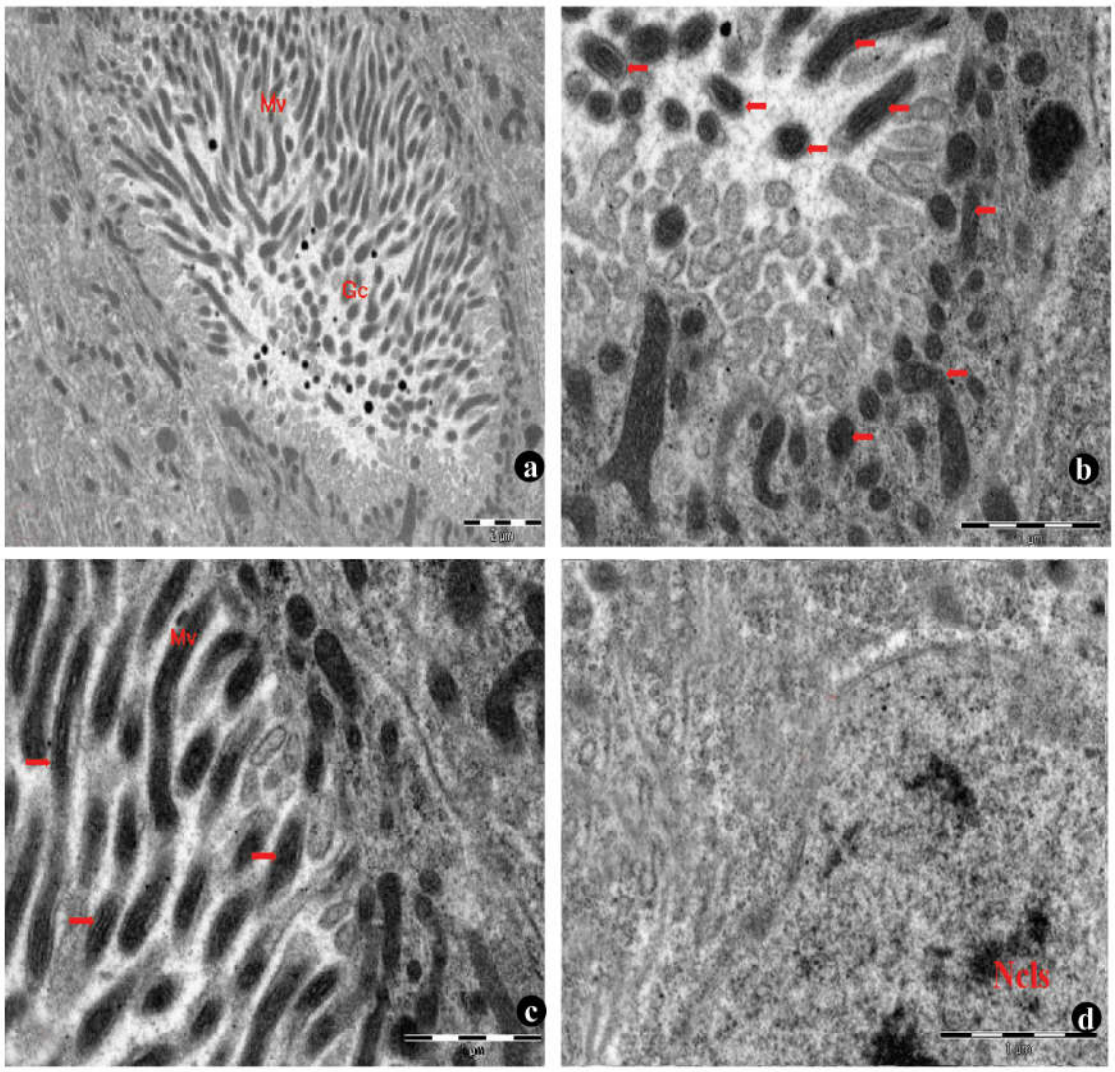


Figure 15. a. Ultrastructural aspects of goblet cells (6800X). **b.** Showing numerous microvilli inside goblet cells (arrows).(23000X). **c.** Magnified portion of microvilli rich in mitochondria (arrows). (23000X).**d.** Enlarged portion of nucleus of goblet cells.

Abbreviations: - Gc- Goblet cell; Mv- Microvilli; Ncls- Nucleus.

PLATE V. 11

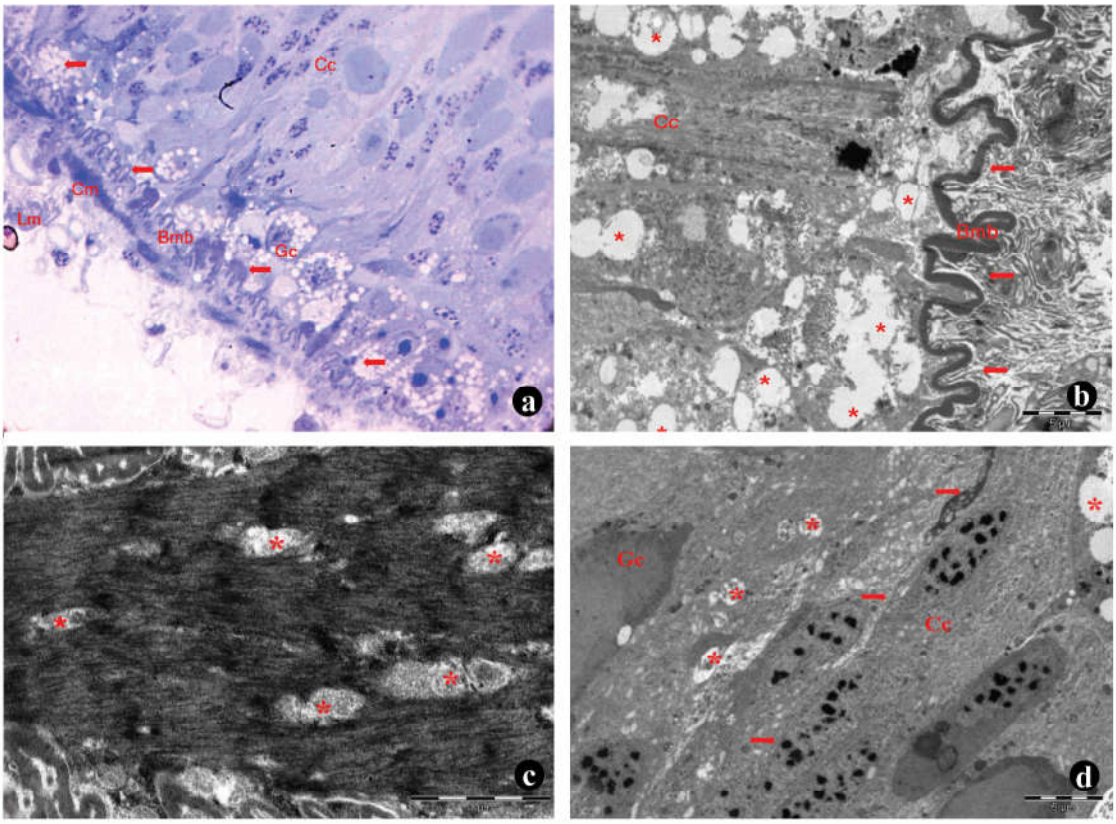


Figure 16. a. Semi thin section of larval midgut treated with 5% of *Clerodendrum infortunatum* showing excessive epithelial vacuolation in the basal region (arrows) (1000X). **b.** Ultrastructural view of vacuolation (asterisks) and detachment of epithelial cells from the muscle layer (arrows). (2900X) **c.** Separation of myofilaments and vacuole like formation in the circular muscle layer (asterisk).(23000X). **d.** Showing the excessive elongation of epithelial cells (arrows) and cytoplasmic vacuolization (asterisks). (2900X).

Abbreviations:- Bmb- Basement membrane; Cc- Columnar cells; Cm- Circular muscle layer; Gc- Goblet cells; Lm- Longitudinal muscle layer.

PLATE V. 12

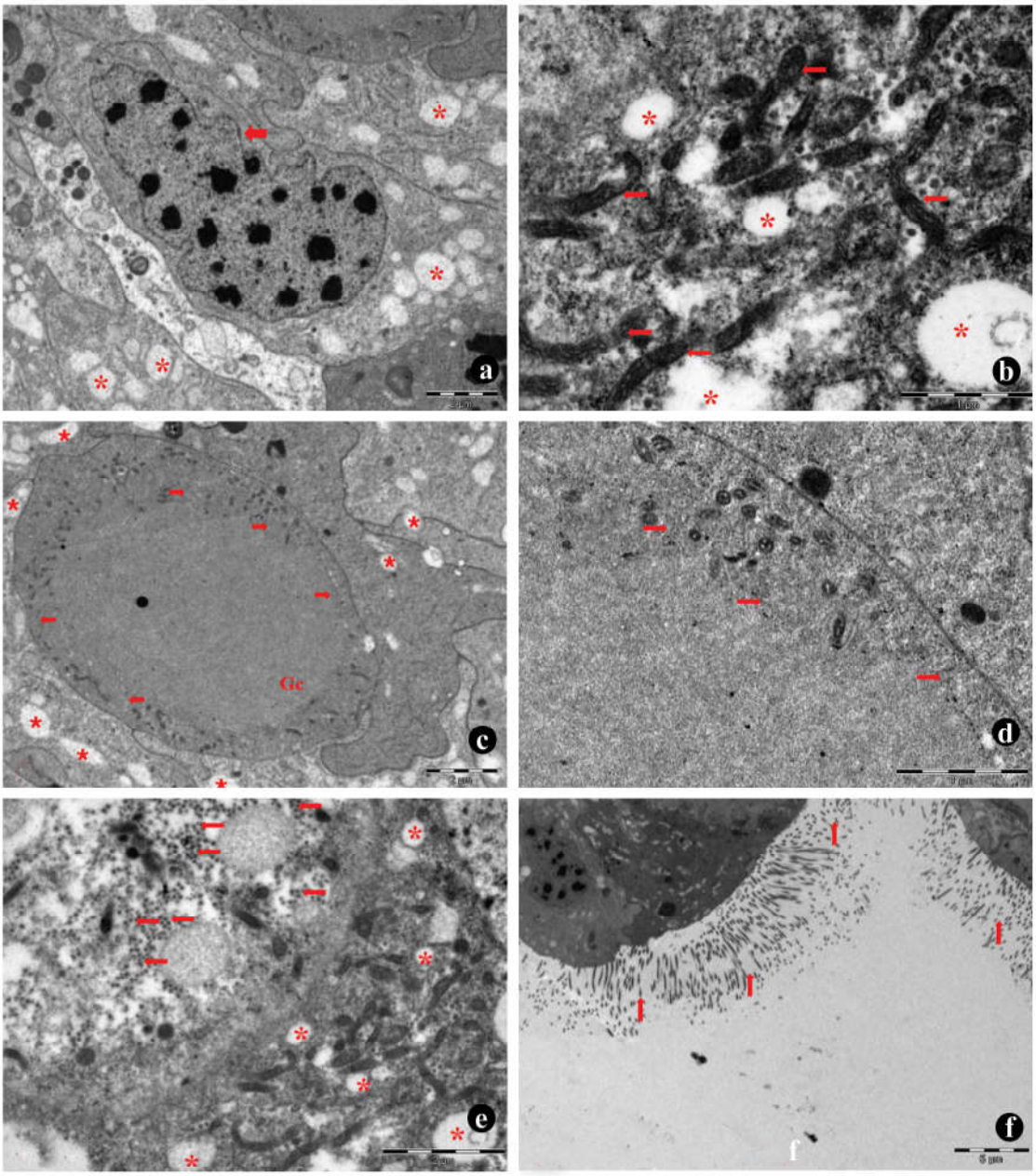


Figure 17 a. Exessive vacuolization in columnar cytoplasm (asterisks) and shrunken nucleus (arrow) due to the treatment of *Clerodendrum infortunatum*. (6800X). **b.** Showing shape of mitochondria were changed (arrows) and vacuole formation (asterisk).(23000X). **c.** Showing the effect on goblet cells with ruptured microvilli inside the goblet cells (arrows) and vacuolization (asterisk).(6800X). **d.** Magnified view of ruptured microvilli with shrunken mitochondria inside the goblet cells (arrows).(23000X).**e.** Excessive vacuoles and secretory granules present in just near to the goblet cells. **f.** Loss of microvilli in the apical region of columnar cells (arrows). (2900X).

Abbreviations: - Gc- Goblet cell.

PLATE V. 13

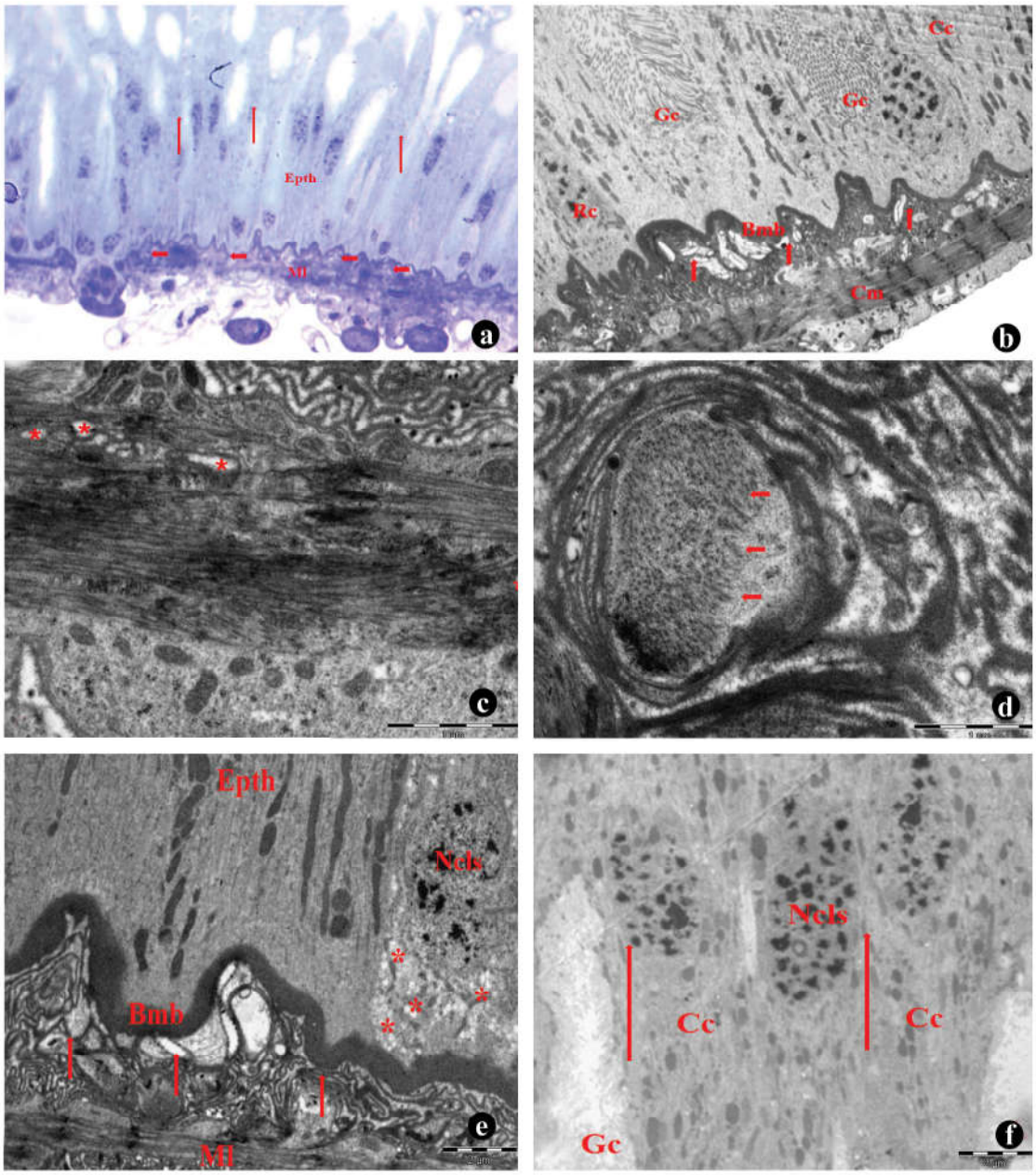


Figure 18. a. Semi thin section of midgut tissue of *O. exvinacea* treated with 4% of *C. odorata* showing elongation (upward arrows) and detachment of epithelial cells from the basement membrane (arrows).(1000X). **b.** Ultrastructuaral view of detachment of epithelium from muscle layer (arrows) and change in shape of regenerative cells (2900X). **c.** Separation of myofilaments in circular muscle layer and vacuole like formation (asterisks).(23000X). **d.** Showing shrunken myofilaments in longitudinal muscle layer (arrows). **e.** Magnified portion of separation of basement membrane (arrows) and some vacuoles (asterisks) (6800X). **f.** Showing excessive elongation of epithelial cells (arrows).(1900X).

Abbreviation :- Bmb- Basement membrane; Cc- Columnar cells; Cm- Circular muscle layer; Epth- Epithelial cells; Gc- Goblet cell; Ml- Muscle layer; Ncls- Nucleus; Rc- Regenerative cell.

PLATE V. 14

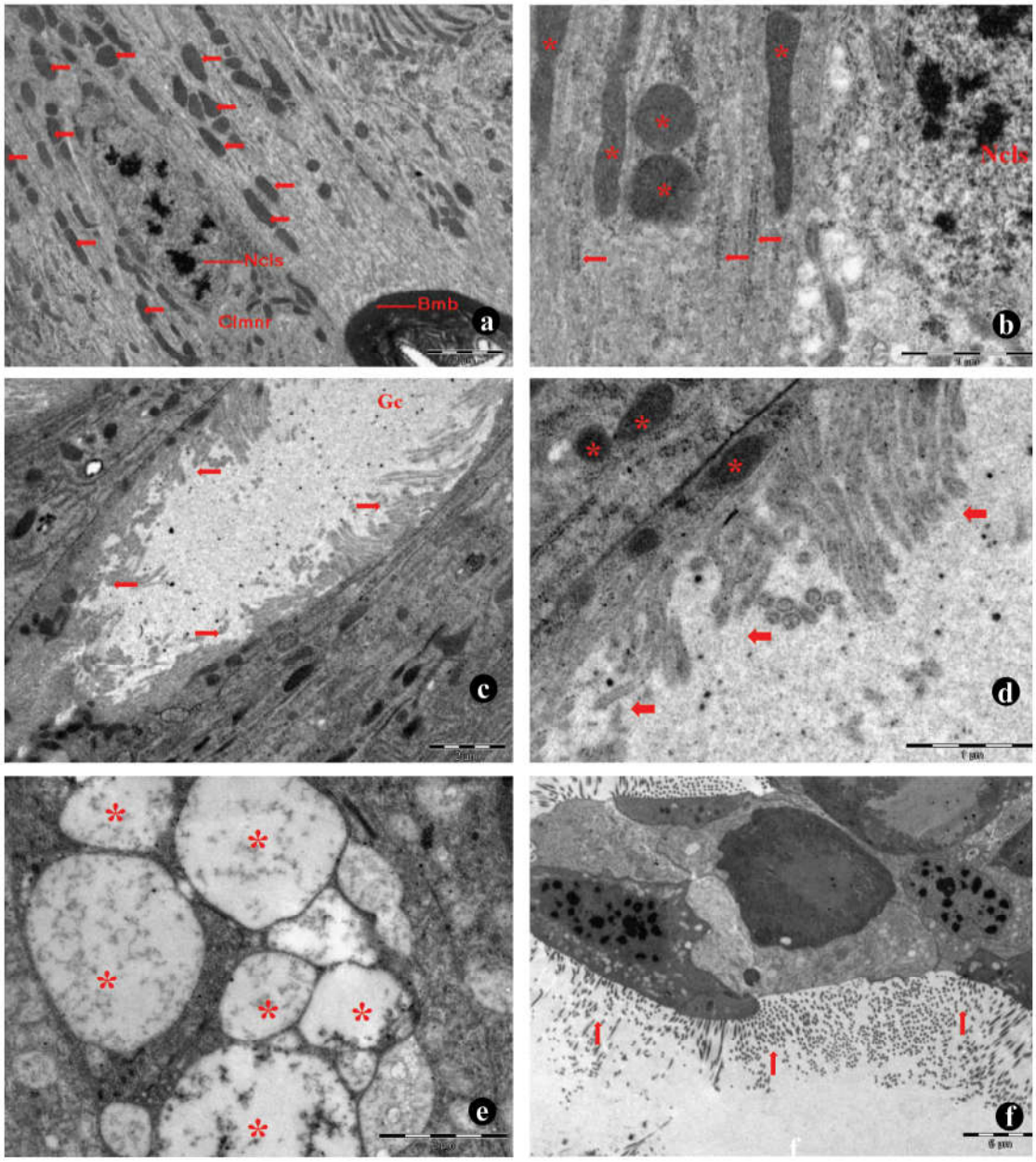
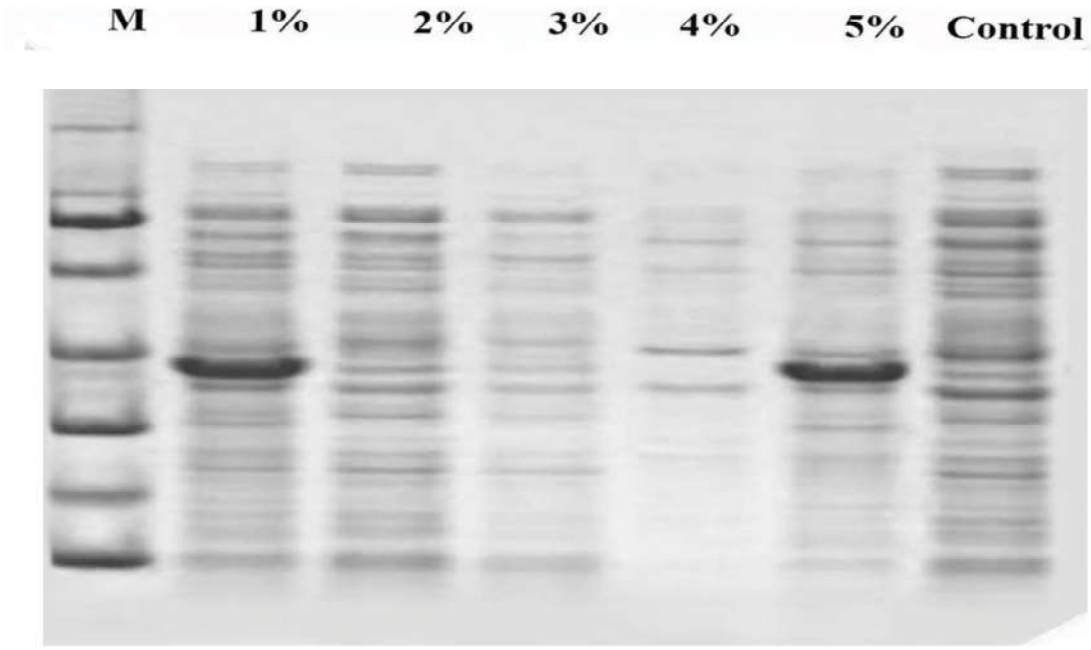


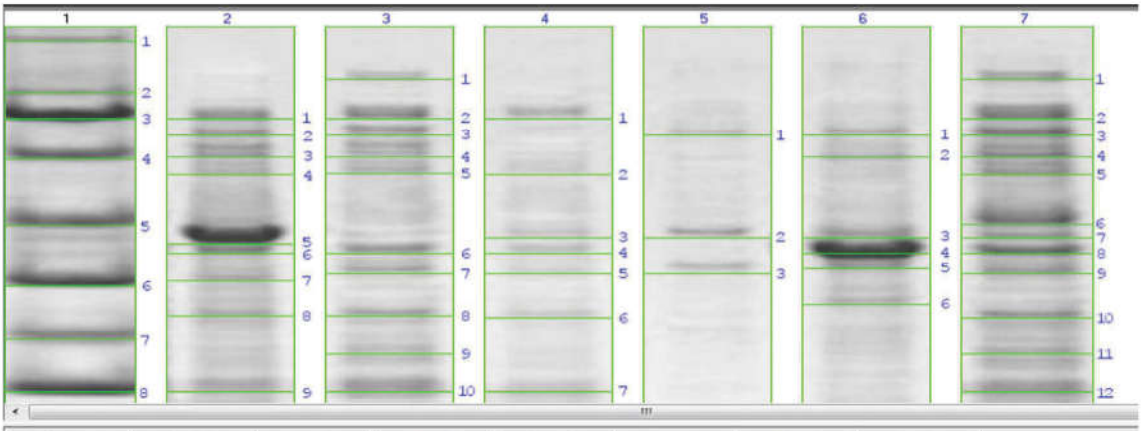
Figure 19.a Numerous secretory vesicles present beside columnar nucleus (asterisks).(6800X). **b.** Magnified view of secretory vesicles (asterisk) and reduced number of RER (arrows).(23000X).**c.** Showing goblet cells with ruptured microvilli (arrows).(6800X). **d.** An enlarged portion showing ruptured microvilli (arrows) and secretory vesicles (asterisks).(23000X).**e.** A magnified portion of vacoules filled with amorphous materials. **f.** Showing loss of microvilli in apical region of columnar cells. (arrows). (2900X).

Abbreviations:- Bmb- Basement membrane; Clmnr- Columnar cell; Ncls- Nucleus

PLATE VI. 1



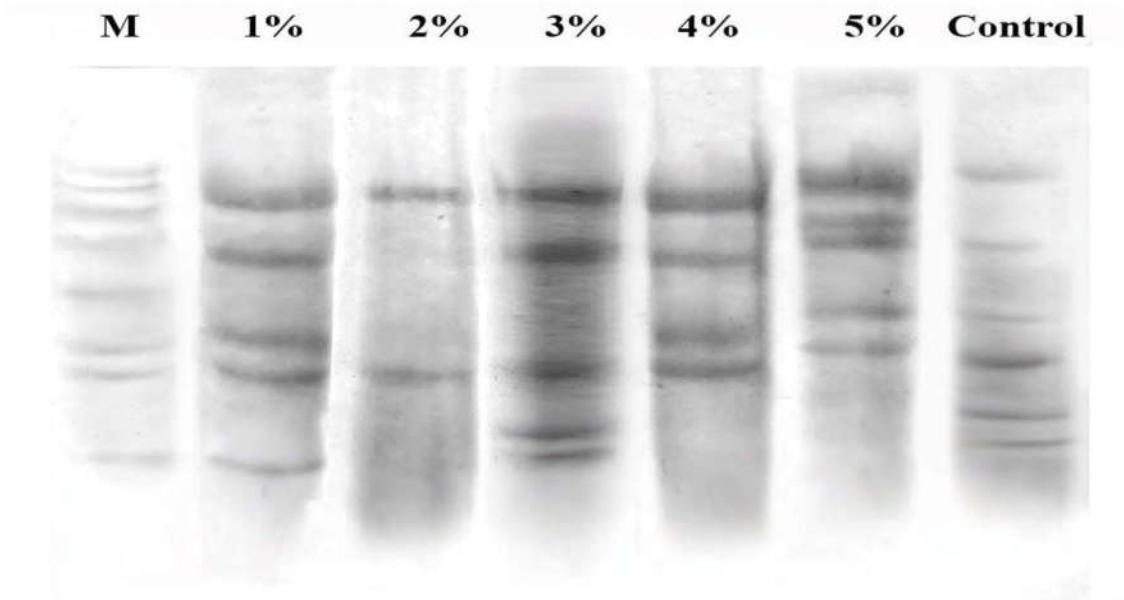
SDS-PAGE gel image showing protein bands of midgut tissue treated with *Clerodendrum infortunatum*.



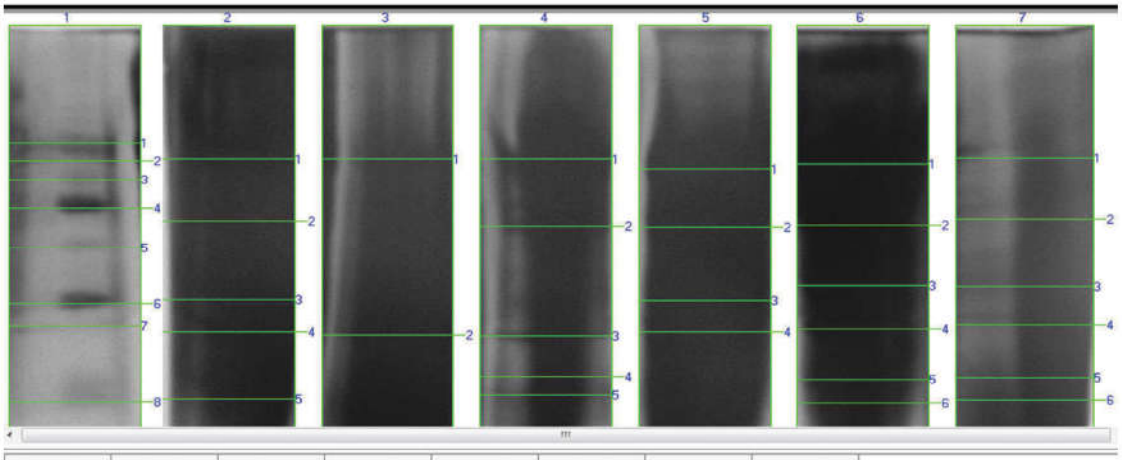
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	116	77.62	102.3	77.62	58.88	58.88	102.3
Band 2	97.4	58.88	77.62	44.77	30.11	50.11	77.62
Band 3	66.2	50.11	58.88	30.11	28.18	30.11	58.88
Band 4	45.2	44.77	50.11	29.9		30.19	50.11
Band 5	31	30.9	44.77	28.18		29.51	44.77
Band 6	25	29.19	29.9	21.37		24.54	35.68
Band 7	18.4	24.54	28.18	14.12			30.11
Band 8	14.4	21.37	21.37				29.9
Band 9		14.12	17.37				28.18
Band 10			14.12				21.37
Band 11							17.37
Band 12							14.12

Gel documentation showing molecular weights of different protein bands of midgut tissue treated with *Clerodendrum infortunatum*.

PLATE VI. 2



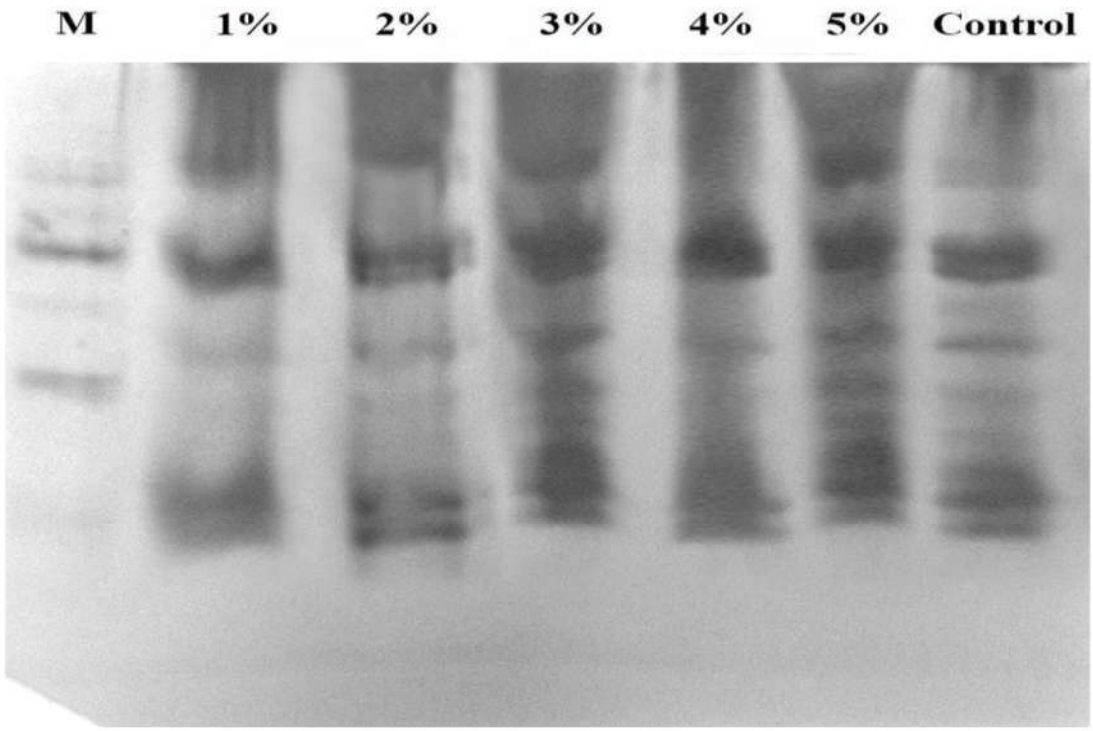
SDS-PAGE gel image showing protein bands of fat body tissue treated with *Clerodendrum infortunatum*.



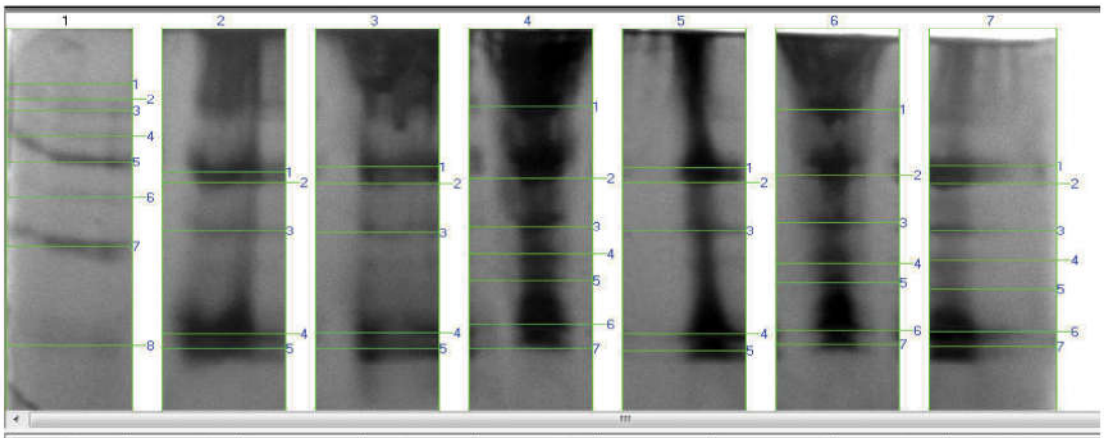
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	91.117	91.117	91.117	85.565	88.321	91.675
Band 2	90.000	66.312	36.831	64.959	64.695	65.225	66.867
Band 3	80.000	51.467		36.527	51.116	55.368	55.146
Band 4	70.000	37.799		30.369	37.799	38.854	40.403
Band 5	60.000	29.997		29.998		30.249	30.325
Band 6	50.000					29.868	29.997
Band 7	40.000						
Band 8	30.000						

Gel documentation showing molecular weights of different protein bands of fat body tissue treated with *Clerodendrum infortunatum*.

PLATE VI. 3



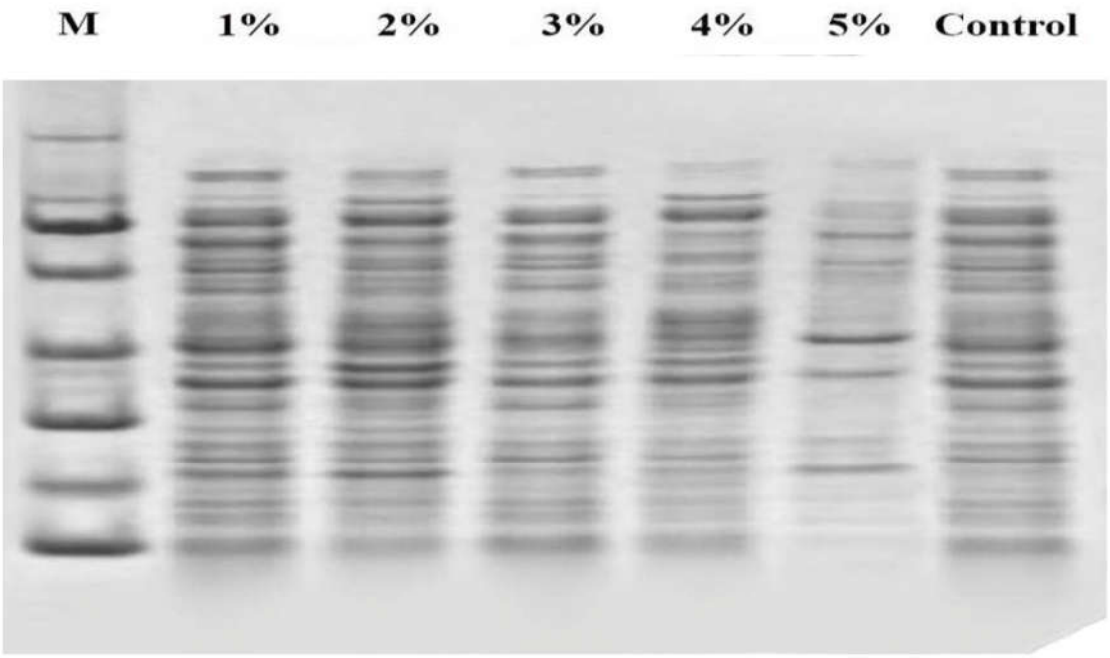
SDS-PAGE gel image showing protein bands of haemolymph treated with *Clerodendrum infortunatum*.



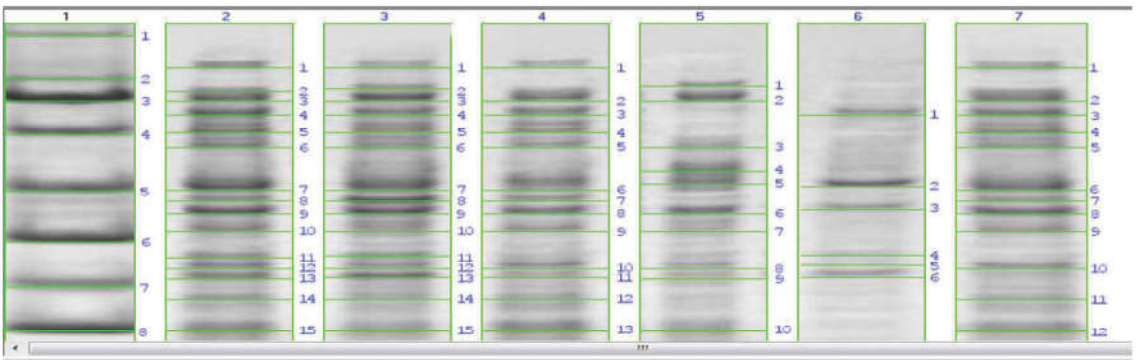
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	56.931	58.461	83.287	58.154	80.764	58.768
Band 2	90.000	53.934	53.641	55.119	53.934	56.021	53.641
Band 3	80.000	42.681	42.298	43.472	42.681	44.297	42.681
Band 4	70.000	30.856	30.934	38.894	30.856	37.593	38.012
Band 5	60.000	29.681	29.681	35.580	29.468	35.352	34.696
Band 6	50.000			31.574		31.092	31.013
Band 7	40.000			29.681		30.077	29.894
Band 8	30.000						

Gel documentation showing molecular weights of different protein bands of haemolymph treated with *Clerodendrum infortunatum.*

PLATE VI. 4



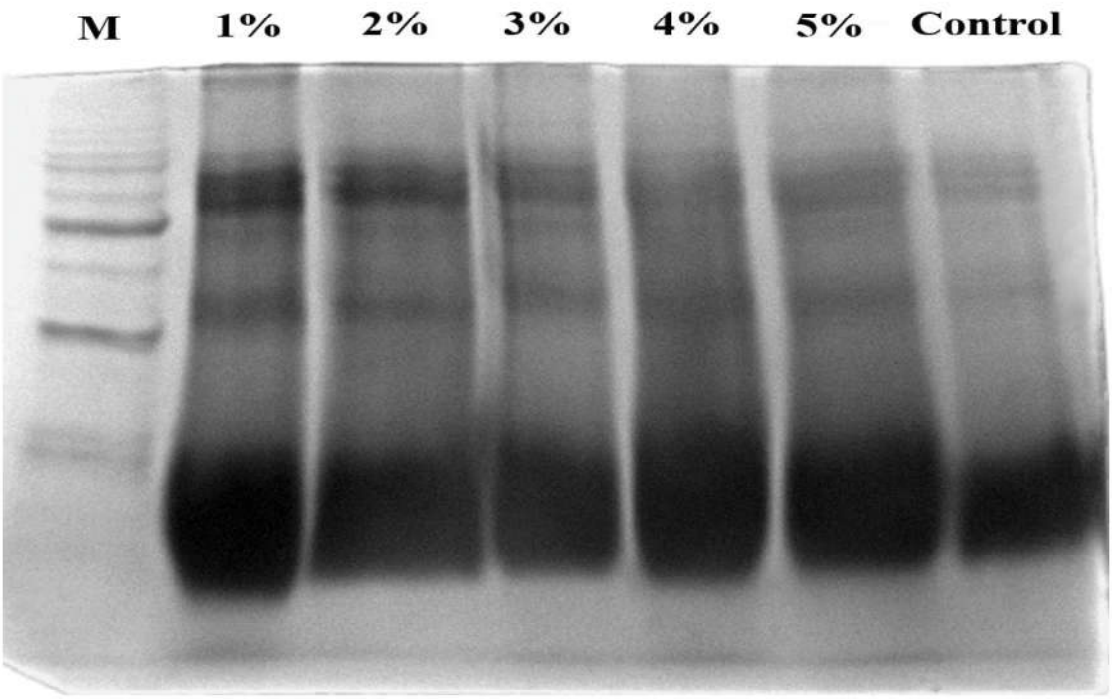
SDS-PAGE gel image showing protein bands of midgut tissue treated with *Chromolaena odorata*.



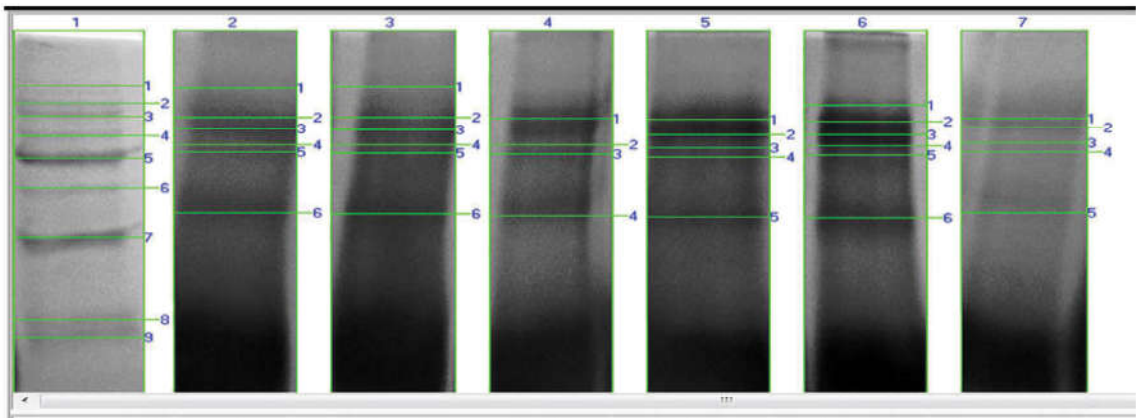
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	116	102.3	102.3	102.3	95.49	58.88	102.3
Band 2	97.4	79.43	89.12	77.62	77.62	44.77	77.62
Band 3	66.2	77.62	77.62	58.88	44.77	33.88	58.88
Band 4	45.2	58.88	58.88	50.11	36.3	28.84	50.11
Band 5	31	50.11	50.11	44.77	34.67	21.37	44.77
Band 6	25	44.77	44.77	35.68	29.9	19.49	35.68
Band 7	18.4	35.68	35.68	30.11	28.18		30.11
Band 8	14.4	30.11	30.11	29.9	21.37		29.9
Band 9		29.9	29.9	28.18	19.05		28.18
Band 10		28.18	28.18	21.37	14.12		21.37
Band 11		22.9	23.44	19.9			17.37
Band 12		21.37	21.37	17.37			14.12
Band 13		19.05	19.49	14.12			
Band 14		17.37	17.37				
Band 15		14.12	14.12				

Gel documentation showing molecular weights of different protein bands of midgut tissue treated with *Chromolaena odorata*.

PLATE VI. 5



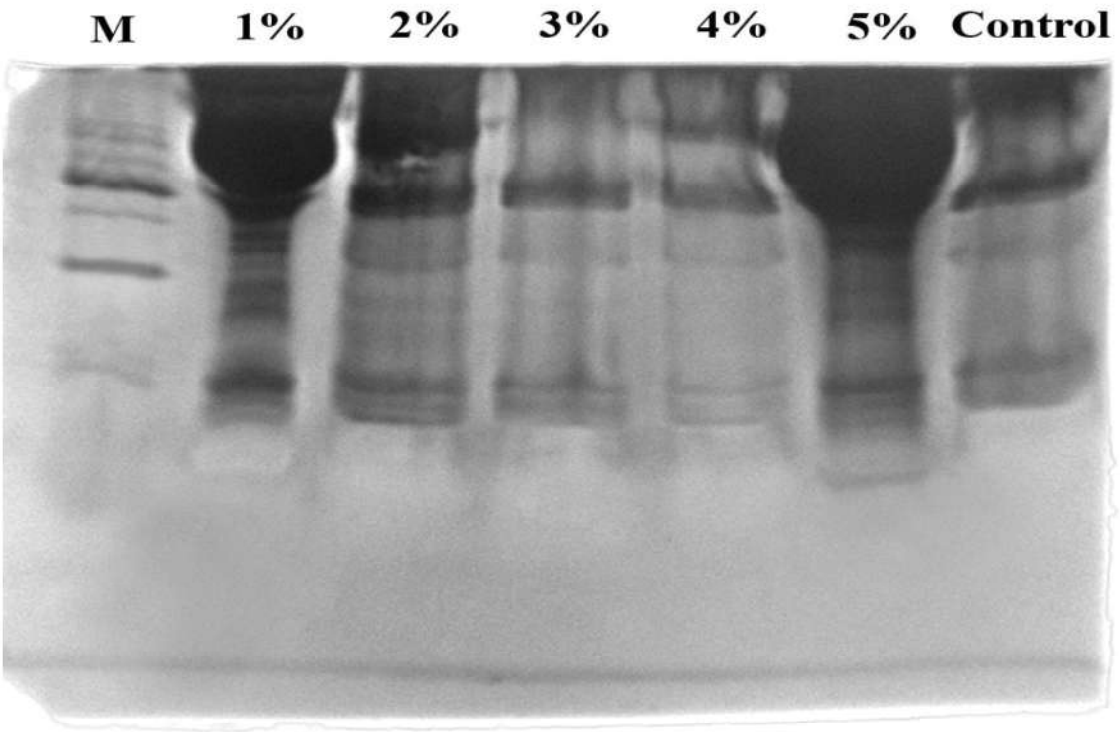
SDS-PAGE gel image showing protein bands of fat body treated with *Chromolaena odorata*.



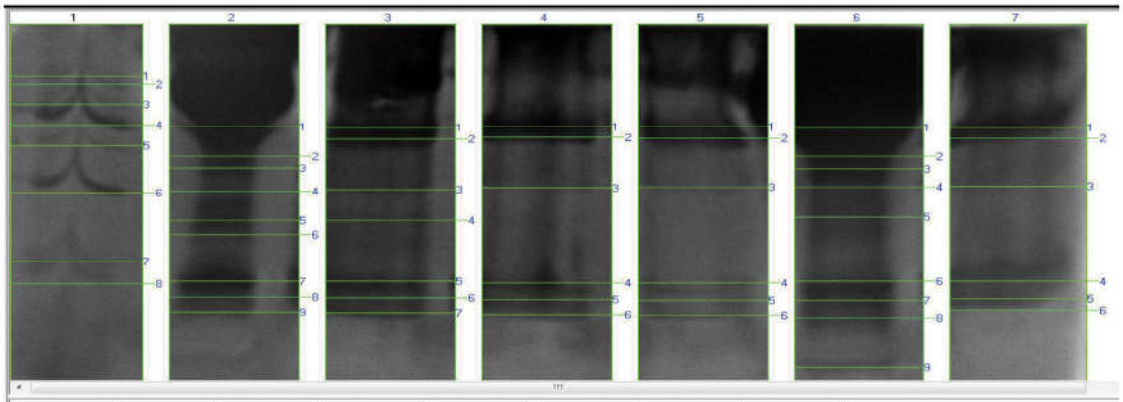
M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	98.958	99.480	78.924	78.386	88.365	78.924
Band 2	90.000	79.462	79.462	65.767	70.504	77.313	74.124
Band 3	80.000	73.599	73.076	61.705	64.385	70.504	66.698
Band 4	70.000	65.767	65.767	43.241	60.419	65.304	62.584
Band 5	60.000	62.584	62.142		43.038	61.272	43.873
Band 6	50.000	43.873	43.659			42.838	
Band 7	40.000						
Band 8	30.000						
Band 9	20.000						

Gel documentation showing molecular weights of different protein bands of fat body treated with *Chromolaena odorata*.

PLATE VI. 6



SDS-PAGE gel image showing protein bands of haemolymph treated with *Chromolaena odorata*.



M.W. Values	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
Band 1	100.000	69.130	68.696	69.130	69.130	68.696	68.696
Band 2	90.000	57.302	62.880	63.663	63.269	57.302	63.269
Band 3	80.000	54.554	50.459	50.708	50.837	54.368	50.970
Band 4	70.000	50.222	47.531	30.407	30.407	50.837	31.220
Band 5	60.000	47.531	31.220	22.963	22.593	47.795	23.333
Band 6	50.000	46.091	23.704	16.296	15.926	31.220	18.148
Band 7	40.000	31.220	17.037			22.593	
Band 8	30.000	24.074				14.815	
Band 9		17.407					

Gel documentation showing molecular weights of different protein bands of haemolymph treated with *Chromolaena odorata*.