Antitumor and Anthelmintic Potentials of *Pleurolobus gangeticus* (L.) J. St. - Hil. ex H. Ohashi & K. Ohashi and *Tragia involucrata* Linn.

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

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Faculty of Science

University of Calicut

By

MADHURI MENON M.Sc.

Under the supervision of

Dr. LEYON VARGHESE Ph.D.
Assistant Professor
Department of Zoology
Christ College (Autonomous)
Irinjalakuda, Kerala, India-680125

2023

DECLARATION

I, MADHURI MENON, hereby declare that the thesis entitled "**Antitumor and Anthelmintic Potentials of *Pleurolobus gangeticus* (L.) J. St. - Hil. ex H. Ohashi & K. Ohashi and *Tragia involucrata* Linn.**" is based on the original work carried out by me at Department of Zoology, Christ College, Irinjalakuda, Thrissur, Kerala, under the guidance of Dr. Leyon Varghese, Assistant Professor, Department of Zoology, Christ College, Irinjalakuda, Thrissur, Kerala. The thesis has been subjected to plagiarism check and no part thereof has been presented for the award of any other degree, diploma or other similar titles of any University.

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DEPARTMENT OF ZOOLOGY (DST-FIST)

CHRIST COLLEGE (AUTONOMOUS), IRINJALAKUDA **(NAAC - A++)**

IRINJALAKUDA NORTH
THRISSUR, KERALA
INDIA – 680 125

PHONE : +91 480 282 5258
CELL : +91 949 500 9645
EMAIL : leyon@christcollegeijk.edu.in

Date... 07-06-2024

CERTIFICATE

This is to certify that the thesis entitled 'Antitumor and Anthelmintic Potentials of *Pleurolobus gangeticus* (L.) J. St. - Hil. ex H. Ohashi & K. Ohashi and *Tragia involucrata* Linn.' submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is an authentic record of the work carried out by Ms. Madhuri Menon, under my supervision in the Immunology and Toxicology Research Lab, Department of Zoology, Christ College (Autonomous), Irinjalakuda, affiliated to University of Calicut and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any University. It is further certified that, that the corrections/suggestions, recommended by the adjudicators have been incorporated in the thesis and that the contents in the thesis and the soft copy are one and the same.

Dr. Leyon Varghese PhD
Assistant Professor
(Supervising Teacher)

Dr. LEYON VARGHESE
ASSISTANT PROFESSOR & PhD GUIDE
IMMTOX RESEARCH LAB, DEPT. ZOOLOGY
CHRIST COLLEGE (AUTONOMOUS) IRINJALAKUDA-680125

Department of Zoology
Irinjalakuda
Christ College (Autonomous)

CHRIST COLLEGE, IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

NOVEMBER 2023

CERTIFICATE

This is to certify that **Ms. MADHURI MENON** has completed the research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis "**Antitumor and Anthelmintic Potentials of *Pleurolobus gangeticus* (L.) J. St. - Hil. ex H. Ohashi & K. Ohashi and *Tragia involucrata* Linn.**" embodies the results of her investigations conducted during the period at which she worked as a research scholar. I recommend the thesis to be submitted for the evaluation for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

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ASSISTANT PROFESSOR & PhD GUIDE
IMMTOX RESEARCH LAB, DEPT. ZOOLOGY
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Madhuri Menon

Dedicated To My Parents

ABSTRACT

ABSTRACT

Search for therapeutically potent natural products goes on and plants are the precious storehouse of bioactive molecules that have the potential to be developed into new drugs. The broad objective of the current study was to test and scientifically validate the pharmacological potentials of two herbs *viz. Pleurolobus gangeticus* and *Tragia involucrata.* Initial *in vitro* assays to evaluate the anticancer potentials of solvent extracts of these plants were conducted in lymphoma cell lines such as DLA and YAC-1. Lowest IC_{50} values were obtained for ethanol extracts, among other extracts, of both plants in inhibiting cell proliferation. Further, a higher mitotic index was observed in extract treated PBMC cells, when compared to untreated controls or reference drug colchicine. The GC-MS and LC-MS analysis of the ethanol extracts revealed the presence of several pharmacologically important compounds, and in order to ascertain the possibility of these molecules to interact with the mitotic spindle fibers, *in silico* methods were used. It was revealed that several of these molecules are having high affinity binding with the colchicine binding site of the αβ tubulin. This indicates that extract components could be binding to or interfering the microtubule assembly in the division initiated cells thereby arresting its cell cycle eventually leading to apoptosis. Flow cytometry analysis revealed a dose dependent increase in the early apoptotic cells by the treatment of these extracts. Considering the promising results in the *in silico* and *in vitro* systems, antitumor studies that followed the toxicity studies were performed in mice model. Ethanol extract treated animals showed a significant decrease in tumour burden compared to the vehicle controls and histopathological examinations of the tumour tissues showed clear evidence for apoptosis.

In our next efforts the anthelmintic potential of the extracts were evaluated using *in vitro* nematode and trematode models. The nematicidal effects were evaluated by egg hatch and larval paralysis assays using the eggs and larvae of *Haemonchus contortus*. A dose dependent egg hatch inhibition and larval paralysis was observed by the treatment of ethanol extracts of both plants. The trend

was similar in the trematocidal assay using the parasite *Fischoederius cobboldi.* The histological sections of flukes treated with the ethanol extracts showed disrupted muscle layers, in comparison to intact tissues in vehicle controls. Masson's trichrome staining that particularly stains the collagen fibers and muscles also confirmed the disruption of the collagen and muscle fibers by the extract treatment. In conclusion, the biological activity elicited by the extracts may be attributed this individual or synergistic activity of the phytocompounds present in the extracts and further studies are therefore highly desirable in this regard.
Keywords: Anticancer, Anthelmintic, *Pleurolobus gangeticus*, *Tragia involucrata*, Tumor reduction

സംഗ്രഹം

ചികിത്സാശക്തിയുള്ള പ്രകൃതിദത്ത ഉൽപ്പന്നങ്ങൾക്കായുള്ള തിരച്ചിൽ ഒരു തുടർപ്രക്രിയയാണ്. അതിൽത്തന്നെ പുതിയ മരുന്നുകളായി വികസിപ്പിക്കാൻ സാധ്യതയുള്ള ബയോആക്ടീവ് തന്മാത്രകളുടെ വിലയേറിയ ഒരു കലവറയാണ് സസ്യങ്ങൾ. നിലവിലെ പഠനത്തിന്റെ വിശാലമായ ലക്ഷ്യം രണ്ട് ഔഷധസസ്യങ്ങളുടെ ഔഷധസാധ്യതകൾ പരിശോധിച്ച് ശാസ്ത്രീയമായി സാധൂകരിക്കുക എന്നതായിരുന്നു, പ്ലൂറോലോബസ് ഗാഞ്ചെറ്റികസും ടെജിയ ഇൻവോലൂക്രെയറ്റയും. ഈ സസ്യങ്ങളുടെ ലായക സത്തുകളുടെ കാൻസർ പ്രതിരോധ സാധ്യതകൾ വിലയിരുത്തുന്നതിനുള്ള പ്രാരംഭ ഇൻവിട്രോ പരിശോധനകൾ DLA, YAC-1 തുടങ്ങിയ ലിംഫോമ സെൽ ലൈനുകളിൽ നടത്തി. കോശങ്ങളുടെ വ്യാപനത്തെ തടയുന്നതിൽ രണ്ട് സസ്യങ്ങളുടെയും മറ്റ് സത്തുകളിൽ നിന്നും എത്തനോൾ സത്തിൽ ഏറ്റവും കുറഞ്ഞ IC50 ലഭിച്ചു. കൂടാതെ ചികിത്സിക്കാത്ത അല്ലെങ്കിൽ റഫറൻസ് മയക്കുമരുന്നായ കോൾചിസിൻ ചികിത്സ എന്നിവയുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ എക്സ്ട്രാക്ട് ചികിത്സിച്ച പിബിഎംസി സെല്ലുകളിൽ ഉയർന്ന മൈറ്റോട്ടിക് സൂചിക നിരീക്ഷിക്കപ്പെട്ടു. തുടർന്ന് എത്തനോൾ സത്തിന്റെ ജി സി - എം എസ്, എൽ സി - എം.എസ് വിശകലനം, ഔഷധശാസ്ത്രപരമായി പ്രധാനപ്പെട്ട നിരവധി സംയുക്തങ്ങളുടെ സാന്നിധ്യം വെളിപ്പെടുത്തി. ഈ തന്മാത്രകൾ മൈറ്റോട്ടിക് സ്പിൻഡിൽ നാരുകളുമായി ഇടപഴകാനുള്ള സാധ്യത കണ്ടെത്തുന്നതിന്, ഏറ്റവും പുതിയ കമ്പ്യൂട്ടർ പഠനരീതികൾ ഉപയോഗിച്ചു. ഈ തന്മാത്രകളിൽ പലതും $\alpha\beta$ ട്യൂബിലിൻ എന്ന മാംസ്യകണത്തിലെ കോൾചിസിൻ ബൈൻഡിംഗ് സൈറ്റുമായി ഉയർന്ന ബന്ധമുള്ളതായി വെളിപ്പെട്ടു. വിഭജനം ആരംഭിച്ച കോശങ്ങളിലെ മൈക്രോട്യൂബ്യൂൾ അസംബ്ലിയുമായി എത്തനോൾ സത്തിന്റെ ഘടകങ്ങൾ ബന്ധിപ്പിക്കുകയോ തടസ്സപ്പെടുത്തുകയോ ചെയ്യാമെന്നാണ് ഇത് സൂചിപ്പിക്കുന്നത്. അതുവഴി അതിന്റെ കോശചക്രം തടഞ്ഞുനിർത്തി ഒടുവിൽ അപ്പോപ്ടോസിസ് എന്ന സ്വാഭാവിക കോശനാശം സംഭവിക്കുന്നു. എത്തനോൾ സത്ത് നൽകിയ ലിംഫോമ കോശങ്ങളുടെ ഫ്ലോസൈറ്റോമെട്രിക് വിശകലനം ഈ സാധ്യത ഉറപ്പിച്ചു. ഇൻ സിലിക്കോ, ഇൻ വിട്രോ സിസ്റ്റങ്ങളിലെ വാഗ്ദത്തമായ ഫലങ്ങൾ കണക്കിലെടുത്ത്, വിഷാംശപഠനങ്ങളും തുടർന്ന് അർബുദചികിത്സയും എലികളിൽ പരീക്ഷിച്ചു. ചികിത്സിക്കാത്ത എലികളെ അപേക്ഷിച്ച് എത്തനോൾ സത്ത് ചികിത്സിച്ച മൃഗങ്ങൾ ട്യൂമർ ഭാരത്തിൽ ഗണ്യമായ കുറവ് കാണിച്ചു. ട്യൂമർ ടിഷ്യൂകളുടെ ഹിസ്റ്റോപാത്തോളജിക്കൽ പരിശോധനകൾ അപ്പോപ്റ്റോസിസിനുള്ള വ്യക്തമായ തെളിവുകളും കാണിച്ചു.

ഞങ്ങളുടെ അടുത്ത ശ്രമങ്ങളിൽ ഇൻ വിട്രോ നെമറ്റോഡ്, ടെമാറ്റോഡ് മോഡലുകൾ ഉപയോഗിച്ച് ഈ സസ്യങ്ങളുടെ ലായക സത്തുകളുടെ ആന്തെൽമിന്റിക് (ദഹന നാളത്തിലെ വിരകളെ തുരത്തൽ) സാധ്യതകൾ വിലയിരുത്തി. ഹീമോങ്കസ് കോണ്ടോർട്ടസിന്റെ മുട്ടകളും ലാർവകളും ഉപയോഗിച്ച് മുട്ട വിരിയിക്കുന്നതും ലാർവ പാക്ഷാഘാത പരിശോധനകളും വിലയിരുത്തി. രണ്ട് സസ്യങ്ങളുടെയും എത്തനോൾ സത്തിൽ ചികിത്സിക്കുന്നതിലൂടെ മുട്ട വിരിയിക്കുന്നതിൽ തടസ്സവും ലാർവയിൽ പക്ഷാഘാതവും നിരീക്ഷിക്കപ്പെട്ടു. ഫിഷോഡെറിയസ് കോബ്ബോൾഡി എന്ന പരാന്നഭോജിയെ ഉപയോഗിച്ചുള്ള ടെമാറ്റോസൈഡൽ പരിശോധനയിലും ഈ പ്രവണത സമാനമായിരുന്നു. ചികിത്സിക്കാത്ത വിരകളുടെ കേടുകൂടാത്ത ടിഷ്യൂകളുമായി താരതമ്യപ്പെടുത്തുമ്പോൾ എത്തനോൾ സത്ത് വികസിച്ച വിരകളുടെ കലകളിൽ പേശീപാളികൾ തകരാറിലായതിലായി കാണിച്ചു. പ്രത്യേകിച്ച് കോളാജൻ നാരുകൾ വ്യക്തമാക്കുന്ന മാസൻട്രൈക്രോം സ്റ്റെയിനിംഗ് പരീക്ഷണങ്ങളിൽ, എത്തനോൾ സത്ത് ചികിത്സയിലൂടെ കോളാജൻ, പേശീനാരുകൾ എന്നിവയുടെ തടസ്സം സ്ഥിരീകരിച്ചു.

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ABBREVIATIONS

ACE	-	Acetyl Choline Esterase
AIDs	-	Acquired Immunodeficiency Syndrome
ALB	-	Albumin
ALP	-	Alkaline Phosphatase
AML	-	Acute Myeloid Leukemia
APX	-	Ascorbate Peroxidase
AS	-	Anterior Sucker
ASE	-	Accelerated Solvent Extractor
ATPs	-	Adenosine Triphosphate
BL	-	Blebs
Bm	-	Basement Memberane
BUN	-	Blood Urea Nitrogen
b.wt.	-	Body Weight
CA4	-	Combretastatin 4
CAT	-	Catalase
Cdk	-	Cyclin-dependent kinase
CDK	-	Cyclin Dependent Kinase
Cg	-	Corrugation
CHO	-	Cholesterol
CHOD-POD	-	Cholesterol oxidase phenol 4-aminoantipyrine peroxidase method
CNS	-	Central Nervous System
COX	-	Cyclooxygenase
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
CT	-	Condensed Tannins
DALYs	-	Disability Adjusted Life Years
DC	-	Differential Count
DEE	-	Desmodium ethyl acetate extract
DHAP	-	Dihydroxyacetonephosphate
DLA	-	Dalton Lymphoma Ascites

DME	-	Desmodium ethanol extract
DMSO	-	Dimethyl Sulphoxide
dNTP	-	DeoxynucleotideTriphosphate
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
dUTP	-	DeoxyUridineTriphosphate
DWE	-	Desmodium water extract
EAC	-	Ehrlich Ascites Carcinoma
EHA	-	Egg Hatch Assay
ESR	-	Erythrocyte Sedimentation Rate
ET	-	Ellagitannins
FBC	-	Fetal Bovine Serum
FEC	-	Fecal Egg Count
FITC	-	Fluorescein isothiocyanate
FTIR	-	Fourier Transformed Infrared
GABA	-	Gamma-aminobutyric acid
GC	-	Giant Cell
GC-MS	-	Gas chromatography–mass spectrometry
GI	-	Gastrointestinal
GLOB	-	Globulin
GR	-	Grooves
GSH	-	Glutathione
GTG	-	G bands by Trypsin using Geimsa
Hb	-	Haemoglobin
HDL	-	High Density Lipid
HPTLC	-	High Performance Thin Layered Chromatography
I/R	-	Ischemia/reperfusion
IAEC	-	Institutional Animal Ethics Committee
ICAM-1	-	Intercellular Adhesion Molecule – 1
KFRI	-	Kerala Forest Research Institute
LC-MS	-	Liquid Chromatography-Mass Spectrometry
LDH	-	Lactate Dehydrogenase
LDL	-	Low-density lipoprotein
LDL	-	Low Density Lipid
LOX	-	Lipoxygenase

LPA	-	Larval Paralysis Assay
MAPK	-	Mitogen Activated Protein Kinases
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
Mcl-1	-	Myeloid Cell Leukemia - 1
MCV	-	Mean Corpuscular Volume
MI	-	Mitotic Index
MMPs	-	Matrix Metalloprotease
Mu	-	Muscular Layer
nAChR	-	nicotinic Acetylcholine Receptor
NADH	-	Nicotinamide adenine dinucleotide
NC	-	Necrosis
NCCS	-	National Centre for Cell Sciences
NCDs	-	Non-communicable diseases
NCI	-	National Cancer Institute
NF-κB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	-	Nitric Oxide
OECD	-	Organization for Economic Co-operation and Development
PBLC	-	Peripheral Blood Lymphocyte Count
PBS	-	Phosphate Buffered Saline
PCV	-	Packed Cell Volume
PG	-	Propylene Glycol
PI	-	Propidium Iodide
PI-3- K	-	Phosphoinositide 3-kinases
PKC	-	Protein Kinase C
PL	-	Pylorus Ligation
PLT	-	Platelet
PMC	-	Pleomorphic cells
PS	-	Posterior Sucker
PS	-	Phosphatidylserine
RBC	-	Red Blood Cells
ROS	-	Reactive Oxygen Species

ROS	-	Reactive Oxygen Species
Rt	-	Retention time
SABS	-	Small Animal Breeding Station
SCID	-	Severe Combined Immunodeficiency
SD	-	Standard Deviation
SDL	-	State Depended Learning
SGOT	-	Serum glutamic-oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic acid transaminase
SNP	-	Single Nucleotide Polymorphism
SOD	-	Superoxide Dismutase
STH	-	Soil Transmitted Helminth
SW	-	Swollen Tegument
TB	-	Total Bilirubin
TC	-	Total Count
TEE	-	Tragia ethyl acetate extract
TFC	-	Total flavonoid content
Tg	-	Tegument
TGL	-	Triglycerides
TLC	-	Thin Layered Chromatography
TME	-	Tragia ethanol extract
TP	-	Total Protein
TP	-	Total Protein
TPC	-	Total phenol content
TS	-	Tegmental sloughing
TUNEL	-	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TWE	-	Tragia water extract
VCAM-1	-	Vascular Cell Adhesion Molecule – 1
VEGF	-	Vascular Endothelial Growth Factor
VLDL	-	Very Low Density Lipid
VT	-	Viscotoxins
WAAVP	-	World Association for the Advancement of Veterinary Parasitology
WHO	-	World Health Organization

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CHAPTER: 1

GENERAL INTRODUCTION

Natural products play a pivotal role in drug discovery and have contributed tremendously to the field of modern medicine. The discovery of novel biomolecules with therapeutic potentials from terrestrial plants, have been of great interest as it is a unique and renewable source of drugs. According to the WHO, 80% of the people in rural areas rely on medicinal herbs as their primary health care system (Sarkar & Deshmukh, 2011). Ethnopharmocological study facilitates drug discovery and also plays a vital role in rescuing the authentic traditional knowledge of use of medicinal plants (Raza, 2006). Ethno pharmacological knowledge can be an important lead to the discovery of plant based drugs and is defined as 'the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man'. It aims at restoring the traditional and folklore knowledge on medicinal plants, and exploring them to reveal its full potential, which will promote its proper documentation (Mukharjee et al., 2010). Around 2,50,000 plant species have been estimated to be present around the world and 14-28% of this has been used for medicinal purposes. Several biologically active chemical compounds in the plants often work in synergistic mode, thereby giving the plant its medicinal value. Exploring such bioactive compounds in plants will aid in the application of potential constituents in modern medicine (Mamedov, 2012). The role of plants and their products in treating various ailments is gaining a global interest and ethnopharmacology plays an important role in developing modern medicines (Gilani, 2005).

Botanical extracts have been intensely investigated by various research institutes across the world especially in underdeveloped countries. Two major reasons that gave popularity to this area of research are namely; ease of botanical sample preparation with least expense and also the availability of unique plants. In developed countries the research focused on drug development from available fauna is of great interest and there is a global effort in discovering new molecules from plants with therapeutic potential. A multidisciplinary approach is now the most important way of developing plant based drugs. The production of reliable and viable botanical drug products may be enhanced by planning a collaborative study between the scientists (conducting *in vitro* and *in vivo* studies of the plant extracts) and the phytochemists (Liu, 2008).

Diseases are broadly classified as communicable (infectious) and non-communicable (non-infectious/chronic diseases). Helminthiasis or worm infection, a communicable disease, falls under the label of 'Neglected Tropical Diseases'. Inspite of having caused 14 million DALYs (Disability Adjusted Life Years) lost yearly during last ten years, it receives a very low (<1%) global research budget (Quinzo et al., 2022). Over 71% of deaths worldwide and 1.6 billion DALY'S, are caused due to non-communicable diseases (NCDs) (Wu et al., 2022). According to the World Health Organizations, cancers account for 9.3 million NCD deaths, which is second to cardiovascular diseases (WHO, 2022).

Helminths are worm-like parasites. According to their morphology and host organ they inhabit, they are divided into clinically relevant groups. They are broadly classified into Trematodes (flukes), Cestodes (tapeworms) and Nematodes (roundworms). Trematodes have a dorsoventrally flattened body which is bilaterally symmetrical and range in length from a few millimeters to 8 cm. It has a complex tegument and the oral suckers are seen around the mouth. For attaching to the host tissues, they have a ventral sucker called acetabulum. The only bisexual flukes that infect humans are schistosomes. The tapeworms or cestodes are leaf shaped and have a flattened elongated body, with segments called proglottids. The head portion is called scolex, which consists of suckers to attach to the host tissue. The roundworms or nematodes have a cylindrical body, and the body wall is composed of an outer cuticle, hypodermis and musculature. They have a complete alimentary canal with oral and anal openings at the anterior and posterior ends respectively (Castro, 2011).

Plant parasitic nematodes cause severe damages to plants, and an annual loss of $ US 80 billion has been estimated. The root-knot nematode (Meloidogyne spp.) affects the plants by penetrating the roots directly behind the root cap. It causes symptoms like stunted growth, wilting, discolouration of the leaves and root deformation. The cyst nematodes (Heterodera and Globodera spp.) are also known to be destructive to the plants as they penetrate the roots intracellularly upto the inner cortex. Some of the other destructive plant parasitic nematodes are; the root lesion nematodes (Pratylenchus spp.), the burrowing nematode (*Radophylus similis*) *Ditylenchus dipsaci*, the pine wilt nematode (Bursaphelenchus xylophilus), the

reniform nematode (*Rotylenchulus reniformis*), *Xiphinema index*, *Nacobbus aberrans* and *Aphalancoides besseyi* (Jones et al., 2013). In humans, out of the 342 helminth species that cause infections, *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Necator americanus*, *Trichuris trichiura*, *Enterobius vermicularis* and *Strongyloides stercoralis* are of great medicinal importance. Several factors like poor living conditions without proper sanitation in the rural and urban slums of developing countries and lack of proper healthcare facilities in such areas are a leading cause for the helminth infections (Stepek et al., 2006).

With respect to anthelmintic resistance, *Haemonchus contortus* is the most widely studied trichostrongylid nematode as it has the ability to develop resistance to all major classes of drugs (Prichard, 2001). The hematophagous gastrointestinal nematodes lead to economic loss in livestock production, as they cause emaciation, anemia and even death of infected animals. The gastroenteritis caused by *H. contortus* is known to be one of the major impediments to livestock farming as they pose serious threats to small ruminants (Mohanraj et al., 2017). Several studies have drawn in the resistance of *H. contortus* to different classes of synthetic anthelmintic drugs. The population that showed resistance to benzimidazole class of drugs were known to show diminished levels or even loss of isotype- 2 of β-tubulin genes. Similarly, imidazothiazole resistant ones possessed truncated forms of two nAChR (nicotine acetylcholine receptor) subunit genes, Hco-unc-63 and Hco-acr-8 (Kotze & Prichard, 2016). Such development of resistance towards synthetic drugs prompted the exploration of herbal medicines for the treatment of helminthic infections.

The gastro-intestinal parasites of ruminants clinically take a toll on their hosts, and lead to severe economic loss in the livestock industry. The disease complex of nematodes affecting ruminants are generally called as parasitic gastroenteritis. Each portion of the digestive system of the hosts is occupied by specific genera of nematodes. The abomasum is usually attacked by *Haemonchus, Ostertagia, Teladorsagia* and *Trichostrongylus. Cooperia, Nematodirus and Trichostrongylus* are seen in the small intestine and *Oesophagostomum* in the large intestine (Craig, 2018). In tropical areas the ruminants are highly affected by strongyle nematodes of the order Strongylida (Income et al., 2021). Haemonchosis is the parasitic infection caused by Haemonchus species (strongyles). *Haemonchus contortus* specifically causes severe anemia in cattle, due to its blood sucking activity. This ultimately

reduces the growth of young animals, affects its milk production and may also lead to death in severe untreated cases (Arsenopoulos et al., 2021). Several genetic and biological factors like short life cycle, increased reproductive rates, large population and quick evolution in nematodes have made them less vulnerable to the synthetic anthelmintic drugs. Resistance towards anthelmintic drugs have become a major cause of concern as it is both heritable and rarely reversible. The most resistant among nematodes are known to be *H. contortus* (Ahuir Baraja et al., 2021). The use of secondary metabolites derived from plants as a source of anthelmintic drug, is one of the best and most effective alternative methods to fight anthelmintic resistance in nematodes.

Cancer is caused by uncontrolled proliferation of cells. The global burden of cancer is not uniform even though the disease is one of the major causes of deaths worldwide. Several factors like rapidly growing population, aging and increased socioeconomic development have contributed to the growth in cancer burden over a period of time in both developed and developing nations (Cao et al., 2021). Synthetic compounds used as pharmaceutical drugs interfere with a single mechanism, while the phytocompounds derived from plants are capable of targeting different phases of cancer process like tumor invasion, promotion and progression making the latter more chemo preventive. Hence these botanical medicines are called 'pharmacological multitaskers' (Treasure, 2005).

The wide use of plant derived metabolites as anticancer agents in modern medicine, began by isolation of vincristine and vinblastine (vinca alkaloids) from the Madagascar periwinkle *Catharanthus roseus* G. Don, which belongs to the family Apocynaceae. It is used in combinatorial therapy with other chemotherapeutic drugs against lymphoma, leukemia, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma. Paclitaxel clinically introduced in the 1990s to the US market, was derived from Taxus sp, *Taxus baccata* which has already been mentioned in Indian Ayurvedic medicine, for the treatment of cancer. Semisynthetic derivative of camptothecin; Topotecan and irinotecan are used to treat ovarian, small cell lung cancers and colorectal cancers respectively. The roots of Podophyllum sp; *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* were used to isolate an active antitumor agent called epipodophyllotoxin which is an isomer of podophyllotoxin. For treating lymphoma and bronchial as well as testicular cancers,

etoposide and teniposide are used which are semi-synthetic derivatives of epipodophyllotoxin. Another plant-derived derivative is Homoharringtonine from Chinese tree *Cephalotaxus harringtonia* var. is also in clinical use. In France, Elliptinium, a derivative of ellipticine isolated from a mid-plant *Bleekeria vitensis* A.C. Srm is used to treat breast cancer (Shoeb, 2006). Several plant derived metabolites like Bruceanti (nortriterpenoid), Noscapine (phthalideisoquinoline alkaloid), homoharringtonine (alkaloid), vindesine (a semisynthetic derivative of vinblastine) are found to be effective against various types of lymphoma (Lucas et al., 2010).

Ayurveda is a comprehensive traditional system of healthcare in India. It was established during 2500 to 600 B.C. Fifty different pharmacological categories of medicinal plants have been described in the Ayurvedic texts. Lack of standardization is a major obstacle for the scientific credibility of Ayurveda (Mishra et al., 2001). However, a proper scientific validation of these plants are very important. For determining the safety, 'drug quality' is an important factor. The standards for herbal drugs and formulations in India, there is a legally recognized book called 'The Indian Pharmacopoeia'. It is published by the Indian Pharmacopoeia Commission, Ministry of Health and Family Welfare, Government of India. The standards prescribed in the pharmacopoeia are that of the identity, purity and strength of the medicinal plant-based drugs which ensures its quality. In accordance with the Drug and Cosmetics Act 1940 and Rule 1945 the pharmacopoeia standards are enforced by the central, state and union territory drug regulatory authorities of India. The Indian Pharmacopoeia Committee constituted in 1948, post-independence, prepared the Indian Pharmacopoeia in the year 1955 (Prakash et al., 2017).

Pleurolobus gangeticus (L.) J. St. - Hil. ex H. Ohashi & K. Ohashi belongs to the family Fabaceae and is a perennial shrub found all over India. It has a poorly developed taproot system with 15-30 cm long lateral roots. They are uniformly cylindrical with numerous branches that bare transverse light brown lenticels. Bacterial nodules are present frequently and the roots have a sweetish taste with no characteristic odour. It has been traditionally used in the treatment of scabies and ringworm, diarrhea, cataract, fever, oedema, asthma, bronchitis, infections and urogenital diseases (Singh et al., 2015). It is an important ingredient of several

Ayurvedic preparations like 'Dashamoolarishta', 'Indukant Ghrita', 'Amrita Prasa Ghrita', 'Dasamoola Satapalaka Ghrita', 'Dhanvantara Taila', 'Narayana Taila', 'Mahavishgarbh Taila and Mahanarayana Taila' and is known as "Moovila" in Malayalam (API, 2001). The "Bheel and Bhilala" tribes of Jhabua district, Madhya Pradesh, are known to apply juice of *Pleurolobus gangeticus* leaves, topically to treat ringworm and scabies (Singh et al., 2015). The root and stem decoctions of *P. gangeticus* were used ethnobotanically by the traditional medicinal system of a developing country Benin to treat Cancer (Toyigbenan et al., 2018). *Tragia involucrata* Linn. belongs to the Family Euphorbiaceae and is widely distributed in South Asian countries and the whole plant, leaves and root decoctions have been used as analgesic, antidiabetic, anti-inflammatory, antimicrobial, antinociceptive, antioxidant antiparasitic, antitumor, diuretic and hepatoprotective (Duarte & Romero, 2021). The various parts of this plant have tremendous ethnopharmacological importance, as it has been known for use against inflammation, allergy, epilepsy, renal stones, asthma, bronchitis, vomiting, diarrhea and numerous other ailments (Pallie et al., 2020). The roots of *T. involucrata* are 2 to 10 cm long and 0.3 to 1.3 cm in diameter. The presence of secondary roots and root scars gives it a rough appearance and it is hard and cylindrical with no characteristic colour and odour. The stem is elongated, fibrous and light grey in colour. They are moderately hard with whitish internal surfaces. The leaves are stinging to touch, simple, petiolate, broadly ovate with accuminate or acute apex. It has serrated margins and are 1 to 3 cm broad. It is an important ingredient of the Ayurvedic formulations called "Vidaryadi Kwatha Churna", "Vidaryadi Ghrita" and is known as "Terkkada" in Malayalam (API, 2004).

The plants *P. gangeticus* and *T. involucrata* have been used by several tribes of various parts of the world, for treating different types of ailments. Different parts of the plants are used to make formulations to treat specific diseases. Though these plants have been extensively used by traditional systems of medicines, it lacks proper scientific validation as well as a strong scientific backing.

The present study was designed with the following objectives:

1. Solvent extraction and phytochemical profiling of *P. gangeticus* and *T. involucrata* using ethyl acetate, ethanol and water.

2. Evaluation of anticancer potentials of *P. gangeticus* and *T. involucrata* extracts in the *in vitro* systems.
3. Toxicity profiling and evaluation of antitumor potentials of pharmacologically active extracts of *P. gangeticus* and *T. involucrata* in mice.
4. Evaluation of nematicidal and trematocidal potentials of *P. gangeticus* and *T. involucrata* extracts.

CHAPTER: 2

REVIEW OF LITERATURE

Ethnopharmacology is a branch of study that deals with the use of plants, plant extracts or plant derived chemicals for therapeutic purposes. Medicinal plants have been used in the treatment of various diseases since time immemorial. In different cultures/civilizations such as Egypt, Western, Chinese, Kampo (Japan) Greco- Arab and Unani/Tibb (South Asia), plants have been used as a mode of natural resource to combat various ailments. The historical data has strong evidence to show that several herbs were used by primitive humans in much advanced ways. Even before the cause of 'Malaria' was identified, quinine derived from cinchona bark was used as a cure. Though the use of synthetic drugs became dominant as a part of growing pharmaceutical industries, the use of herbal medicines was never out of sight. The use of herbal medicines gained profound importance by the middle of the 19th century. At least 25% plant derived drugs can be seen in any pharmacy in the West. The folk knowledge of indigenous people, and the study of traditional cures couldn't be substituted, despite appreciable advancements in synthetic chemistry (Gilani, 2005). The discovery of new techniques by communities and individuals, transformed practices, thus making traditional medicine an evolutionary process. In search of new drugs, people have been exploring the floral world since time immemorial, which led to the discovery of numerous medicinal plants with excellent curative potentials. In the traditional medicinal systems like Ayurveda, Homeopathy, Siddha and Unani 95% of the prescriptions are plant based (Savithramma et al., 2011). In order to get a deeper insight into the medicinal potentials of a plant, a good knowledge of its chemical composition is necessary (Hussein & Ansarry, 2019).

Ayurvedic medicinal plants have been put to extensive research on various aspects like pharmacognosy, chemistry and clinical therapeutics (Patwardhan, 2005). This traditional system of medicine had a pharmacological approach, as it recommended the use of complex herbal mixtures of multicompound extracts. Research in the field of 'drug discovery' from plants focus on the isolation of bio-able compounds from these available natural sources whereas herbal medicine and phytotherapy research are based solely on characterization of more complex pharmacological interaction shown by an extract with several chemical entities (Leonti, 2013). The discovery of quinine from *Cinchona* bark, to treat malaria, was made by the French scientists Caventor and Pelletier in the 19th century. Primary metabolites play a vital role in

the basic life functions of a plant such as growth, cell division, respiration and reproduction. On the other hand, secondary metabolites produced by the plants are low in abundance with great therapeutic potentials (Bourgaud et al., 2001).

Several natural products from higher plants were used for treating various ailments prior to World War II, and some of them are used till date. Antibiotics isolated during this era were also from natural sources like species of *Penicillium, Cephalosporin* and *Streptomyces.* Several other drugs discovered, like quinine from *Cinchona* bark, morphine and codeine from latex of opium poppy, digoxin from the leaves of *Digitalis*, atropine and hyoscine from species of Solanaceae are still in clinical use. The post-war period had relatively fewer discoveries with reserpine (a tranquilizer) and vinblastine (a chemotherapeutic drug) isolated from *Rauwolfia* sp. and *Catharanthus roseus* respectively, being an exception. As years passed by, a multidisciplinary approach in this field by including pharmacology and synthesis led to the emergence of successful clinical agents. Atenolol (a beta blocker) and Captopril (ACE inhibitor) for treating hypertension, salbutamol (adrenoceptor stimulant) for asthma and benzodiazepine for insomnia and anxiety attack are some of the examples. Research has been prioritized in the field of cancer and AIDS as they are the deadliest diseases that affect humans. The National Cancer Institute (NCI) are working in collaboration with local botanical institutes and are focusing their research on plant based drug development (Ming et al., 2003). Researchers have examined the pharmacological potentials of very few proportion of plants from a total of 5,00,000 existing plant species around the world. The sophisticated screening methods and advanced knowledge in the field of chemistry have made it possible to isolate bioactive compounds from secondary metabolites obtained from plants. The detailed structural elucidation of the isolated compounds, also paves way for a more targeted therapeutic pharmacological action (Velu et al., 2018).

2.1 *Pleurolobus gangeticus*

2.1.1 Systematic position

Kingdom	Plantae
Subkingdom	Tracheobionta

Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Genus	Pleurolobus
Species	gangeticus

2.1.2 Botanical Description

P. gangeticus belongs to the family Fabaceae. It is known as ‘Shalparni' in Hindi. The plant is endangered and is one among the ‘Dashamoola’ (ten roots) of Ayurveda (Singh et al., 2015). It is a small perennial shrub with height ranging from 60 to 130 cm. The branches are angular, pubescent or glabrous. Leaves are somewhat angular and pubescent with 1–2.5 long petioles. It is simple, variable, ovate, oblong or rounded, 3–14 cm×2–7 cm, acute to acuminate and glabrous above. The inflorescence is terminal or axillary, many flowered, slender, elongate racemes, 10-30 cm long often combined to panicles. Flowers are purplish or white and are 4-7 cm long. Calyx 4–5 cm long and pubescent. The keels are incurred and the Vessilum is 5-6 cm long. Pods are 6-8 joined, curved and subfalcate, the upper portion is straight, lower deeply indented, hooked-hairy. The lateral roots are very strong, light yellow in colour and have a smooth texture. However, the tap root is poorly developed. It is during the months of March to December that it is known to bear flowers and fruits. It is distributed through-out the warmer parts of India, tropics of Africa to Indo-Malaysia (Rastogi et al., 2011).

2.1.3 Ethnomedical Uses

Ethnobotany is the study and collection of data regarding indigenous medicinal plants from local healers, that are widely prescribed in particular area to cure various ailments (Heinrich, 2000). In Bulamogi county, Uganda the people greatly relied on

traditional medical practitioners who had tremendous indigenous knowledge on medicinal properties of plants. According to the information collected from these practitioners, the roots of *P. gangeticus* were chewed to prevent premature ejaculation (Tabuti et al., 2003). The ethnobotanical survey of Sitamata wildlife sanctuary, Rajasthan, India documented several medicinal plants and their properties which the tribals used for treating various ailments. Among them half cup of root decoctions of *P. gangeticus* was consumed orally as an antidote in snake bites (Jain et al., 2005). The decoction of the whole plant is used in the treatment of digestive disorders, diarrhea, edema, intermittent fevers, malaria, and urinary tract infection. It has been used as a bitter tonic, febrifuge, anti-catarrhal, antiemetic, digestive and also in various inflammatory conditions including that of the chest. The plant harbors calcium, phosphorus, magnesium, vitamin A and C and has excellent wound healing capacity too. The Assamese use leaves of *P. gangeticus* to cure eczema (Saikia et al., 2006). The roots of *P. gangeticus* are chewed for around 20 minutes to relieve toothache as per the ethnobotanical information collected regarding oral health from the Nandurbar district of Maharashtra, India (Badgujar et al., 2008). Considering the fact that the flora of Chandauli district of Uttar Pradesh, India is less explored for its ethnobotanical values and therapeutic roles, a survey was conducted to gather indigenous knowledge on the medicinal properties of various plants from tribes such as 'Kharwas', 'Polehero', 'Kevat' and 'Dhobi', as the people depended on them for treatment due to the lack of government health facilities in the district. According to these traditional healers the paste prepared from the leaves of *P. gangeticus* along with *Aloe vera* when applied on the scalp helped reduce hair fall (Singh & Singh, 2009). The 'Pawar' tribes of Satpuda hills, Maharashtra, India are known to use the root powder of *P. gangeticus* mixed with honey to cure mouth ulcers. This ethnobotanical information was collected from the local traditional medicine men called 'Badwa' (Kosalge & Fursule, 2009). The information on the traditional ethnobotanical knowledge collected from the 'Bheel' and 'Bhilala' tribes of Jhabua district, Madhya Pradesh, revealed the use of fresh leaves juice of *P. gangeticus* to cure scabies and ringworm by topical application (Wagh & Jain, 2010). The Rudrakod sacred groves in Nallamala hill ranges of southern Andhra Pradesh, are known to house several plants of medicinal values similar to the ones found in the Himalayas. Among the information collected about various plants, the people of Nallakaluva village near to the hills used powdered leaves of *P.*

gangeticus ground with a pinch of salt to heal boils and blisters. They also consumed a spoon full of root extract twice a day to cure whooping cough (Rao & Sunitha, 2011). The knowledgeable 'Paliyar' and 'Muthwar' healers in Theni district, Tamil Nadu used the roots of *P. gangeticus* to cure asthma (Jeyaprakash et al., 2011).

In Traditional Chinese Medicine, the roots were given as a sedative agent to children and also applied for toothache (Ma et al., 2011). The elucidation of medicinally important herbs used traditionally by the villagers of Sivagangai district, Tamil Nadu, India brought into limelight the importance of the leaf decoctions of *P. gangeticus* in treating piles, dysentery and diarrhea. According to the villagers, the dosage for curing dysentery and diarrhea were oral administration of the decoction twice a day for 2-3 days and that for piles was the application of leaf paste in the anal area once a day for two weeks (Shanmugam et al., 2012). The local healers of Wayand district of Kerala, India prescribed the use of *P. gangeticus* roots for treating type 2 diabetes mellitus. They recommended consumption of 2 ml of extract prepared by mixing 10 g of crushed roots of *P. gangeticus* and Pseudarthria *viscida* wrapped in a cotton cloth and boiled in 200 ml water for 3 minutes, thrice a day after meal, for several days (Kumar & Janardhana, 2012). The 'Bhil', 'Pawara', 'Tadwai' and 'Vanjara' tribes of Jalgaon district, Maharashtra, India are known to consume the roots of *P. gangeticus* boiled in milk for seven or more days for flatulence (Shubhangi, 2012). The survey conducted in the tribal areas of Jhalod taluka of Dahod district, Gujarat, India gave the information that they used the whole plant of *P. gangeticus* to treat various gynecological ailments (Maru & Patel, 2012). The ethno medical study on the indigenous uses of plants in the less floristically studied regions of central Gangetic plain, particularly the Buxar district of Bihar revealed the use of leaf decoctions of *P. gangeticus* mixed with black pepper being used as a blood purifier and also to cure fever (Singh et al., 2013). In the Salugu panchayat of Paderu Mandal, Visakhapatnam, Andhrapradesh, India the locals used roots of *P. gangeticus* to treat rheumatic trouble (Padal et al., 2013). In Santhal Pargana district, Bihar, the roots are used to cure snake bites and scorpion stings. Meanwhile, in Tharus-Basti district, Uttar Pradesh, the roots are used to cure jaundice. The leaves of the plant were used to treat acidity, whooping cough and also for boils and blisters by tribes of Srikakulam district, Andhra Pradesh. The whole plant was used

as a febrifuge, bitter tonic, diuretic and anti-catarrhal by tribes of Amravati Tahsil, Maharashtra (Singh et al., 2015).

2.1.4 Phytochemical Profile

The preliminary investigation on the phytochemical composition of *P. gangeticus* was carried out earlier in the year 1955 by Avasthi and Tewari. This plant was unexplored during those days, and it was revealed by the examination that its root constitutes lactones, alkaloids, phytosterols and other colour imparting compounds. The qualitative analysis also revealed the presence of organic acid, saponin, reducing sugars and essential oils (Avasthi & Tewari, 1955). The amount of phytochemicals present in the plant may vary according to the geographic condition and the soil type. The densitometry quantification using HPTLC showed that the amount of lupenol was higher in the methanol root extracts of *P. gangeticus* collected from higher altitude than ones collected from the plains (Jayanthi et al., 2013). The FTIR analysis of the leaf extract revealed the presence of various functional groups like alcohols, phenols, aldehydes, carboxyl, primaryamine, alkyl halides, alkanes, alkenes and carboxylic acid (Vanaja et al., 2019).

Table 2.1: Phytochemicals identified from different parts of *P. gangeticus*.

Plant parts	Phytochemicals	References
Whole plant	Trans-5-hexadecenoic acid, 1-Tritriacontanol, 1-heptadecanol, β-amyrone, Gangetin, b-sitosterol, glycosphingolipid, 5 methoxy N, N-dimethyltryptamine, 8-C-prenyl-5,7,50-trimethoxy 30, 40-methylene dioxyflavone, Salicylic acid, 5-O-Methyl genistein 7-O-b-D-glucopyranoside, 3,4dihydroxy benzoic acid, Kaempferol 7-O-β-d-glucopyranoside, 5 methoxy N, N-dimethyltryptamine Nb-oxide, 3-O-b-D-glucopyranoside-b-sitosterol, rutin, quercetin 7-O-b-D-glucopyranoside and uridine triacetate.	Mishra et al., 2005
Roots and aerial plant parts	Lupeol, lauric acid, mixture of β-sitosterol and Stigmasitosterol, Gallic acid, protocatechuic acid, salicylic acid, chlorogenic acid, caffeic acid, rutin, quercetin and kaempferol	Niranjan & Tiwari, 2008

Aerial plant parts and roots	Indole-3-alkyl amines, β carbolines, pterocarpans, gangential, sterols, 5 methoxy N, N-dimethyltryptamine, N-dimethyltryptamine	Rastogi et al., 2011
Whole plant	5-methoxy N, N-dimethyltryptamine, N-methyl-H-Harman, N -methyl serotonin Bufotenine, N-oxide Hypaphoirine, 6 Methoxy-2-methyl-β-Carbolinum, hordenine, uridine acetate, β-amyrone, Tritriacontanol, 1-Heptadecanol, Aliphatic β-lactone, Trans-5-hexadecanoic acid, N-methyltryamine, β-phenylethylamine, 3,4-Dihyroxyphenethyltrimethyl ammonium hydroxide, 24-Ethylcholesta-5, 22-dien-3β-ol, 24-Methylcholesta-5-en-3β-ol, β-sitosterol, lupeol, stigmasterol, 3,4 Dihyroxybenzoic acid, Vanillic acid, Chlorogenic acid, 4,7-Trohydroxy-8-prenyl flavone, 4-O-α-L-rhamnopyranosyl-(1-6)-β-glucopyranoside, 8-C-prenyl-5,7,5-trimethoxy-3,4-methylenedioxy flavone, Kaempferol 7-O-β-d-glucopyranoside, quercetin 4-O-β-d-glucopyranoside, gangetin, gangetenin, desmodin, desmocarpin, 5-O-methylgenistein, 7-O-β-glucopyranoside, salcicilic acid, 3-4-dihyroxybenzoic acid	Bhattacharjee et al., 2013
Leaves	Methyl salicylate β-D-glucopyranoside, syringarenisol-4-O-β-D-glucopyranoside, leunuriside	Dat et al., 2015
Leaves	(6S,9R)- roseoside, Kaempferol-3-O-rutinoside, Quercetin-3-O-rutinoside, β-sitosterol-3-O-glucopyranoside, protocatehuic acid	Xuan & Noi, 2018
Aerial plant parts	Gangeticoside, leonuriside A, methylbenzoate 2-O- β-glucopyranoside	Dang et al., 2020
Root	Diisobutyl phthalate, 3α,12α-dihydroxy-5β-chol-8(14)-en-24-oic Acid, 8αSeco-6,8α-deoxy-5-oxoavermectin, gingerdione, amphibine H, alverine, trans-1,2-diphenylcyclobutane , acetyleugenol, acetyl tributyl citrate, cinncassiol D3, 2-alpha-methyl17beta-[(tetrahydro-2H-pyran-2-yl)oxy]-5alpha-androstan-3-one and deoxymiroestrol	Daniel et al., 2022

2.1.5 Pharmacological review

Lipid peroxidation causes damage to the cell membrane, as the free radicals 'steal' electrons from the lipids of the cell membrane. Hydrogen peroxide penetrates the cell by crossing the cell membrane making toxic effects by forming hydroxyl radicals. The hydro alcoholic extract of *P. gangeticus* possesses highly potent antioxidant activities, and was also found to be capable of reducing the levels of hydrogen peroxide*, in vitro*, in a dose dependent manner (Govindarajan et al., 2003). The phenolic fractions of *P. gangeticus* showed significant inhibition of lipid peroxidation in the liver, and also increased glutathione content. Further analysis of the phenolic fractions revealed the presence of two important antioxidants cholinergic acid and caffeic acid (Govindarajan et al., 2006). Oxidative stress is the major reason for cardiac hypertrophy, where the cardiac muscles thicken, thereby reducing the ventricular chamber size. Myocardial ischemia reperfusion causes metabolic stress, calcium overload and also an increase in the production of ROS. The increased ROS production causes degradation of mitochondrial integrity eventually leading to necrotic and apoptotic cell death. The methanol extracts of *P. gangeticus* roots showed radical scavenging activity and also brought the activities of the mitochondrial respiratory enzymes to near normal levels in ischemic myocardium pretreated with the extract. This further confirmed the antioxidant and cardio protective nature of the extract (Kurian et al., 2008). The I/R injury exhibits oxidative stress, and the aqueous extract of *P. gangeticus* roots were found to possess free radical scavenging activity, thereby reducing the stress. An alkaloid named cactine was discovered from the extract which is used to treat irregular heartbeat, angina pectoris and cardiac neuralgia (Kurian et al., 2009). The leaf extracts of the plant have free radical scavenging activity and also a potent primary antioxidant (Venkatachalam & Muthukrishnan, 2012). Increased hydrogen peroxide levels in the parietal cells is cytotoxic and it is rapidly scavenged by peroxidases using gastric glutathione. Hence, when the level of gastric glutathione decreases, the mucosal lining gets damaged. The role of ethanol extracts of the roots of *P. gangeticus* is to increase the level of glutathione levels thereby being gastroprotective (Mahesh et al., 2012). The methanolic extracts of *P. gangeticus* The methanolic extracts of *P. gangeticus* was found to be an effective antihypertrophic,

as well as protective against oxidative stress and mitochondrial alterations (Sankar et al., 2013).

Nociceptors are sensory neurons that respond to damaging signals and thereby induce the sensation of pain. The anti-nociceptive activity of water decoctions of *P. gangeticus* was studied and it was found that it modulates secondary messengers, opioid receptor subtypes and neurotransmitters (Rathi et al., 2004). The aqueous extract of *P. gangeticus* was found to decrease the levels of acetylcholinesterase, thereby preventing the breakdown of the neurotransmitter acetylcholine and increasing the memory of both old and young mice (Joshi & Parle, 2006). Scopolamine is a drug which when administered continuously, causes memory deficits. The aqueous extracts of *P. gangeticus* were found to have neuroprotective function, as they are known to protect mice from memory deficits induced by scopolamine. The aqueous extracts were also found to increase the SDL (state dependent learning) values in mice (Hanumanthachar & Milind, 2007). A more detailed study on the antiamnesic properties of *P. gangeticus* revealed that the chloroform extracts of the plant contained very potent alkaloid fractions which showed significant antiamnesic activity in mice. They were found to be capable of reversing the amnesia induced by scopolamine and the antiamnesic activity was statistically equivalent to the standard drug Piracetam (Mahajan et al., 2015). The alkaloid fractions of chloroform extracts of *P. gangeticus* roots, showed antidepressant and anxiolytic activity when experimented using forced swim test and elevated plus maze model respectively in Laca mice (Mahajan et al., 2017). The hydro-alcoholic whole plant extract of *P. gangeticus* showed the ability to reverse the $AlCl_3$ induced spatial memory deficit in mice, thus emphasizing its neuroprotective activity (Changdar et al., 2019).

The ethyl acetate extracts of roots of *P. gangeticus* has the ability to inhibit lipid peroxidation, and henceforth was found to protect the myocardium against ischemia-reperfusion-induced damage in rats. The extracts when administered before ischemia was also found to improve cardiac functioning. The release of lactate dehydrogenase (a pathologic marker for myocardial ischemia) from the coronary effluent and levels of malondialdehyde (a bio-marker of oxidative stress) in myocardial tissues were found to be lowered by these extracts (Kurian et al., 2010a). Acetylcholine (Ach) in frog hearts is found to show negative ionotropic and chronotropic effects, i.e.,

reduce the heart rate, by its action through Ach/muscarinic receptor, a G-protein receptor activator. The methanolic extracts of *P. gangeticus* roots were found to show similar effects as shown by Ach. The route of action was also confirmed to be the same (G-protein receptor) as the physiological response was reversed by the addition of Ach/muscarinic receptor antagonist called atropine. The acetylcholine-like action was also confirmed by increased Na^{+}/K^{+} ATPases. Improved Ca^{2+} sensitivity of contractile protein is due to reduced cardiac marker enzyme and improved Ca^{2+} ATPase activity induced by the extracts. This highlights its role as a cardioprotective one (Kurian et al., 2010b). There was also a decrease in the release of lactate dehydrogenase in the coronary effluent which indicated the cardioprotective function of the ethanol extract of leaves. (Kurian et al., 2010c). Pretreatment of Wistar rats with aqueous extracts of roots of *P. gangeticus* were found to reduce the extent of mitochondrial and sarcoplasmic damage during IR, as the cardiac mitochondrial enzymes and sarcoplasmic ATPases were significantly found to have improved (Kurian & Paddikkala, 2010).

Anti-inflammatory potentials were also studied by carrageenan induced paw oedema in rats. Carrageenan is a bi-phasic sulphated polysaccharide as it induces inflammation and pain in two phases. The first is release of serotonin and eventually by releasing inflammation causing bradykinin, prostaglandins and lysosomes. The anti-inflammatory property of the plant *P. gangeticus* has been attributed to its ability to inhibit the mediators responsible for inflammation and pain (Rathi et al., 2004). The cyclooxygenase (COX) protein exists in two different isoforms COX-1 and COX-2. The former is a housekeeping enzyme found in almost all tissues, and the latter is involved in inflammation. Synthetic drugs like aspirin, ibuprofen etc. inhibit COX proteins in general, and so have various side effects when used for a prolonged period of time. The search for natural compounds that specifically targets COX-2 inhibition, lead to the discovery of the potentials of sialicin isolated from *P. gangeticus* extracts to bind to COX-2 protein and inhibit them. The arachidonic acid and VEGF pathway enzymes play a vital role in tumor angiogenesis and metastasis along with COX proteins. It is therefore estimated that these proteins may be actively involved in interaction with salicin (Srivastava et al., 2013).

The methanol extracts of stem of *P. gangeticus* was found to possess anti-nociceptive activity, as they relieved acetic acid induced abdominal pain in mice

(Jahan et al., 2010). The ethanolic extracts of *P. gangeticus* helped in reducing the acid output measured after pyloric ligation. The cytoprotective ability of the extract was proved due to enhanced mucin secretion and it also protected the gastric mucosa by inhibiting gastric secretions. *P. gangeticus* also showed immense anti - ulcer property in a dose dependent manner, in a pylorus ligation (PL) induced ulcer model in rats. These PL ulcers cause accumulation of gastric acids, and pepsin, thereby degrading the gastric mucosa (Dharmani et al., 2005).

The aqueous extracts of *P. gangeticus* were capable of reducing the activities of cardiac specific enzymes like LDH, SGOT, and ALP in the serum, which elevates during a myocardial damage, thus being cardio protective in nature. The extract also lowered the levels of total cholesterol, LDL and triglycerides thereby confirming its hypocholesterolemic action (Kurian et al., 2005). *P. gangeticus* methanol root extracts were found to protect the myocardium when administered, post a global ischemia. The role of acetylcholine (Ach) is to bind to the muscarinic receptor, a g-protein coupled receptor to reduce I/R (ischemia reperfusion) injury. The *P. gangeticus* root extracts were interestingly found to mimic the Ach action on muscarinic receptors thereby being an effective cardio protective agent (Kurian & Paddikkala, 2012). Excessive collagen deposition in the ventricles of the heart, is a strong indicator of development of fibrosis. The roles of metalloproteases (MMPs), like MMP-2, MMP-9 and MMP-13 are to increase the collagen synthesis thereby creating an imbalance between its synthesis and degradation eventually, leading to the deposition of collagen and cardiac fibrosis. The aqueous root extracts of *P. gangeticus* were found to reduce the levels of ventricular MMP-2 and MMP-9 in isoproterenol induced cardiac hypertrophic rats, thereby exhibiting its antifibrotic activity (Hitler et al., 2014).

The chloroform extract of roots of *P. gangeticus* decreased the levels of bilirubin SGOT and SGPT levels, which is an indication of regeneration of damaged hepatocyte cell membrane (Prasad et al., 2005).

The glycolipids isolated from chloroform and N-butanol fractions of ethanol extracts of *P. gangeticus* were found to increase the NO production in macrophages thereby emphasizing its immunostimulatory action and also the intracellular leishmanicidal activity in peritoneal macrophages (Mishra et al., 2005).

The extracts of aerial parts of *P. gangeticus* were found to possess antidiabetic potential, as it increased the insulin secretion from MIN6 cells, which display the characteristics of pancreatic beta cells by secreting insulin. The increased insulin production helped reduce the glucose levels, *in vitro* and also *in vivo* in diabetic rats treated with the extracts of *P. gangeticus*. The extracts of *P. gangeticus* also had the potential to stimulate the β-cells directly to secrete insulin and thereby confer protection against hyperglycemia (Govindarajan et al., 2007). The oral and intraperitoneal administration of insulin in rats, along with aqueous extract of roots of *P. gangeticus* helped insulin to get absorbed across the GI tract in active form. The increased levels of insulin also threw light on the secretagogue action of the extract (Kurian et al., 2010d)

The wound healing capacity of aqueous extracts of *P. gangeticus* was remarkable when experimented on incisions, excisions and dead space wound model in rats. The extract was mixed with ointment base and applied, and the results were impressive and comparable to those of a standard drug povidone iodine ointment (Jain et al., 2010).

The renal protective ability of ethanol extracts of *P. gangeticus* was investigated, and it was found that the extracts had the ability to remove oxidative stress and decrease blood glucose levels of diabetic rats, which in turn helped the protection of the renal system (Yasmeen et al., 2011).

The methanolic extracts of *P. gangeticus* was also found to possess antimicrobial activity against gram -ve pathogenic bacteria like, *Proteus mirabilis*, Pseudomonas *aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi*, *Salmonella typhinurium*, *Escherichia coli* and also a naturally occurring fungus, Candida *albicans*. The antibacterial activity was attributed to the presence of phenolic, glycosidic and flavonoid contents present in the crude extract (Vijayalakshmi et al., 2011). The antibacterial activity of chloroform, alcohol, methanol and aqueous extracts of *P. gangeticus* were studied against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi*, *Streptococcus mutans* and *Pseudomonas aeruginosa*. All the extracts except aqueous extract showed significant antibacterial activity. However, the highest potential was shown by methanol extract, followed by ethanol and chloroform extract (Karthikeyan et al., 2012).

The leaf extracts of the plant were subjected to the synthesis of biologically active nanoparticles and it produced ecofriendly nanoparticles that had significant antibacterial activity against selected bacterial strain of *E. coli* (Thirunavoukkarasu et al., 2013). The metal oxides with nanostructures are widely being used in various technological areas. Titanium dioxide (TiO_2) is a metal oxide used as nanoparticles, were earlier considered safe but now is reported to have pulmonary and renal toxicity. In order to confirm this, experiments were conducted in wistar rats and it revealed the toxicity of TiO_2 synthesized by conventional sol-gel technique. Meanwhile, the same when synthesized with the aqueous extracts of roots of *P. gangeticus* did not show any signs of renal toxicity and also was found to maintain the levels of mitochondrial enzymes which prevented the risk of mitochondrial toxicity and eventual cell death (Ansari & Kurian, 2017). According to Rohit et al. (2015), copper oxide nanoparticles synthesized using aqueous extracts of *P. gangeticus* roots, were found to be much less toxic when compared to the ones synthesized without it. The copper oxide nanoparticles accompanied with the extracts also showed impressive antibacterial potentials. Similarly, the silver nanoparticles synthesized from methanol extract of the stem of *P. gangeticus* are known to possess antioxidant and antibacterial properties (Azad & Banarjee, 2014). The synthesis of $NiCl_2$ nanoparticles using aqueous extracts of roots of *P. gangeticus* not only helped in discovering a non-toxic nanoparticle but also revealed its potential to be a good antioxidant and antimicrobial agent owing to the presence of phenolic compounds in the extract (Sudhasree et al., 2015). The mechanism of action was further discovered to be the inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. The COX promotes the conversion of arachidonic acid to prostaglandins which are intimately involved in inflammation. The lipoxygenase enzyme levels are also known to be high in the case of inflammation, asthma and allergies. Therefore, a dose-dependent inhibitory effects of aqueous extracts of aerial parts of *P. gangeticus* and petroleum ether extracts of roots of *Abroma augusta* on COX and LOX were confirmed to be one of the reasons for its anti-inflammatory activity (Bisht et al., 2014). The gold nanoparticles biosynthesized from the leaves of *P. gangeticus* showed significant antioxidant potentials (Ghosh et al., 2020).

The compound salicin isolated from the leaves of *P. gangeticus* and the methanol and ethyl acetate extracts of the plant was found to possess anti-cancer properties and also increased the life span of the EAC tumor bearing host (Srivastava et al., 2015). The methanolic extracts of *P. gangeticum* were found to possess antimutagenic properties, as they reduced the structural aberrations induced cyclophosphamide in bone marrow cells (Hasmukhlal et al., 2016). The crude aqueous extract of *P. gangetcius* was found to inhibit growth of A549 human lung adenocarcinoma cells. The extract was known to arrest the cell cycle in the G1 phase by decreasing the levels of cyclin A, cyclin B1, and Cdc2 expression and increasing p21 and p27 expression in a concentration-dependent manner (Chen et al., 2022).

2.2 *Tragia involucrata*

2.2.1 Systematic position

Kingdom	Plantae
Phlyum	Tracheobionta
Class	Equisetopsida
Order	Malphigiales
Family	Euphorbiaceae
Genus	Tragia
Species	involucrata

2.2.2 Botanical Description

The plant is a slender twining perennial herb, with stinging hairs distributed throughout. Leaves are 2.5-12.5 cm long and 2-4.5 cm broad. It is simple, serrate, alternate and stipulated with a densely hispid-pubescent. The stem is long, slender and twining. The flowers are borne at the terminal axillary end and are apetalous, unisexual and regular. The fruit is a 3-lobed capsule of about 8 mm diameter. The seeds have slight mottling and are smooth, sub globose and grayish brown in colour (Pallie et al., 2020).

2.2.3 Ethnobotanical Uses

Identification of the medicinal importance of *T. involucrata* dates back to the 1st century AD. The plant has been mentioned in the ancient scriptures of Ayurveda like

Charaka samhita, *Sushruta samhita* and *Vagbhata samhita* by the name 'Vrishchakali', for the treatment of epilepsy, fever and respiratory tract disorders (Pallie et al., 2020). According to Surya et al. (2014), the entire plant of *T. involucrata* has the potential to act as an antidiabetic. In Sri Lanka the locals prepared decoctions of *T. involucrata* whole plant using equal amount of water and administered it orally to treat diabetes mellitus (Ediriweera & Ratnasooriya, 2009). The foot ulcer pathogens from diabetic patients were also successfully killed using *T. involucrata* (Reddy et al., 2017).

The aqueous leaf extracts of *T. involucrata* showed potent antibacterial activity against *Proteus vulgaris*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Ignacimuthu & Sen, 1998). The diaphoretic potentials of its roots have been exploited to bring down elevated body temperature and thereby curing high fever (Reddy et al., 2017). The whole plant is used as a mouthwash to cleanse the oral cavity. It is used in the treatment of gastric ailments and as an antiemetic, and also to cure diarrhoea, vomiting and dysentery (Pallie et al., 2020). The aqueous extracts of *T. involucrata* roots have immense potential to cure constipation and also when mixed with coconut oil and applied on the skin it relieves itches due to allergy (Rahmatullah et al., 2012). The root decoctions of *T. involucrata* are known to cure asthmatic problems (Savithramma et al., 2007). The indigenous system of medicine used root decoctions of *T. involucrata* along with pepper in treating bronchitis (Shanmugam et al., 2012).

The herbal composition comprising the extracts of *T. involucrata* are used for treating chronic inflammatory skin disorders like psoriasis, eczema and seborrheic dermatitis (Solanki, 2011). Pruritus is a skin disease that causes itchy skin and scratching sensation. The root extract of *T. involucrata* are known to cure pruritus (Sundarrajan & Arumugam, 2017). The powdered leaf and root bark of *Crotalaria pallida*, along with the leaves *Wrightia tinctoria* and *T. involucrata* are used to cure skin diseases by applying the paste made from these plant materials mixed with water (Ayyanar & Ignacimuthu, 2005). The ethnobotanical information collected from Niyamgiri hills of Kalahandi district, Odisha, India revealed that the stem and leaves of *T. involucrata* are fried in ghee and consumed to cure rheumatic ailments. The sun dried leaves of the plant, powdered and mixed with til oil was also used to treat scabies and ringworm (Mallik et al., 2012). Similarly, the

ethnobotanical survey conducted in Rajshahi districts of Natore found that the locals used several plants with various medicinal properties to treat a variety of ailments. They used the root and stem of *T. involucrata* along with 0.25 g of black pepper to warm the body. The smashed stem was mixed with mustard oil and applied on the skin to treat dermatitis (Mawla et al., 2012).

The information collected from local traditional health practitioners of Bangladesh which included predominant tribes such as Marma, Tanchangya, Chakma, Khumi, and Tripura was an eye opener about the innumerable medicinal properties of various plants. The decoction prepared from the roots of *T. involucrata* was known to treat painful micturition (Kadir et al., 2014). The presence or absence of endogenous inhibitors and complex formers increase the concentration of unbound calcium and phosphorus that causes formation of urinary calculi in renal tubules leading to a multifactorial disorder called urolithiasis. The urease producing bacteria seen in urinary tract infections produces ammonium magnesium phosphate known as struvite. The struvites account for around 17% of all the urinary stones. Calcium oxalate stones make up 79% of kidney stone diseases. The aqueous leaf extracts of *T. involucrata* showed inhibitory effect on the growth of struvite crystals *in vitro*. The inhibition was found to be more effective when the silver nanoparticles prepared from the extract were used. This was also found to inhibit the calcium oxalate stones *in vivo*. Therefore, the aqueous extracts from the leaves of *T. involucrata* and the silver nanoparticles synthesized from it proved to be very effective in the treatment of urolithiasis (Velu et al., 2017).

The roots of *T. involucrata* are known to be used as a blood purifier, in treating vomiting, giddiness, and also conditions like pitta, melagia and brachialgia (Dash et al., 2000). The whole plant of *T. involucrata* is crushed and the paste is applied on the forehead to cure pain in the head and forehead. This was recorded on a survey conducted to collect ethnobotanical information on indegenous plants in Bengal part of Manbhum region, Chota Nagpur, India (Dey et al., 2017). The ethnic group Yanadis in Cuddapah district of Andhra Pradesh, is known to administer the shoot extract of *T. involucrata* orally to treat epilepsy (Sharma et al., 2013). The floristic study of various plants conducted in the Vedha Giri Hills of Erode, revealed the medicinal properties of various plants, and the study brought into light the role of *T. involucrata* fruit paste in treating one sided headaches by topical application on the

forehead (Ravi et al., 2016). The survey conducted on the ethnomedicinal plants from Paderu division of Visakhapatnam district, Andhra Pradesh, revealed the medicinal properties of various plants that have been traditionally used in the area. Approximately 13 tribal groups are living in this area, and the indigenous information collected from them revealed that the whole plant of *T. involucrata* was highly effective in treating fever (Padal et al., 2010). Similar studies conducted in Paderu forest division of Andhra Pradesh, particularly in a place called Munchingiputtu mandal, helped to collect information regarding the use of *T. involucrata* by 'Kondadora' tribes to control predominant disease like Malaria and also in treatment of ophthalmic ailments (Padal et al., 2012).

The Yandi tribe of Chandragiri reserve forest, Andhra Pradesh used *T. involucrata* as an aphrodisiac. According to them, 2 capsules of *T. involucrata* when consumed for 5-6 days along with a glass of milk and honey were known to act as an aphrodisiac. The oral intake of 2 capsules for 3 consecutive days were also recommended by the Yandi tribe to get relief from scorpion or snake bites (Savithramma et al., 2016). The ethnobotanical survey conducted in the Kolli hills of Eastern Ghats, Tamil Nadu, revealed that the tribals here used the whole plant of *T. involucrata* to treat snake bites (Prabu & Kumuthakalavalli, 2012). The tribals of Malda district, West Bengal India, used the rhizome of *Zingiber cassumunar* Roxb. crushed along with ginger and mixed with powdered roots of *T. involucrata* are applied for healing ankle sprains (Saha et al., 2014).

2.2.4 Phytochemical Profile

The chemotaxonomic evaluation of *T. involucrata* has justified its inclusion in the family Euphorbiaceae. The phytochemical analysis of aqueous leaf extracts of *T. involucrata*, when subjected to TLC revealed 9 partially pure compounds. Further investigation on these compounds by GC-MS analysis revealed their identity (Samy et al., 2006a). The butanol fractions of aqueous alcoholic extracts of the whole plant of *T. involucrata* when subjected to further fractionation on silica gel yielded six sub fractions. Among them two subfractions contained components that helped in DPPH radical scavenging activity (Sulaiman & Balachandran, 2016). The petroleum ether, ethyl acetate and methanol extracts of *T. involucrata* were subjected to column chromatography using n-hexane: ethyl acetate as the mobile phase. The fractions (5

nos) were collected and tested against fungi *Malassezia furfur* and *Trichophyton rubrum.* showed potent antifungal activity. These fractions also showed antibacterial activity against certain gram +ve (*Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis, Bacillus brevis, Eschericia coli*) and gram -ve bacteria (*Vibrio cholera, Shigella dysenteriae, Pseudomonas aeruginosa*) (Panda et al., 2012). The phytochemical research on the methanol extract of leaves of *T. involucrata* revealed an important piperidine based alkaloid like phenyl methyl piperidine derivative that proved to be an effective muscle relaxant and bronchodilating agent. It also showed anti-allergic effects on histamine induced muscle contraction in guinea pigs, and further *in silico* investigation revealed that it contains good inhibition against histamine H1 receptor due to the hydrogen bond interaction of the ligand (Yadav et al., 2015). The table below shows the various phytochemicals identified from different parts of the plant *T. involucrata*.

Table 2.2: Phytochemicals identified from different parts of *T. involucrata*

Plant parts	Phytochemicals	References
Leaves	Shellsol, Vinylhexyl ether, 2 methylnonane, 2,4 dimethyl hexane, 2,6 dimethyl heptane	Samy et al., 2006a
Whole plant	Ar- Tumerone; 9, 10 Anthracenedione 1,8-dihydroxy-3-methyl; Friedelane-3-one	Sundaram et al., 2009
Roots	Stigmasterol, quercetin, rutin, 10, 13-dimethoxy-17-(6-methylheptan-2-yl)-2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetra decahydro-1Hcyclopenta [a] phenanthrene and 3-(2,4-dimethoxyphenyl)-6,7-dimethoxy-2,3- dihydrochro-men-4-one	Panda et al., 2012
Leaves	Steroids, terpenoids, glycosides, carbohydrates, alkaloids and flavanoids	Sahaya et al., 2013
Leaves	5-hydroxy-1-methylpiperidine-2-one	Yadav et al., 2015
Whole plant	Iridine, Quercetin derivatives like dihexosyl quercetin, quercetin-3-O-rutinoside, caffeoyl hexoxyl-O-hexoside, orientin and Tricin 7-O-hexosyl-O-hexoside	Sulaiman & Balachandran, 2016

2.2.5 Pharmacological review

The methanolic root extracts of *T. involucrata* were found to possess anti-inflammatory and antinociceptive activities. The possibility of anti-oedema activity was suspected to be due to the inhibition of lipoxygenase pathway. Lipoxygenases are enzymes that generate pro-inflammatory mediators (Dhara et al., 2000). Anti-inflammatory properties of aqueous leaf extract of *T. involucrata* were discovered, and the reduction in inflammation was comparable to that of the synthetic drug phenylbutazone (Samy et al., 2006b). The enzyme Phospholipase A_2 has a vital role in inflammation, and so herbal anti-inflammatory drugs that inhibit PLA_2 are under study. The hydrocarbons 2,4-dimethyl hexane, 2 methylnonane and 2,6 dimethylheptane derived from *T. involucrata* had the potential to reduce oedema induced by snake venom and also to reduce the human synovial fluid PLA_2 (Samy et al., 2012).

The hydrocarbon esters such as shellsol, vinyl hexyl ether, and 2, 4-dimethyl hexane isolated from *T. involucrata* possessed antimicrobial and anti-inflammatory activities and it could be effective in controlling the growth of certain food-borne and food-spoilage pathogens (Reddy et al., 2017). The fractions of *T. involucrata* were collected using 20% (v/v) n-hexane and ethyl acetate as the mobile phase and were identified by GC-MS analysis to reveal the presence of phytochemicals present in them. The compounds shellsol and vinyl hexyl ether had strong inhibition against *S. aureus* and *P. vulgaris* whereas 2-methylnonane and 2,6 dimethylheptane had moderate inhibitions (Samy et al., 2006a). The ethanol leaf extract also showed antibacterial activity against *Pseudomonas aeruginosa, B. subtilis, V. cholera* and *R. arrhizus* (Sahaya et al., 2013).

Shellsol isolated from *T. involucrara* showed strong wound healing effects on rats. It was found to increase the hydroxyproline levels by two fold, which helped in increasing the levels of collagen that plays a major role in wound healing (Samy et al., 2006a).

The psychopharmacological studies with methanolic root extract of *T. involucrata* gave several interesting findings. The extract, when tested on Swiss albino mice and Charles-Foster rats, had a significant effect on the CNS. A reduction in exploratory behavior was noticed, which resembled the action of CNS-depressant drugs. Several

other behavioural changes were also recorded. Reduction of spontaneous motility, analgesic action against acetic acid induced writhing, increased strength of nerve impulse in pentobarbitone induced time and hypothermia. The mechanism of action was suspected to be similar to the synthetic drugs reserpine and chloropromazine, that induces hypnosis by hypothermic action. The extract was found to increase the GABA content, similar to that shown by synthetic anxiolytics like diazepam, thereby revealing its anxiolytic property. It also exhibited motor incoordination and muscle relaxant activity and therefore a significant CNS-depressant activity (Dhara et al., 2002). The methanolic leaf extract of *T. involucrata* showed significant antifungal properties against *Rhizopus stolonifer, Aspergillus niger and Alternaria solani* (Gupta et al., 2019). The ethyl acetate and methanol fractions of the whole plant of *T. involucrata* had potential antinociceptive activity, which indicated the presence of analgesic components in the extracts. The acetic acid induced writhing model and radiant heat tail-flick model in mice, was conducted for studying their peripheral and central antinociceptive action respectively. Both the extracts proved to have peripheral antinociceptive potentials, whereas the methanolic extract alone showed central antinociceptive potentials (Alimuzzaman & Ahmed, 2005).

The methanolic leaf extract of *T. involucrata* was also found to increase the levels of antioxidant enzyme APX. Increase in such antioxidant enzymes aids free radical and ROS scavenging, thereby inhibiting the growth of microbial pathogens (Gupta et al., 2019). The aqueous methanolic extract of *T. involucrata* and three fractions obtained from it was proved to have antioxidant potential and brine shrimp lethality potentials (Savithri et al., 2010). The petroleum ether, chloroform and aqueous extracts of roots of *T. involucrata* were tested for anti-diuretic, anti-inflammatory, anthelmintic and analgesic activity and all the extracts showed potential anti-inflammatory and anthelmintic activities in carrageenan induced paw models and earthworms respectively. However, the diuretic and analgesic activity was prominently shown by the aqueous extract, and the latter may be due to the inhibition of lipoxygenase and cyclooxygenase pathway of arachidonic acid metabolism (Rao et al., 2007a). The methanolic extracts of leaves of *T. involucrata* possessed anthelmintic activity as it was found to have dose dependent paralytic effect on earthworms (Patil et al., 2015). The decrease in GSH levels intracellularly, in acetaminophen induced rats are an indication of reactive oxygen species production, which causes cellular damage. The

ethanol extracts of *T. involucrata* was found to increase the GSH (glutathione), SOD (superoxide dismutase), and CAT (catalase) levels, and thereby protecting the hepatic and nephrotic tissues (Palani et al., 2009).

The aqueous, petroleum ether and chloroform extracts of *T. involucrata* showed antidiabetic effects in the decreasing order respectively. The pancreatic β cells are known to get regenerated with the help of flavonoids, and insulin secretion from these cells are known to get stimulated by glycosides, thus lowering the blood glucose levels. The presence of both these compounds were found in these extracts and hence the antidiabetic potential (Rao et al., 2007b). The aqueous-ethanolic extract of *T. involucrata* was examined for its antidiabetic property in rats, by experimentally inducing hyperglycemia with streptomycin and nicotinamide that destroy the pancreatic β cells. The treatment of these rats with the extract, was found to regenerate the β islets of pancreas, which was evident from histopathological examination. This leads to decrease in the blood glucose levels (Farook & Atlee, 2011). The methanolic extracts of *T. involucrata* was found to be effective against *Escherichia coli, Proteus vulgaris* and *Staphylococcus aureus* isolated from pus samples of diabetic foot ulcer and also from urine samples of diabetic patients (Chelladurai & Suresh, 2016). The blood glucose levels of alloxan monohydrate induced rats were lowered by ethanolic extracts of the whole plant of *T. involucrata* (Kar et al., 2003).

The hexane and ethyl acetate extracts of *T. involucrata* were reported to have anti-fertility effects on Wistar rats. The extracts had anti-implantation effects on rats, when administered orally. It was found that these extracts had the ability to increase the estrogen levels, and so an imbalance between estrogen and progesterone levels did not support implantation thereby causing infertility. The histopathological examination confirmed an unfavorable uterine milieu (Joshi & Gopal, 2011). The ethanolic extracts of *T. involucrata* possessed free radical scavenging activity, and the detection of phenolic compounds in it proved its DNA protection, DNA repairing and metal chelating activity with the help of DNA nicking assay (Prakash et al., 2009).

The hexane and ethyl acetate extracts of *T. involucrata* were found to reduce tumor volume in EAC tumor bearing mice. The life span of EAC tumor-bearing mice was also increased, as the extracts helped deplete the ascites fluid, depriving the tumor

cells of its nutritional requirements thereby delaying the cell division (Joshi et al., 2011). The methanol and hexane extracts of *T. involucrata* have been previously known to show cytotoxic effects on KB and MCF-7 cell lines and the chloroform and ethyl acetate extracts of *T. involucrata* showed antiproliferative effects on K562 cell lines (Thomas et al., 2021).

2.3 Helminthiasis and the role of plants in their treatment

It is estimated that globally around 2 million people are affected by soil-transmitted helminths (STH) thereby suffering from helminthiasis, and another 4.5 billion people are estimated to be at a high risk of getting infected. Among various infectious diseases worldwide, intestinal helminth infections are the most common ones. Globally more than 200 million people are said to be affected by a common helminthiasis called schistosomiasis and over 90% of the cases are in sub- Saharan Africa (Alemayehu et al., 2017).

Numerous plants have been previously reported for their anthelmintic efficacy by the indigenous system of medicine. The essential oils of *Ocimum gratissimum*, demonstrated potent anthelmintic effects, by inhibiting egg hatch of *Haemonchus contortus*. The essential oils from this plant constituted 14 components, out of which the main ones were eugenol (43.7%) and 1,8- cineole (32.71%) (Pessoa et al., 2002). The aqueous extracts of *Vernonia amygdalina* and *Annona senegalensis* showed anthelmintic activity by inhibiting egg hatch in the case of adult female *H. contortus* harvested from the abomasa contents of sheep (Alawa et al., 2003).

The anthelmintic activity is greatly associated with the condensed tannins (CT) present in the plant extracts. The fractions with high molecular weight CT showed greater anthelmintic activity, compared to the ones with low molecular weight (Spiegler et al., 2017; Williams et al., 2014). The plants with high amounts of condensed tannins, like *Havardia albicans* and *Acacia gaumeri* were found to possess anthelmintic potential as they inhibited both the larval migration and exsheathment. However, the plants *Brosimum alicastrum* and *Leucaena leucocephala* did not significantly inhibit larval migration, but affected the exsheathment in a dose-dependent manner (Alonso-Diaz et al., 2011). The reduction in fecal egg count (FEC) was observed in tannin treated nematode species, in which *H. contortus* in the abomasum of ruminants were more susceptible to tannins,

compared to *T. colubriformis* residing in the intestines. The difference in response shown by the nematodes can be attributed to the different sites occupied by them in the GI tract (Max et al., 2005). The rhizomes of *Zingiber officinale*, barks of *Ficus religiosa* and the bulb of *Allium sativum* showed strong anthelmintic activity against *H. contortus* larvae. Tannins and saponins from the leaf extracts of *S. mombin* showed significant anthelmintic activity against nematode eggs collected from goat fecal samples (Athanasiadou et al., 2001). The importance of condensed tannins in providing anthelmintic property to the plants, was studied using bioactive fractions of extract from the leguminous forage *Onobrychis viciifolia* commonly known as sainfoin. The fractions containing tannins and flavonol glycosides were tested against the infective L3 stage of *H. contortus* larva, by larval migration assay, and was found effective in causing larval mortality (Barrau et al., 2005). The tannins/polyphenolic compounds present in the plant extracts of *Arachis pintoi*, *Cratylia argentea (C.a) Yacapini*, *C.a Veranera* inhibited the exsheathment of L3 larval stage of *H. contortus larvae* and *C.a veranera* and *Gliricidia sepium* reduced their motility (Von Son-de Fernex et al., 2012). Tannins are known to interfere with the physiological functions of helminth worms such as motility, feed absorption and reproduction by attaching to glycoproteins on the cuticle. Chemically tannins and polyphenolic compounds were also known to uncouple oxidative phosphorylation, thereby interfering with the energy generation mechanism in helminth parasites (Aggarwal et al., 2016).

The aqueous extract of stem bark of *Nauclia latifolia* showed dose dependent egg hatch inhibition of strongyle eggs (Onyeyili et al., 2001). Thymol from *Lippia sidoides* showed ovicidal and larvicidal activities against *H. contortus*. Similarly, activity was also observed in the essential oils from *Croton zehntneri* (Camurca-Vasconcelos et al., 2007). A herbal formulation, prepared using aqueous extracts of leaves of *Azadirachta indica* and *Nicotiana tabaccum*, and flowers and seeds of *Calotropis procera* and *Trachyspermum ammi* respectively were tested against *H. contortus* following standard procedures of egg hatch and adult motility assay. The formulation proved to have stronger ovicidal and wormicidal effects *in vitro* against *H. contortus* when compared to levamisole, the reference drug that was used (Zaman et al., 2012). The mechanism of anthelmintic activity by secondary metabolites like tannins and alcohols present in the plant, *Vernonia amygdaina* was known to be

similar to that of benzimidazole. They affect the microtubule system, which interferes with the feeding ability of worms due to paralysis, thus leading to its mortality (Oyeyemi et al., 2018).

The acetone extract of the plant *Vernonia amygdalina* Del. showed significant dose dependent anthelmintic effects against *H. contortus* eggs and larva. This plant was believed to have been used for wild chimpanzees and gorillas as self-medication for parasites. The plant contains several bioactive compound like vernolanin, vernolide, hydroxyvernolide, vernomydine and some novel sigmastane type steroid glycosides (Ademola & Eloff, 2011).

The polarity of secondary metabolites presents in plants have an important role in determining its anthelmintic activity. This was studied by Eugale et al. (2006) in eggs and adult worms of *H. contortus* by treating them with hydro-alcoholic and aqueous extracts of the plants *Croton macrostachyus* and *Ekebergia capensis*. The hydro-alcoholic extracts showed better activity in adult worms when compared to aqueous extracts, and the reason could be easy transcuticular absorption of hydro-alcoholic extracts as they may possess some non-polar organic chemicals with a wide range of polarity when compared to aqueous extracts. The extracts of *Turnera ulmifolia* (leaves and roots), *Parkia platycephala* (leaves and seed), and *Dimorphandra gardneriana* (leaves and bark) showed potent anthelmintic activity against various larval stages of *H. contortus* (Oliveira et al., 2017). The acetone-water plant extracts of *Lysiloma latisiliquum, Laguncularia racemose, Rhizophora mangle, Avicennia germinans* were known to have nematicidal activity against *H. contortus*. They seemed to block the larval elusion from eggs of *H. contortus* (Vargas – Magana et al., 2014).

In the pharmaceutical market, the use of therapeutic proteins plays a very important role and has been subjected to several stages of development to attain viable commercial products. The high levels of proteases and chitinase activity of the extracts of *Leucaena leucocephala* was found to possess potential anthelmintic activity against nematode eggs (Soares et al., 2015). The proteins present in the fractions of *Spigelia anthelmia* were known to possess strong anthelmintic activity against *H. contortus* by inhibiting the egg hatching. Plant defense proteins including proteases, protease inhibitors and chitinases were identified in the fraction, and secondary metabolites were absent in these fractions (Araujo et al., 2017). The ethyl

acetate and methanol extracts of leaves of *Annona squamosa, Eclipta prostrata, Solanum torvum and Catharanthus roseus* and the seed extracts of *Terminalia chebula* showed egg hatch and larval developmental inhibition (Kamaraj & Rahuman, 2011). The extracts of plant *Acacia nilotica* and *Acacia radiana* showed anthelmintic effect properties against *H. contortus*. The former rich in condensed tannins (CT) showed no effect on inhibition of egg hatch and little effect on adult mortality. Significant effect on inhibition of larval exsheathment was observed. However, *A. raddiana*, which was rich in total tannins and total phenols, proved to be effective against all the stages of nematode (Zabre et al., 2017). Extracts of *Balanites aegyptiaca* and *Sesbania sesbania* showed very high anthelmintic effects on *C. elegans* (Ibrahim, 1992).

The essential oil of citronella showed a potential anthelmintic activity on live *Fasciola gigantica.* The results were comparable with that of the synthetic drug oxyclozanide. The essential oil had effects on the uterus as they showed shrinkage of yolk, and disintegration of eggs. The absence of villi in the intestinal caeca, desquamation of the gut epithelial cells, and total disappearance of spines from the cuticle confirmed the mechanistic action of the oil on *F. giganticum.* The anthelmintic effects of aqueous extracts of *Allium sativum*, *Lawsonia inermis* and *Opuntia ficus indica* were examined in adult *F. gigantica.* The extracts of *O. ficus indica* caused paralysis of the adult flukes at 2.5 and 5% concentration while that of *A. sativum* and *L. inermis* were effective at 5% concentration. Microscopic examinations revealed the detachment of spine, rupture of intestinal caeca and blebbing of tegument (Jeyathilakan et al., 2012). *Artemisia Mexicana, Bocconia frutescem, Lantana camara, Piper auritum and Cajanus cajan* showed significant fasciolicidal activity when examined against *Fasciola hepatica.* The presence of alkaloids in all these plants except *A. mexicana and C. cajan* were estimated to be the reason behind its anthelmintic effects. However, some non-nitrogenous compounds in the plants *A. mexicana and C. cajan* have been attributed to its fascolicidal activity (Alvarez-Mercado et al., 2015).

The anthelmintic activity of the whole plant of *Chenopodium album* (L) and *Caesalpinia crista* (L) were confirmed by *in vitro* and *in vivo* studies. The chlorinergic effect was believed to be the reason for the cessation of movement of adult worms leading to its death. This action could be due to direct effect on the

muscle, activation of the transmission process in the neuromuscular junction or both (Jabbar et al., 2007). The aqueous extracts of *Vernonia amygdalina* were tested against ascarids (Toxocara canis) and hookworm (Ancylostoma caninum). The experiment was conducted in puppies by observing the fecal samples before and after treatment with these extracts. A significant reduction in the helminth count was observed, thus highlighting it's *in vivo* anthelmintic activity (Adedapo et al., 2007). The ethanol extracts of pumpkin seeds (*Cucurbita pepo*) were found to have nematicidal properties. The *in vitro* studies revealed that the extracts had a nematicidal effect on the egg larval as well as the adult stages of *Heligmosoides bakeri*, a nematode that affects the gastrointestinal tract of mice. The *in vivo* studies on mice infected with *H. bakeri* also showed satisfactory results as the extract helped in reducing the fecal egg counts and also the number of adult stages of the nematode (Grzybek et al., 2016).

In the Amazon area, some species like Ficus glabrata (Moraceae) are pharmacologically known to be potent against intestinal worms. Picopendri P, a terpene isolated from *Picrodendron baccatum, was* found to have immense nematicidal activity (Kone et al., 2005). The crude ethanol extracts from the leaves of *Cassia alata, Cassia angustifolia* and *Cassia occidentalis* were studied for them *in vitro* anthelmintic potentials against helminths like *Heterakis gallinarum, Raillietina tetragona* and *Catatropis sp.* from domestic fowl. The results showed that *C. alata* induced early paralysis in worms followed by *C. angustifolia*. Also, it was found that the combination of *C. occidentalis* and *C. alata* had stronger worm paralyzing ability than the combination of *C. alata* and *C. angustiflora.* A broad spectrum vermifugal activity was seen in all the plant extracts tested (Kundu et al., 2014). The ethanolic and aqueous extracts of *Vernonia amygdalina* and *Secamone africana* have good anthelmintic potential, as they inhibit *Ascaris suum* motility, in a dose-dependent manner. Extracts of *S. africana* did not contain saponins and anthocyanosides as in *V. amygdalina* and that was attributed to be one of the reasons for the former extracts to be less potent than the later ones (Nalule et al., 2013). Notoginsenoside R_2 and 4,6 Digalloyl glucose isolated from ethyl acetate extracts of aerial parts of *Chenopodium album*, were known to possess good anthelmintic potentials (Choudhary et al., 2021).

The plants used in Turkish folklore medicine were examined for their anthelmintic properties. The highest inhibitory effects on pinworms (*Syphacia obvelata* and *Aspiculuris tetraptera*) that are commonly found in laboratory mice, was shown by ethanol and aqueous extracts of *Mentha longifolia* and *Jasminum fruticans*. Except the *Zea mays* seeds, ethanol extracts were found to have high anthelmintic potential. Although effective against both the pinworms, the aqueous extracts of *Pinus nigra sp* and *pallasiana* cones had only statistically significant activity on *S. obvelata* (Kozan et al., 2006).

2.4 Cancer and the use of plant derived metabolites in its management

Emerging number of new cancer cases in a particular population, over a specific period of time is called 'cancer incidence'. The exposure to etiological factors, individual susceptibility, screening practices and quality and acceptability of health care are the factors that determine the incidence. Cancer incidence is one among several other factors that cause cancer mortality, the others being individual biological factors, tumor characteristics, stage of diagnosis and response to treatments (Kamangar et al., 2006). Considering specific cancer types the colorectal and breast cancer cases are estimated to reach 4.7-4.4 million globally, by the year 2070. This is predicted to be followed by lung and prostate cancer approximately affecting 3.8-2.9 million (Soerjomataram & Bray, 2021). The side effects both major and minor which are an inevitable part of conventional therapeutic methods like chemotherapy and radiotherapy used to treat cancer discourage patience to preserve consistently through the medical protocols, which in turn leads to progression of the disease and associated complications. The minor complications include nausea, vomiting, diarrhea and constipation while the major ones are myelosuppression and organ toxicity. There is a strong need for constant research in an alternative therapeutic method to treat the disease in a more cost-effective, potent and less toxic manner. Natural products isolated from the plant kingdom account for almost 60% of the currently used anti-cancer drugs and globally more than 3000 plants have been reported to have anticancer potential. The use of plant derived products for cancer treatment reached 50% in Asiatic patients (Alonso-Castro et al., 2011). The aqueous extracts of *Selaginella tamariscina* were found to be effective against

human leukemia cell line U-937 and human ovarian cell line A-2780. The extracts were found to induce apoptosis by DNA fragmentation, as a result of increased p53 gene expression and cell cycle arrest at the G1 phase. The hot aqueous extracts of bark of *Acer nikoense* was effective against mouse leukemia P-388 cell lines, induced apoptosis. Apoptosis of prostate (PC-3, DUI-45), stomach (MKN-45), liver (QSY-7721, SK HEP-1), breast ((MDA-MB-435), ovarian (OVCAR), colon (HT-29) and lung (NCI-H-209) and some normal cells in culture was found to be induced by extracts of *Solanum muricatum*. Similarly, apoptosis inducing ability of European mistletoe (*Viscum album*) is widely exploited in Europe as an alternative method of cancer therapy (Taraphdar et al., 2001).

Alkaloids contain nitrogen, derived from amino acids, and from soluble salts in aqueous environment. In plant vacuoles, they remain as N-oxide in free state, or coupled with phenolic acid like chlorogenic acid or caffeic acid. Several alkaloids are present in the plant kingdom, and they are having anticancer potentials against various types of cancer. Vincristine, vinblastine, morphine and ajmaline are examples for combinatorial biosynthesis of alkaloids which are used to treat cancer. Thalicarpine and tetrandrine isolated from *Thalictrum dasycarpum Fisch*. and *Cyclea peltata Diels* respectively have antitumor potentials and were found to be active against walker carcinoma – 256 (WM) in rats. Emetine, an isoquinoline alkaloid, is a widely used amoebicide, however it is known to have activity against lung carcinoma at high dose levels. It is isolated from the plant *Cephaelis accuminata* (Sofi et al., 2018). Rebbamycin and staurosporine, from *Streptomyces albus* belongs to the family indolocarbazole alkaloids, and possess anti-tumor properties (Sanchez et al., 2005).

Naturally occurring phenolic phytochemicals are known to possess anti-cancer potentials and hence this means of natural resource is gaining greater interests in the field of research, for developing safe and cost effective chemotherapeutic drugs to combat cancer. The extracts of six commonly consumed berries (Cranberry, raspberry, blueberry, blackberry, strawberry and red berry) were tested for its anti-proliferative effect on human colon (HT-29), prostate (LN Ca P) breast (MCF-7) and oral (KB, HCT-116, CAL 27) cell lines and was found to inhibit the growth of these cancer cells in a dose-dependent manner. Several classes of polyphenolic compounds like anthocyanin, ellagitannin, gallotannins, kaempferol and quercetin

were found to have contributed to this property of the berry extracts. Blackberry and strawberry extracts also showed pro-apoptotic activities (Seeram et al., 2006).

The cell cycle transit is controlled by waves of cyclin/Cdk (cyclin-dependent kinase) activation. Hence Cdks play a very important role in regulating cell cycle and thereby proliferation. Flavonoids like quercetin, flavopiridol and 2-thio flavopiridol were found to possess good anticancer activities. Flavopiridol is said to have advanced Cdk inhibitory activity and hence a very potent naturally available anticancer agent (Sielecki et al., 2000). The phosphoinositide -3- kinases (PI-3-kinases) are a family of enzymes which help in generating new intracellular second messengers, thereby playing a vital role in cell-signaling. This in turn regulates other cellular activities like proliferation, apoptosis and cytoskeletal organization. Protein kinase C (PKCs) are receptors for tumor-promoting phorbol esters, and thus have a role in tumorigenesis. Flavonols are good inhibitors of PI-3-kinase and PKCs followed by flavones. This leads to the conclusion that they may have anti-proliferative and proapoptotic potentials (Gamet – Payrastre et al., 1999).

Tannins are polyphenolic phytochemicals that are known to possess anticancer activity. Condensed tannins isolated from black beans showed anti-proliferative effects on Caco-2-colon, MCF-7 and Hs578T breast and DU 145 prostatic cancer cells. However, no inhibitory effects were observed on normal human fibroblast lung cells. The tannin treated cancer cells showed reduced ATP levels and this was considered as one of the reasons for its decreased proliferation and migration activity (Bawadi et al., 2005). Terpenoids are a large family of secondary metabolites composed of isoprenoid units. They exhibit significant anticancer properties by acting on different stages of tumor development. The terpenoids like β-elemene, furanodiene, furanodienone, germacrone, curcumol and curdione isolated from the essential oils of *Curcumae rhizoma*, had promising anticancer effects. They exhibited these potentials via various mechanisms like inhibiting cell proliferation, inducing apoptosis, inhibiting migration, invasion, and adhesion by mediating both extrinsic and intrinsic pathways (Chen et al., 2021). D-carvone is an anti-nociceptive monoterpene isolated from oils of peppermint and caraway seeds. They have detoxifying ability and also the ability to regulate oxidative stress thereby acting as a cytotoxic to colon cancer. D-carvone also induced chromatin condensation and nuclear fragmentation in HT-29 and SW480 colon cancer cell

lines in a dose dependent manner thereby highlighting its apoptosis inducing potential. The sesquiterpenes like isobutyroplenolin and arnicolide D from *Centipede minima*, had potentials to inhibit NF-κB and increase ROS production thereby inhibiting cell proliferation and inducing apoptosis (Sharma et al., 2017).

Several anticancer agents derived from plants are in research and clinical trials. Some of them are: Sulphoraphane from cruciferous vegetables are known to have inhibitory effects on tumor growth in breast cancers and also antiproliferative effects. Paclitaxel (taxol) from *Taxus brevifolia* L. are known to induce apoptosis, inhibit the translation machinery of the cell and disrupt microtubules as well as spindle fiber formation. Epipodophyllotoxin from *Podophyllum peltatum* L is a podophyllotoxin isomer with proapoptotic effects as well as cell cycle interference. Five isolates vincristine, vinblastine, vinorelbine and vinflunine are vinca alkaloids isolated from *Catharanthus roseus* are known to have antitumor activities, pro apoptotic and cell cycle arrest inducing properties. It is also known to be anti-mitotic and binds to β-tubulin thereby being a microtubule inhibitor. Pomiferin is an isoflavonoid from *Maclura pomifera; Dereeis Malaccensis.* It induces cytotoxicity of cancer cells, has apoptosis inducing effects, causes DNA fragmentation and inhibits histone deacetylase. It has shown growth inhibition in six human cancer cell lines (Greenwell & Rahman, 2015).

Since 1940s upto 2006, 47% of 155 anticancer drugs in the pharmaceutical market in North America, Western Europe and Japan were either natural products or a progeny of semisynthetic natural product lead compounds. The high throughput screening of natural compounds and crude extracts have led to the isolation of targeted therapeutic molecules from these natural sources. From the leaves of *Alvaradoa haitiensis* Urb., belonging to the family Picramiceae, several anthracenone C-glycoside, alvardoins E-N, were isolated. Among them the most cytotoxic ones were alvardoin E and F. Alvardoin e was found to induce apoptosis, chromatin condensation and membrane depolarisation in human prostate cancer cells. Narciclasine and pancratistatin, isolated from the bulbs of a Narcissus species and *Hymenocallis littoralis* Salisb. are iso carbostyril alkaloids which showed *in vivo* growth inhibitory activity against sarcoma and lymphocytic leukemia models. Strong cytotoxic activity against a group of human cancer cell lines, like prostate (DU 145), lung (A549), and oral epidermoid carcinoma were exhibited by a

quassinoid, 2-'(R-)-O-acetyl glaucarubinone, isolated from the bark of *Odyendyea gabonensis.* Quassinoids are triterpenoid derivatives found in several plants belonging to the family Simaroubaceae. Similarly, diterpene quinone derivatives isolated from the rhizhomes of *Salvia miltiorrhiza* Bunge, of the family Lamiaceae, were found to have tremendous therapeutic efficiencies. These diterpenes known as "Tanshen" are namely; 1) Tanshinone, which had *in vitro* as well as *in vivo* inhibitory effects on breast cancer cells through regulation of adhesion molecules like ICAM-1 and VCAM-1. It also had apoptosis inducing potential 2) Tanshinone ⅡA, which had the ability to induce G2/M phase arrest in cervical cancer cells by disrupting microtubule formations. It was also found to be effective against hepatocellular carcinoma cells by interfering with NF-κB signaling and 3) Cryptotanshinone which also had the ability to arrest cell cycle at G1-G0 phase and also inhibit cyclin D1. From the tropical plant *Aglaia foveolata* silverstrol and its isomer 5" epimer, episilvestril were isolated. In human prostate cancer cell lines, silverstrol was known to induce apoptosis via caspase 2,9 and 10 regulation and also bring about a p53 independent cell cycle arrest at G2/M checkpoint. It was also known to have strong antileukemic potentials, as it reduced Mcl-1 expression in chronic lymphocytic leukemia cells (Pan et al., 2012).

The mistletoe plants (*Viscum album)* are a good source of anticancer agents. Viscotoxins (VT) and lectins isolated from them had potent cytotoxic effects. The crude extracts obtained from mistletoes were therapeutically more potent, when compared to isolated lectins and VTs. Camptothecin, a quinoline alkaloid, is a pentacyclic compound derived from the plant *Camptotheca accuminata*. Camptothecin derivatives were designed to remove its freely hydrolysable lactone ring, which made it unstable, and such derivatives proved to have commendable activity against topoisomeraseⅠ. Camptothecin prevents the DNA strands of a single chain from getting reassembled by binding to the DNA- topoisomerase Ⅰ complex. Camptothecin derivatives are used to treat different types of cancers either individually or in combination with other drugs to ensure greater efficacy. For metastatic and small-cell lung cancers, a camptothecin derivative called topotecan is used as a chemotherapeutic agent. Irinotecan (another derivative) on the other hand is used individually against gastrointestinal cancers and in combination with 5-fluorouracil and leucovorin in metastatic colorectal cancers. A stilbene derivative

called combretastatin from the *Combretum caffrum* tree was known to have cytotoxic potentials. This tree is also called 'African willow' as it is seen in the southeastern regions of Africa. It exists in the trans as well as cis forms. The CA4 (combretastatin 4) is a cis form of the stilbene derivative and is known to effectively put a check on cell proliferation and induce apoptosis by binding to the colchicine site on β-tubulin, thereby destabilizng the microtubules and inhibiting its polymerisation. Combretastatin is used alone or in combination with other anticancer compounds like paclitaxel, manumycin A or carboplatin to treat anaplastic thyroid cancers. Podophyllotoxins isolated from *Podophyllum emodi* is a toxic lignan. The podophyllotoxin as well as its derivative possess good cytostatic effects. It is known to inhibit tubulin polymerisation by binding to β-tubulin as well as induce both single and double stranded breaks in DNA causing a G2 phase cell cycle arrest. Since these podophyllotoxins are highly toxic in nature, their semisynthetic derivatives like etoposide and teniposide are commonly used to effectively treat various cancer types. These semisynthetic derivatives, however, have a different mechanism of action as it is phase specific and works in the interphase of the cell. It stabilizes the intermediate covalent complexes formed topoisomerase Ⅱ and the cleaved DNA. 12-O-tetra-decanoylphorbol-13-acetate induced skin tumors in female CD-1 mice were inhibited by geniposide, a compound isolated from 40 species of plants belonging to different families, the most famous one being fruits of *Gardenia jasminoides* Elli. of the family Rubiaceae. The derivative of geniposide, an aglycone called genipin, had strong apoptosis inducing effects in non-small cell lung cancer H1299 cells. It was known to initiate mitochondrial death cascade by increasing Bax levels in response to p38 MAPK signaling. Colchicine, an alkaloid from the plants of Colchicum genus, stabilizes the microtubules at low concentration while depolymerised it at higher concentrations. In the treatment of hepatocellular and choli angiogenic carcinoma, low concentrations of colchicine are clinically acceptable. Colchicine adversely affects mitochondrial metabolism in cancer cells by modifying the voltage dependent anion channels. Artemisinin, an antimalarial agent, was isolated from a Chinese plant called *Artemisia annua* L. belonging to the family Asteraceae. A semisynthetic derivative of this compound called artesunate was known to have anti-angiogenic potentials. In neuroblastoma cells artesunate was known to be effective against

chemoresistant neuroblastoma cells, by inducing apoptosis. Another anticancer drug, Homoharrigtonine isolated from several plants of Cephalotaxus genus, were used in Traditional Chinese Medicine. The drug was known to induce apoptosis in the cell by blocking the synthesis in the peptidyl transferase center. It is particularly used in treating breast cancer and leukemia, though used in treating several other cancer types. It was possible to achieve remission in 92% of chronic myelogenous leukemia patients. A modified diterpene quinone derivative called salvicine from the Chinese herb *Salvia prionitis* has shown good tumor inhibition in a wide range of human tumor cells in vitro and human tumor xenografts in mice. It was known to induce DNA damage leading to apoptosis in human cancer cells. An alkaloid in Apocynaceae species called elipticine was known to form strong covalent bonds with DNA, thereby disrupting the cell cycle. It particularly regulates the expression of cyclin B1 and cdc2. It was known to have commendable cytotoxic activity against breast cancer cells, leukemia, neuroblastoma and glioblastoma. Roscovitine, another anticancer compound, is a semisynthetic derivative of R-roscovitine (30) derived from olomoucine which is isolated from cotyledons of *Raphanus sativus* L. The semi-synthesized roscovitine was more effective in treating cancers when compared to olomoucine, as the former induced an irreversible inhibition on cell proliferation. The efficacy of the drug may be attributed to its targeted action on CDK 1, CDK 2 and CDK 5. In human cancer cells, it was known to inhibit RNA synthesis. In glioblastoma A172 it showed a dose dependent inhibition of cell proliferation and also pro-apoptotic effects. Maytansine from Ethiopian plant *Maytenus serrata*, and its derivatives have the ability to disrupt microtubule assembly by binding to tubulin. Hence it is considered a good antimitotic agent. Due to its poor efficacy and severe side effects, maytansine has ceased from being used clinically despite its cytotoxic potentials. However, maytansinoids, a derivative of maytansine, is effectively being used as a chemotherapeutic drug mostly in association with antibodies.

Another antineoplastic drug from the roots of *Thapsia garganic* L. called Thapsigargin was isolated. It is a lactonic sesquiterpene capable of inducing apoptosis. This drug binds to sarcoplasmic/endoplasmic reticulum calcium adenosine in the sarco/endoplasmic reticulum, and prevents its ability to pump calcium from the cytoplasm to sarcoplasmic and endoplasmic reticulum. This

disrupts the calcium homeostasis of the cell, and induces stress which leads to caspase activation. As a result of this, the apoptotic factors are released from the mitochondria ultimately leading to cell death. Bruceantin isolated from *Brucea antidysenterica* showed anticancer activities against numerous cancer cells like B16 melanoma, colon 38, and L1210 and P388 leukemia, in mice. Bruceantin showed tumor regression in early as well as advanced stages when studied *in vivo* using RPMI 8226 human-SCID xenografts. The mechanism of action was found to be its interaction with peptidyl transferase, blocking the formation of peptide bonds, thereby inhibiting protein synthesis of the cell (Lichota & Gwozdzinski, 2018). Several plants like *Luffa aegyptiaca, Beta vulgaris, Capsicum frutescens, Solenostemma argel and Colocasia antiquorum* are known to have anticancer potentials against Acute Myeloid Leukemia (AML) and lymphocyte leukemia. Crocetin, a carotenoid isolated from *Crocus sativus* L. induced apoptosis and also inhibited nucleic acid synthesis. It showed cytotoxicity and antiproliferative effects on both human lung cancer cell lines and animal models (Gezici et al., 2019).

CHAPTER: 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Acetic anhydride	: MERCK Specialities Pvt Ltd., Mumbai
Albendazole	: MERCK Specialities Pvt Ltd., Mumbai
Aluminum chloride	: MERCK Specialities Pvt Ltd., Mumbai
Ammonium hydroxide	: NICE chemicals Pvt Ltd., Kochi
Ammonium oxalate solution	: NICE chemicals Pvt Ltd., Kochi
Anisaldehyde	: Sisco Research Laboratories Pvt Ltd
Annexin V	: Thermo Fisher Scientific, USA
Bismuth carbonate	: MERCK Specialities Pvt Ltd., Mumbai
Bouin's solution	: Hi- Media laboratory; Mumbai
Bovine serum albumin (BSA)	: MERK specialities Pvt Ltd., Mumbai
Chloroform	: MERCK Specialities Pvt Ltd., Mumbai
Copper sulfate (CuSO4.5H2O)	: MERCK chemical Ltd. India.
Cyclophosphamide	: NEON laboratory Ltd., Mumbai
Dimethyl sulfoxide (DMSO)	: MERCK Specialities Pvt Ltd., Mumbai
Disodium hydrogen phosphate	: MERCK Specialities Pvt Ltd., Mumbai
DPX	: NICE chemicals Pvt Ltd., Kochi
EDTA	: MERCK Specialities Pvt Ltd., Mumbai
Eosin	: Hi-Media laboratories; Mumbai
Ethanol	: MERCK Specialities Pvt Ltd., Mumbai
Ethyl acetate	: NICE chemicals Pvt Ltd., Kochi
Ferric chloride	: MERCK Specialities Pvt Ltd., Mumbai
Fetal Bovine serum (FBS)	: Gibco Life technology., Bangalore
Folin ciocalteu reagent	: MERCK Specialities Pvt Ltd., Mumbai

Formaldehyde	: MERCK Specialities Pvt Ltd., Mumbai
Gallic acid	: Sigma Aldrich, USA
Giemsa stain	: MERCK Specialities Pvt Ltd., Mumbai
Glacial acetic acid	: NICE chemicals Pvt Ltd., Kochi
Hematoxylin	: Hi-Media laboratories, Mumbai
Hydrochloric acid	: MERCK Specialities Pvt Ltd., Mumbai
Lead acetate	: MERCK Specialities Pvt Ltd., Mumbai
Leishman's stain	: Hi- Media laboratory; Mumbai
Lugol's iodine	: Hi- Media laboratory; Mumbai
Mercuric chloride	: MERCK Specialities Pvt Ltd., Mumbai
Methanol	: MERCK Specialities Pvt Ltd., Mumbai
Penicillin	: Hi- Media laboratory; Mumbai
Potassium acetate	: MERCK Specialities Pvt Ltd., Mumbai
Potassium dihydrogen phosphate	: MERCK Specialities Pvt Ltd., Mumbai
Potassium hydroxide	: MERCK Specialities Pvt Ltd., Mumbai
Potassium iodide	: MERCK Specialities Pvt Ltd., Mumbai
Propidium iodide Stain	: Thermo Fisher Scientific, USA
Propylene glycol	: MERCK Specialities Pvt Ltd., Mumbai
Roswell Park Memorial Institute Medium	: Life technologies, Bangalore
Sodium carbonate	: NICE chemicals Pvt Ltd., Kochi
Sodium chloride	: NICE chemicals Pvt Ltd., Kochi
Sodium hydroxide	: MERCK Specialities Pvt Ltd., Mumbai
Sodium iodide	: MERCK Specialities Pvt Ltd., Mumbai
Sodium sulphate	: MERCK Specialities Pvt Ltd., Mumbai
Streptomycin	: Hi- Media laboratory; Mumbai
Sulphuric acid	: MERCK Specialities Pvt Ltd., Mumbai

Toluene : MERCK Specialities Pvt Ltd., Mumbai

Toluidine blue : MERCK Specialities Pvt Ltd., Mumbai

Trypan blue : Spectrum private limited; India

Tyrosin : Hi- Media laboratory; Mumbai

Xylene : NICE chemicals Pvt Ltd., Kochi

3.1.2 Diagnostic reagent kits

Albumin : Beacon Diagnostics Ltd., Gujarat India

Alkaline phosphatase : Agappe diagnostics Ltd., Kochi

Bilirubin total : Agappe diagnostics Ltd., Kochi

Cholesterol HDL : Agappe diagnostics Ltd., Kochi

Cholesterol Total : Agappe diagnostics Ltd., Kochi

Creatinine : Euro Diagnostic Systems Pvt Ltd., Chennai

Drabkin's reagent : Agappe diagnostics Ltd., Kochi

Globulin : Agappe diagnostics Ltd., Kochi

Hemoglobin (Hb) : Agappe diagnostics Ltd., Kochi

SGOT : Agappe diagnostics Ltd., Kochi

SGPT : Agappe diagnostics Ltd., Kochi

Total protein : Agappe diagnostics Ltd., Kochi

Triglycerides : Agappe diagnostics Ltd., Kochi

Urea : Agappe diagnostics Ltd., Kochi

3.1.3 Instruments

Accelerated solvent extractor : Thermoscientific (Dionex ASE 150), USA

Atomic absorption spectroscopy : Shimadzu (AA-7000), Japan

Centrifuge : REMI (column oven CTO-20A), India

Cyclo vortex mixer : Rotex Instruments Pvt Ltd., India

Deep freezer (-20°c)	: Remi laboratory instruments, Mumbai
Deep freezer (-80°c)	: Thermo Fisher Scientific, USA
Electronic weighing balance – ATX224	: Shimatzu Corporation Ltd., India
Flow cytometer	: Beckman Coulter, USA
Fluorescent microscope	: Leica, German Radicle., Ambala
Fluorescent microscope	: MetaSystems, India Pvt. Ltd
GC-MS analyser	: Agilent (7890 A GC system fitted with -a DB 5MS column), USA
Hatching chambers	: Rotek Instruments Pvt. Ltd., India
High performance TLC	: CAMAG System, Switzerland
Horizontal Laminar flow hood	: Clean Air., Chennai, India
Hot air Oven	: Rotex instruments Pvt Ltd., India
HPTLC Scanner	: CAMAG System, Switzerland
Incubator	: Rotex instruments Pvt Ltd., India
Light microscope	: Leica (DM 500) German Radicle, Ambala
Magnetic stirrer	: Spinit Tarsons Products Pvt. Ltd. Kolkata
pH meter	: Elico Limited, Hyderabad, India
Phase contrast microscope	: Magnus, INVI, New Delhi, India
Spectrophotometer	: Agilent (Carry 60 UV vis), USA
Stereo zoom microscope	: Leica (M205 C) German Radicle, Ambala
Vortex mixer	: Rotek Instruments Pvt. Ltd., India
Water bath	: Rotek Instruments Pvt. Ltd., India

3.1.4 Animals

Male and Female Swiss albino mice (25-30 g) procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Science University, Mannuthy, Thrissur were used for *in vivo* studies. The animals were maintained in the animal house facility of Amala Cancer Research Centre following standard conditions (24 – 28°C, 60-70% humidity, 12 h dark/light cycle), and were fed with standard mice feed from Sai Druga Feeds, Bangalore, India and water *ad libitum.* They were kept in polypropylene cages with a bedding of paddy husk and covered with a stainless steel grill on the top which had the provision for providing food and water. All the animal experiments were conducted after getting approval from the Institutional Animal Ethics Committee (IAEC) (Approval No: ACRC/IAEC/20(1)-P06) and also by strictly abiding the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by Ministry of Environment and Forest, Government of India.

3.1.5 Cell lines and its maintenance

YAC-1 cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in RPMI medium supplemented with FBS (10% v/v), Streptomycin (100 µg/ml) and Penicillin (100 U/ml) and incubated at 37°C with 5% CO_2 in an incubator in a 25 ml T25 culture flask. Sterile conditions were maintained throughout. The old medium was regularly replaced with a fresh medium and also constant monitoring of the medium for any colour change was done. The cells were sub cultured when 70% - 80% confluence was attained. After discarding the spent medium, cells were washed thrice in PBS and were added to a fresh set of sterile culture flasks with appropriate amounts of fresh RPMI medium. The Dalton's Lymphoma Ascites cell lines were obtained from Adayar Cancer Institute, Chennai. These cells were maintained in the intraperitoneal cavity of mice at Amala Cancer Research Centre. The cells were aspirated from the intraperitoneal cavity and 1×10^6 cells were inoculated into the peritoneal cavity of another healthy mouse at around 14 days' interval.

3.1.6 Plant Material

Collection and authentication of *P. gangeticus* and *T. involucrata*

Pleurolobus gangeticus and *Tragia involucrata* are the plants selected for the study. The plants were collected from Thrissur district, Kerala, India and herbarium was prepared. The herbarium was used for identification and authentication by Dr. Sreekumar, HOD, Department of Botany, Kerala Forest Research Institute (KFRI), Peechi, Thrissur District, Kerala. A voucher specimen was maintained at KFRI (Voucher No. 17682 and 17683 for *T. involucrata* Linn. and *P. gangeticus* (L.) respectively (Figure 3.1)

3.2 Methods

3.2.1 Extraction of Plant material

The plant materials were collected and dried separately in a hot air oven at 47°C, and was finely powdered with the help of a mixer grinder. The powdered material was further used for extraction. The extraction of the plant materials was done using an accelerated solvent extractor, Thermoscientific (Dionex ASE 150), USA (Mottaleb et al., 2012). This instrument had a single extraction cell, and solvents of varying polarities could be used for extraction. Adsorbents (filter paper) were used while extracting which ensured a high degree of selectivity in the procedure. Initially an adsorbent was placed at the bottom of the extraction cell, prior to sample loading. The samples were ground well to reduce its particle size to less than 1 mm using a mortar and pestle. Dispersal of the samples is a vital step to prevent its aggregation, which would otherwise interfere with the extraction procedure. The sample mixture (15 g) was mixed well with an equal amount of an inert substance called diatomaceous earth, which will prevent the compacting of the samples and also absorb the moisture content if any. The stainless steel extraction cell was then filled with the sample, and cellulose filters 1.91 cm in diameter were placed on both the ends of the cell. The cell was tightly closed and placed in the instrument for extraction. The solvent used for extraction was filled (200 ml) in the allotted chamber above the instrument. Temperature ranging from 75-125°C depending on the solvent used; 1500 psi pressure was provided. Following extraction, the thimble was flushed with 60% solvent and purged with nitrogen. The extracts were collected in small 60 ml vials. In between each extraction procedure, the complete system was

rinsed to avoid any extract carry over. The extract was then transferred into a rotary vacuum evaporator and stored in air tight containers at 4°C in a refrigerator until further use.

3.2.2 Qualitative Phytochemical Screening

The whole plant extracts of *P. gangeticus* and *T. involucrata* taken in ethyl acetate, ethanol and water were tested for the presence of various phytochemicals like phenols, tannins, terpenoids, steroids, flavonoids and alkaloids using prescribed methods (Trease & Evans, 1989; Sofowora, 1996; Harborne, 1999).

3.2.2.1 Test for alkaloids

The extract was dissolved in 5 ml HCl and kept in a water bath for approximately 5 minutes, and filtered. The filtrate was allowed to cool and transferred into 3 test tubes. Each test tube containing the filtrate was treated in the following way:

a) Dragendorff's test - To the first test tube among the three, freshly prepared Dragendorff's reagent was added and presence of brownish precipitate indicates alkaloids.

   [Preparation of Dragendroff's reagent - In 50 ml of glacial acetic acid, 5.2 g of Bismuth carbonate and 4g of Sodium iodide were added and boiled for a few minutes. The precipitated sodium acetate crystals were filtered off after 12 h. To the filtrate (40 ml), 160 ml of ethyl acetate and 1 ml of water was added and thoroughly mixed. The solution was stored in amber coloured bottles and the working solution was prepared by mixing 10 ml of the stock with 20 ml of glacial acetic acid.]

b) Mayer's test - To the second one, one drop of Mayer's reagent was added and white precipitate indicates presence of alkaloids.

   [Preparation of Mayer's reagent - Mercuric chloride (1.358 g/60 ml water Potassium iodide (5 g/20 ml water) were mixed thoroughly and made up to 100 ml with distilled water]

c) Wagner's test - To the third one, one drop of Wagner's reagent was added and reddish brown precipitates indicate presence of alkaloids.

[Preparation of Wagner's reagent - iodine (1.27 g) and potassium iodide (2 g) were dissolved in 5 ml of sulphuric acid and made up to 100 ml]

3.2.2.2 Test for phenols

a) Ferric chloride test: To 1 ml of the crude extract dissolved in water, equal volume of ferric chloride was added and the presence of deep blue colour indicates the presence of phenols

b) Lead acetate Test: 10% lead acetate solution was added to the extracts and white precipitate indicates the presence of phenols.

3.2.2.3 Test for tannins

a) Ferric chloride test: 5% of ferric chloride was added to 2 ml of the extract, after the latter was heated using a water bath and filtered. Presence of dark green precipitate is an indication of tannins.

b) Lead acetate test: 2 to 3 drops of 1% lead acetate solution was added to 1 ml of the extract and yellowish precipitate formation is an indication of presence of tannins.

3.2.2.4 Test for flavonoid

a) Ferric chloride test: The extract (0.5 g) was boiled with water and filtered, and to the filtrate (2 ml), few drops of freshly prepared ferric chloride was added. Bluish green or violet colour indicated the presence of flavonoids.

b) Sulphuric acid test: Addition of concentrated sulphuric acid to the extracts and appearance of yellow colour is an indication of flavonoids.

3.2.2.5 Test for terpenoids

a) Salkowski test: 2 ml of the extracts were first treated with 2 ml of chloroform and then 3 ml of concentrated sulphuric acid and was added to it. A reddish-brown coloured ring formation indicated the presence of terpenoids.

3.2.2.6 Test for saponins

a) Foam test: Frothing upon addition of 5 ml of distilled water to the 2 ml of extracts is a clear indication of presence of saponins.

b) Sodium bicarbonate test – To 1 ml of extracts, 0.25 ml of $NaHCO_3$ and 0.25 ml of distilled water was added. A stable honeycomb like froth indicates the presence of saponins

3.2.2.7 Test for steroids

a) Salkowski test: The test is similar to that performed for analyzing the presence of terpenoids. 2 ml of chloroform and 2 ml of concentrated H_2SO_4 was added to 2 ml of the extract and shaken well. The presence of sterols was confirmed by the appearance of red colour in the chloroform layer and greenish yellow fluorescent colour in the acid layer.

b) Liebermann Burchard Test: To 1 ml of chloroform, 1 ml of acetic anhydride was added and cooled to 0°C. To this concentrated sulphuric acid (approx. 100 µl) was added, followed by 1 ml of the extract. Appearance of green, red or orange colour that changes according to time indicates the presence of steroids.

3.2.2.8 Test for glycosides

a) Borntrager's test: A mixture was prepared by mixing 2 ml of the extracts with 5 ml of dilute HCL and boiled for an hour in a water bath. In order to separate the chloroform layer, the above prepared mixture was initially treated with 2 ml of chloroform and mixed thoroughly. An equal amount of dilute ammonium solution was added to the chloroform layer, and an appearance of pink colour while adding ammonium solution indicates the presence of glycosides.

3.2.2.9 Test for coumarins

a) In a test tube containing 2 ml of the extracts, 3 ml of 10% of NaOH was added and incubated in a water bath (38ºC). The presence of yellow fluorescence when observed under UV light indicates the presence of coumarins

3.2.3 Quantitative phytochemical screening

3.2.3.1 GC-MS analysis for identification of volatile compounds

The bioactive extracts of both the plants were examined using the Shimadzu GC-MS instrument (model no: QP2010S). The GC was performed on an ELITE 5-MS column (30 m×0.25 mm (inner diameter), with 0.25 µm film thickness). The following conditions were provided while running the sample. Injection volume 1 µl; temperature; 260°C and pressure 61.5 kPa. The samples were injected in the split

mode and the mass spectra was recorded every 0.5 seconds over a range set with an m/z of 50-500 Da. The oven heating was initiated with an initial temperature of 70.0°C for 2 minutes, then increased to 200°C at the rate of 10°C/min, and then maintained at 280°C for an additional 15 minutes. It was possible to compute the phytochemical components from GC peak areas normalization, automatically done by the software. The NIST 11 and WILEY 8 library were used for performing the searches. Comparison of the mass spectrum with the reference spectrum enabled the identification of exact names of fifteen components.

3.2.3.2 LC-MS analysis for identification of non- volatile compounds

Ultra Performance Liquid chromatography coupled to UPLC-Q-ToF-MS (Quadrupole time-of-flight mass spectrometry) was carried out for chromatographic separation and detection of nonvolatile analytes. The Acquity UPLC system (Waters) were equipped with a TUV detector (J12TUV750A), sample manager FTN (K12 SD1069G) a column chamber (J12 CHA730G) and a solvent manager (H12Q SM632A). For chromatographic separation, reverse phased BEH C18 column (50 mm×2.1 mm×1.7 μm) with 0.3 $mLmin^{-1}$ flow rate were used. The mobile phase (mixture of water and acetonitrile with 0.1% formic acid) was used in a gradient mode. The samples were studied using ESI ionization mode and 10 μl of sample was injected. The m/z range scanned was between 50 and 1000. The dissolution gas flow was at the rate of 900 L/h at 350°C. The collision energy ranged from 5 to 30eV. Masslynx software (4.1) was used for data acquisition.

3.2.3.3 Estimation of Total Phenolic Content (TPC)

Folin-Ciocalteu spectrophotometric technique was used to determine the total phenolic content (Singleton & Rossi, 1965). To 1 ml of the extracts 3.9 ml of distilled water and 0.5 ml of the Folin -Ciocalteu reagent was added and kept intact for 3 minutes. Following this, 2 ml of 20% sodium carbonate solution was added to the tubes and allowed to stand in a water bath (40ºC) for 60 seconds. Agilent Carry 60 UV vis spectrophotometer was used to measure the absorbance at 650 nm. The standard used was gallic acid and the results were expressed as mg of gallic acid equivalents (mg GAE/g) of the extract.

3.2.3.4 Estimation of Total Flavonoid Content (TFC)

Aluminium chloride colorimetric method assisted in the quantitative estimation of TFCs in the plant extracts (Chang et al., 2002) The extracts were diluted by adding methanol to a concentration of 100 mg/ml. This was then mixed with 0.1 ml of 0.1 mM potassium acetate solution and 0.1 ml of 10% (w/v) aluminium chloride solution, incubated for half an hour at room temperature. After this, the absorbance was measured using Agilent Carry 60 UV vis spectrophotometer at 450 nm. Quercetin was maintained as the standard and data was expressed as mg of quercetin equivalents (mg QCE/g) of extracts.

3.2.4 Trypan blue dye exclusion method for cytotoxicity analysis

The evaluation of short term cytotoxicity was conducted according to Strober (1997), with minor modifications. This assay is based on the principle that live cells exclude trypan blue dye as they have an intact cell membrane, whereas the dead cells that have lost its membrane integrity will allow the entry of this dye, thus giving a dark blue appearance. The cells were suspended in PBS/RPMI and the number was adjusted to approximately $1x10^5$ cells/ml. These cells were treated with plant extracts at different concentrations and vehicle control was also kept for reference. For short term treatment, the cells were incubated in test tubes for three hours at 37°C, while for a longer time period cells were seeded in six well plates with RPMI medium and incubated at 37°C in a CO_2 (5%) incubator. After incubation trypan blue was added and kept at room temperature for 3 minutes. The observations were made in a haemocytometer under a microscope (40×). The percentage cytotoxicity was calculated by using the formula:

$$\% \text{ cytotoxicity } = \frac{\text{No.of dead cells}}{\text{No.of live + dead cells}} \times 100$$

3.2.5 Peripheral Blood Lymphocyte culture and Giemsa Staining

Peripheral blood lymphocyte culture was done according to Moorhead et al. (1960), with modifications. Triplicates were maintained for each sample. Initially, 4.5 ml of RPMI medium was added into 15 ml culture tubes followed by 0.5 ml of fresh blood. The culture was incubated at 37°C, for 72 h. 0.01% of colchicine solution (0.016 mg ml^{-1}), was added 2 h prior to the 72^{nd} hour in the reference sample, while 15 µg of DME or TME were added to the treatment samples. A control group

without colchicine or extracts were also maintained. After completion of 72nd hour, the cells were subjected to centrifugation at 1,000 rpm for five minutes. The supernatant was carefully removed using a micro pipette. The pellets were then suspended in 5 ml of pre warmed, hypotonic solution (KCl - 0.0075 M) and incubated at 37°C for 5 minutes. It was then subjected to centrifugation at 1000 rpm for 10 minutes. The hypotonic solution was further removed completely and freshly prepared fixative solution 3 part methanol and 1 part glacial acetic acid was added and shaken vigorously. The mixture was then made up to 10 ml using the fixative. It was incubated overnight at 2-8°C, followed by centrifugation at 1000 rpm for 10 minutes. The cell suspension was then dropped onto slides cleaned in 100% ethanol followed by distilled water. The slides were then dried on a hot plate at 40°C-50°C for 10 minutes and treated in trypsin solution for 5-10 seconds. The slides were dipped in PBS and then stained using Giemsa stain, by keeping the slides in the stain for 3-4 minutes. The slides were rinsed in double distilled water to remove the excess stains and were observed for cell density and metaphase spread, under Metasystems Zeiss fluorescent microscope (10x magnification). Due to the degree of chromosome condensation, metaphase is the most visible phase of cell division and mitotic index (MI) was calculated using the formula (Bulla et al., 2014).

$$MI\% = \frac{\text{No: of Metaphases}}{\text{Total No: of cells counted}} \times 100$$

3.2.6 Annexin V/PI staining for Apoptosis analysis

The apoptosis analysis using Annexin V/PI (propidium iodide) was done according to Crowely et al. (2016) with minor modifications. Approximately 1×10^6 YAC-1 cells were seeded in a T25 cell culture flask (triplicates) and treated with extract at three different concentrations (5, 10 and 15 µg/ml). The spent medium along with the cells were collected after 24 h and transferred to 15 ml tubes. Washing was done twice with PBS by centrifugation (670× g for 5minutes). The supernatant was discarded and the pellets were resuspended in 400 μl PBS. The cells were then incubated with 100 μl of PBS buffer and 2 μl each of Annexin-V (1 mg/ml) and propidium iodide (PI) (1 mg/ml). The cells were then analyzed using a flow cytometer.

3.2.7 Nematicidal activity of Plant extracts

3.2.7.1 Fecal egg collection by floatation and centrifugation

Naturally infected goats were selected for the study and fecal pellets were collected from its rectum. The fecal egg floatation assay was performed according to Coles et al. (1992) with minor modifications. The fecal pellets (3 g) were soaked in approximately 42 ml of water and kept aside for a few minutes, until it softened. The pellets were homogenized using a mortar and pestle. This solution was filtered through a sieve of 20 cm diameter with a mesh having 0.15 mm aperture, into a beaker. Centrifugation step followed in a bench top (REMI) centrifuge at 2000 rpm for 2 minutes. The supernatant was removed and to the remaining sediments at the bottom of the 15 ml centrifuge tubes, saturated saline was added and made up to 15 ml. The solution was mixed thoroughly and again centrifuged at 2000 rpm for 2 minutes. After the second wash step, it was kept aside undisturbed for 3 minutes. The next step involved aspiration of 1-2 ml of the supernatant from the top layer of the solution, and to this aspirated solution distilled water was added and centrifuged again at 2000 rpm for 2 minutes. The supernatant was discarded and the remaining portion was mixed thoroughly and observed under a light microscope (40x magnification) for the presence of helminth eggs.

3.2.7.2 Egg hatch assay (EHA)

The World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines with minor modifications were used for egg hatch assay (Coles et al. 1992). The extracts were serially diluted (0.625, 1.25, 2.5, 5 and 10 mg/ml) and added to each of the six well plates. To this approximately 100 strongyle ova in 0.5 ml distilled water was added. Albendazole at concentrations 6.25, 12.5, 25, 50 µg/ml was kept as the standard, while 1% DMSO served as negative control. All the multi well plates were incubated for 48 h at 27°C. Six replicates were kept for each concentration. After 48 h incubation, Lugol's iodine solution was added to inhibit hatching. The unhatched eggs and hatched larvae (dead or alive) were counted using a stereozoom microscope (40× magnification).

3.2.7.3 Larval paralysis assay (LPA)

The protocol previously used by Varady & Corba (1999) was used with modifications. Naturally infected goats were subjected to fecal sample collection,

and it was cultured at 27°C for a week to obtain third instar *H. contortus* larvae. The larval paralysis assay was conducted in 96-well microtiter plates. Approximately 100 motile larvae of third instar (L3) *H. contortus* in 100 µL water were prepared and to this equal volumes of plant extracts in concentrations 0.625, 1.25, 2.5, 5 and 10 mg/ml were added in triplicates. Albendazole (6.25, 12.5, 25, 50 µg/ml) was maintained as the standard and 1% DMSO served as negative controls. All samples were then incubated at room temperature for 24 h. After 24 h of treatment, the loss of motility of the larvae were recorded. A stereozoom microscope at 40× magnification was used to count non-motile larvae and the results were expressed as Mean ±SD.

3.2.8 Trematocidal activity of the plant extracts

3.2.8.1 Collection of adult flukes

From a slaughterhouse freshly collected adult *F. cobboldi* from the rumens of infected cattle, were used for the study. The experiment was conducted according to Anuracpreeda et al. (2016) with minor modifications. After washing the flukes collected in 0.85% NaCl solution, the healthy and motile ones were selected for the study.

3.2.8.2 Fluke survival assay

The plant extracts were prepared in various concentrations by dissolving in 1% DMSO. Adult flukes were randomly selected and 25 flukes for each test concentration were used for the study. Group 1 served as the vehicle control (1% DMSO); group 2 with 1 mg/mL of Albendazole (ABZ) served as positive control; groups 3 to 8 were treated respectively with ethyl acetate, ethanol and water extracts of both the plants at various concentrations (25, 12.5, 6.25 and 3.125 mg/mL). Three replicates were kept for each group and the *F. cobboldi* under study was incubated with CO_2 at 37° C. It was then observed under a stereo zoom microscope for motility at 0, 0.5, 1, 1.5, and 2 h of incubation times. The time required for total inactivity or paralysis followed by the death of fluke was recorded and the tegumental changes were examined after microtomy.

3.2.8.3 Morphology and histology of flukes after treatment

For studying the morphological changes to flukes post-treatment, stereo zoom microscope analysis was done. *Fischoederius cobboldi* treated in different

concentrations of extracts and controls were studied using Leica M205 C stereo zoom microscope. Images were taken by a Leica DMC4500 digital camera attached to Leica M205 C stereomicroscope with the software package LAS, version 4.3.0. The fluke specimens were prepared for histology examination by the method proposed by Anuracpreeda et al. (2015). Dead *F. cobboldi* from each group were fixed in Bouin's fixative solution for 12 h, and then was transferred to 10% formalin for further histological processing. Sections of the tissues were taken and stained using hematoxylin and eosin and observed for abnormalities and photographed under a light microscope (Leica DM 500). The tissues fixed in 10% formalin were also subjected to Masson's trichrome staining, which is used for the detection of collagen fibers. The collagen fibers will be stained purplish-blue in colour. The formalin fixed tissues were re-fixed in Bouin's solution (saturated picric acid, 25 ml of formaldehyde (37-40%) and 5 ml glacial acetic acid are mixed thoroughly to prepare Bouin's solution) for 1 hour at 56°C to improve staining quality. The yellow colour of bouin's solution was washed off by rinsing it in running tap water for 5-10 minutes. The rinsed tissues were immersed in Weigert's iron hematoxylin working solution (Stock solution A (Hematoxylin 1g and 95% alcohol 100 ml) and Stock Solution B (29% Ferric chloride in water 4 ml, distilled water 95 ml and concentrated HCl 1 ml) are mixed thoroughly in equal parts) for 10 minutes and washed well in distilled water. This was then stained using Biebrich scarlet-acid fuchsin solution (90 ml of 1% aqueous biebrich scarlet, 10 ml of 1% aqueous acid fuchsin and 1 ml of glacial acetic acid are mixed thoroughly to prepare this solution) for 10-15 minutes, followed by phosphomolybdic-phosphotungstic acid solution (25 ml of 5% phosphomolybdic acid and 25 ml of 5% phosphotungstic acid are mixed thoroughly) for 10-15 minutes. The sections were then directly transferred to aniline blue solution (aniline blue 2.5 g, acetic acid glacial - 2 ml and distilled water - 100 ml are mixed thoroughly) for 5-10 minutes. After rinsing in distilled water, it was kept in 1% acetic acid solution (1 ml of glacial acetic acid is mixed with 99 ml of distilled water) for 2-5 minutes. Rinsed again in distilled water and dehydrated quickly through 95% ethyl alcohol, absolute ethyl alcohol, and mounted in resinous medium.

3.2.9 Metaphase indexing in *Allium cepa*

The experiment was conducted according to Chakraborty et al., 2021 with minor modifications. Onion bulbs were collected locally and after removing the outer scales they were placed in distilled water at room temperature (27-28°C) overnight for germination to take place. The germinated bulbs were then treated with Colchicine 0.4 mg/ml (reference), 1% DMSO (vehicle control), DME (4 mg/ml) and TME (4 mg/ml) for a duration of 4 h. After 4 h, 1N HCl was used for hydrolyzing the roots and it was then stained with 1% toluidine blue following the standard protocol (Sharma & Sharma, 1999). The meristematic region was visualized under a microscope (40x magnification) for different cell cycle phases. The metaphase index was calculated (Georgieva & Vassilevska, 2021) using the formula:

$$\text{Metaphase index} = \frac{\text{No: of cells in metaphase}}{\text{Total No: of cells in division}} \times 100$$

3.2.10 Acute toxicity studies

According to the OECD (Organization for Economic Co-operation and Development) guideline 425, a limit test was performed with plant extracts in Swiss albino mice. Female Swiss albino mice were grouped into three groups (n=6). Group 1 serves as the vehicle control with 1% propylene glycol administered orally. Group 2: single dose of 2000 mg/kg b.wt of DME dissolved in 1% propylene glycol. Group 3: single dose of 2000 mg/ kg b.wt of TME dissolved in 1% propylene glycol. The experimental set up was closely monitored post oral administration of the extracts, for any signs of toxicity or death. The mice were observed daily for 14 consecutive days. To examine the changes in internal organs, the animals were euthanized post 14 days of study.

3.2.11 Sub-acute toxicity studies

Sub-acute toxicity was conducted in Swiss albino mice of both sexes, according to the OECD 407 with minor modifications. The doses for subacute toxicity were fixed as 1/5th (high) and 1/10th (low) of 2000 mg/kg b.wt of mice. The mice (n=6) were sought into twelve groups. Group 1: males served as untreated control, Group 2: males served as vehicle control (1% propylene glycol), Group 3: males received low dose DME (200 mg/kg b.wt) and Group 4: males received high dose DME (400 mg/kg b.wt). Group 5: males received low dose TME (200 mg/kg b.wt), Group 6:

males received high dose TME (400 mg/kg b.wt), Group 7: females served as untreated control, Group 8: females served as vehicle control (1% propylene glycol), Group 9: females received low dose DME (200 mg/kg b.wt), Group10: females received high dose DME (400 mg/kg b.wt of mice) and Group 11: females received low dose TME (200 mg/kg b.wt) and Group 12: females received high dose TME (400 mg/kg b.wt). The extracts were orally administered continuously for 28 days. The treatment started on day 0 and continued for 28 days at 24 h interval. During the experiment food and water consumption were monitored every three days along with weekly recordings of the body weight of mice. At the end of the experimental period animals were fasted overnight and euthanized in a carbon dioxide chamber, on the following day. Blood was collected by cardiac puncture and used for analyzing hematological parameters. Histological analysis of few internal organs were also done.

3.2.12 Relative organ weight

The observation of vital organs is important in toxicity studies, hence after the 28 day long sub-acute toxicity study, the vital organs likes heart, brain, liver, stomach, intestine, lungs, kidney, spleen, testes and ovary from all the animals were dissected out and washed in 0.9% saline. The weight of each organ was recorded with the help of an electronic balance and from this relative organ weight was derived (Peter et al., 2018). The following formula was used to calculate relative organ weight:

$$\text{Relative organ weight} = \frac{\text{Organ weight}}{\text{Body weight}} \times 100$$

3.2.13 Determination of hematological parameters

3.2.13.1 Estimation of haemoglobin (Hb)

The haemoglobin content in the blood was determined by the method previously described by Drabkin & Austin (1935).

Principle

The Drabkin's reagent contains potassium ferrocyanide and potassium cyanide. The haemoglobin in the blood initially reacts with potassium ferrocyanide and gets oxidised into methaemoglobin, which then reacts with potassium cyanide to form cyanmethaemoglobin. This stable coloured product has an absorbance maximum at

546 nm and its absorbance is directly proportional to the concentration of Hb present in the blood.

Procedure

Blood was collected by cardiac puncture into heparinized tubes. 5 ml of Drabkin's reagent was added to approximately 20 µl of heparinized blood, and after thorough mixing it was incubated at room temperature for 5 minutes. The absorbance of the reaction mixture was measured at 546 nm. The haemoglobin content was calculated using the formula:

Hemoglobin (g /dL) = (Absorbance of the sample/Absorbance of Standard) ×60 × 0.251.

3.2.13.2 Estimation of red blood cells (RBC)

The RBC count in the blood was estimated according to the method proposed by Cheesbrough & McArthur (1976).

Principle

Hayem's solution (0.25 g of mercuric chloride, 2.50 g of sodium sulphate and 0.50 g of sodium chloride dissolved in distilled water) was used as the RBC diluting fluid to dilute the blood sample 200 times. It prevents haemolysis by fixing the RBCs. The RBC count was estimated using a haemocytometer and the values are expressed as the number of RBC/cu mm of whole blood.

Procedure

The RBC pipette was used to draw blood upto 0.5 mark and RBC fluid was used to dilute it upto mark of 100. The RBC pipette was rubbed well between the palms for a few minutes to ensure thorough mixing. The diluted blood was carefully charged to the Neubauer counting chamber, without any air bubbles. A light microscope (40× magnification) was used for counting.

Estimation of RBC count was done using the formula:

RBC count (cells/mm^3) = Number of cells counted x dilution factor/Depth factor x area counted

Dilution factor = 200

Depth of counting chamber = 0.1mm

Area counted = 5/25

So, total RBC count = Number of cells × 10,000/mm^3

3.2.13.3 Estimation of total white blood cell count (TC)

The total leukocyte count in the blood was determined using a method previously described by Cheesbrough & McArthur (1976).

Principle

The Turk's fluid used to dilute the blood, consists of 2 ml glacial acetic acid and 1g/100 ml crystal violet dissolved and made upto 100 ml using distilled water. The acetic acid present in this solution lyses the red blood cells alone and the crystal violet in the solution stains the WBCs.

Procedure

The WBC pipette was used to draw blood upto 0.5 mark and diluted upto the mark 11 using Turk's fluid. The WBC pipette was rubbed well between the palms for a few minutes to ensure thorough mixing. The diluted blood was carefully charged to the Neubauer counting chamber, without any air bubbles and was kept aside for a while for the cells to settle down. A light microscope (40× magnification) was used for counting the WBC's in the four square corners.

Estimation of WBC count was done using the formula:

Total WBC count (cells/mm^3) =Number of cells counted ×50

3.2.13.4 Estimation of differential leucocyte count (DC)

The differential count of leucocytes was estimated according to the method proposed by Wintrobe & Greer (2009).

Principle

For the estimation of different types of leucocytes present, blood smear stained with Leishman stain was used. The shape of the nucleus, size of the cell, and presence or absence of granules are the factors that determine various types of leucocytes. After the leishman's staining is done, identification of different types of leucocytes are possible as the neutrophils show purple coloured nuclei and a pale blue cytoplasm. The eosinophils show a light pink cytoplasm and purple coloured nuclei but they have a dumbbell shape and also orange-red granules are present. The basophils show

purple coloured nuclei with dark blue granules and the monocytes show a purple-coloured horseshoe shaped nucleus and pink coloured cytoplasm. Similarly, the dark blue circular nuclei with light blue cytoplasm are the lymphocytes. Platelets can be identified with their violet-coloured granules.

Procedure

A few drops of blood were added onto a clean and dry slide, and using a spreader, a thin smear of blood was prepared on the slide. Leishman stain (0.15 g/100 ml methanol) was added to these blood smeared slides and kept aside for 2-3 minutes. An equal amount of distilled water was added inorder to dilute the stain. After 2-3 minutes, excess stains on the slides were washed off and air dried. Using a light microscope at 40× magnification, WBC counting was done.

3.2.13.5 Estimation of platelet count (PLT)

The method proposed by Cheesbrough & McArthur (1976) was used for platelet counting.

Principle

The dilution fluid used in the procedure is made up of 1% ammonium oxalate solution, and it exclusively lyses the erythrocytes. This makes the tiny shimmering platelets visible under the microscope.

Procedure

The procedure is similar to that of RBC counting. 1:200 dilution of blood was done by adding 1% ammonium oxalate (dilution fluid). The RBC pipette was used for this, and the solution was gently mixed for 2 minutes. The Neubauer's counting chamber was charged with the solution prepared and it was observed under a light microscope (40x magnification).

The platelet count was determined using the formula: Number of platelets per mm^3 =N ×10000

Where N = Total number of cells counted

3.2.13.6 Estimation of packed cell volume (PCV)

The Packed cell volume was determined by the method proposed by Wintrobe & Greer (2009).

Principle

When the Wintrobe Micro haematocrit tube (Wintrobe tube) was centrifuged with anticoagulated blood in it, at a moderate speed it led to the sedimentation of erythrocytes at the bottom of the tube. This red cell coloumn sedimented at the bottom is called as packed cell volume or haematocrit.

Procedure

The pasteur pipette filled with the anti-coagulated blood was gently introduced into the Wintrobe tube to fill it up to the 10 cm mark. This was followed by a centrifugation step for about 30 minutes at 3000 rpm. Three layers are formed. The top layer of plasma, middle layer of a buffy coat which contains leucocytes and platelets and the bottom layer of erythrocytes. The blood sample was mixed thoroughly and the length of the erythrocyte cell column was measured. The percentage of erythrocyte cell column to the total volume of blood sample was calculated.

3.2.13.7 Estimation of erythrocyte sedimentation rate (ESR)

To estimate the erythrocyte sedimentation rate, a method proposed by (Westergren, 1957) called the Westergren tube method was used.

Principle

In a Westergren's tube kept in a vertical position, undisturbed, anti-coagulated blood was added. The level of the RBC coloumn was observed both initially (0 h) and after 1 h of the experiment. The ESR was expressed as (mm/h), that is the distance travelled by the RBC column.

Procedure

Firstly, a solution was prepared by mixing 1.6 ml of venous blood with 0.4 ml 3.8% sodium citrate solution. This solution was filled up to the level marked as zero in the Westergren's E.S.R tube and kept vertically. The readings were taken at a time interval of 30 minutes, 1 hour and 2 hours after filling Westergren's E.S.R. tube. The ESR value was expressed as distance travelled (mm) per 1 hour.

3.2.14 Liver function analysis

The serum was separated by centrifuging the blood collected through heart puncture at 5000 rpm for 10 minutes in eppendorf tubes. The liver function marker enzymes like SGOT, SGPT, ALP, TB, TP, ALB and GLOB were analyzed. The amount of bilirubin and total protein was also estimated.

3.2.14.1 Serum glutamate oxaloacetic transaminase (SGOT)

The method previously described (Thefeld et al., 1974; Bergmeyer et al., 1976) was used for estimation of Serum glutamate oxaloacetic transaminase activity.

Principle

SGOT is an enzyme that catalyses the transfer of amino groups between L-aspartate and α-ketoglutarate to form oxaloacetate and L-glutamate. In the presence of malate dehydrogenase, the oxaloacetate reacts with NADH to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance and can be spectrophotometrically determined at 340 nm. It is directly proportional to the activity of the SGOT enzyme in the sample.

Procedure

The reagent kit for SGOT analysis was purchased from Agappe Diagnostics Ltd and the kit consisted of two reagents, namely (R1): Tris buffer having pH 7.8 (88 mmol/l), L-Aspartate (260 mmol/L), MDH (>900 U/L) and LDH (>1500 U/L) and (R2): NADPH (0.24 mmol/l) and α-ketoglutarate (12 mmol/l). Working reagent was prepared by mixing 4 volumes of R1 and 1 volume of R2. This remains about a month if kept at 2 - 8ºC. While conducting the assay, 1000 µL of working reagent and100 µl of serum samples were mixed thoroughly and kept incubated for 1 minute at 37 °C. After incubation, variation in absorbance was measured at 340 nm at one-minute time intervals for a total duration of 3 minutes.

The SGOT activity was estimated using the formula SGOT activity (U/L) = (ΔOD/ min) x 1745.

3.2.14.2 Serum glutamate pyruvate transaminase (SGPT)

The Serum glutamate pyruvate transaminase activity was estimated according to the method previously described (Thefeld et al., 1974)

Principle

The conversion of L-alanine and α-ketoglutarate to pyruvate and L-glutamate is catalyzed by the SGPT enzyme. The pyruvate reacts with NADH to form NAD+ in the presence of lactate dehydrogenase. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance and can be spectrophotometrically determined at 340 nm. It is directly proportional to the activity of the SGPT enzyme in the sample.

Procedure

The reagent for SGPT analysis was purchased from Agappe Diagnostics Ltd and the kit consisted of two reagents, namely (R1): Tris buffer having (110 mmol/l), L-alanine (600 mmol/l) and LDH (>1500 U/L) and (R2): NADH (0.24 mmol/l) and α-ketoglutarate (16 mmol/l). A working reagent was prepared by mixing 4 volumes of R1 and 1 volume of R2. This remains about a month if kept at 2 - 8ºC. While conducting the assay, 1000 µl of working reagent and 100 µl of serum samples were mixed thoroughly and incubated for 1 minute at 37 °C. Next, variation in absorbance was measured at 340 nm at a time interval of 60 seconds for a total duration of 3 minutes.

The SGPT activity was estimated using the formula: SGPT activity (U/L) = (ΔOD/ min) x 1746

3.2.14.3 Alkaline phosphatase (ALP) activity

For analysing the alkaline phosphatase activity, the method previously proposed by Schlebusch et al. (1974) was used.

Principle

At a pH of 10.4, ALP enzyme hydrolyzes p-nitrophenyl phosphate to a yellow coloured p-nitrophenol and inorganic phosphate. The change in absorbance can be spectrophotometrically determined at 405 nm and the absorbance is directly proportional to the activity of the ALP enzyme.

Procedure

The reagent kit for ALP analysis was purchased from Agappe Diagnostics Ltd. The kit consists of two reagents, namely reagent 1 (R1): diethanolamine buffer having pH 10.2 (125 mmol/l) and magnesium chloride (0.625 mmol/l) and reagent 2 (R2):

p-nitrophenyl phosphate (50 mmol/l). A working solution was prepared by mixing 4 volumes of R1 and 1 volume of R2. While conducting the assay, 1000 µl of working reagent and 20 µl of serum samples were mixed thoroughly and incubated for 1 minute at 37 °C. Next, variation in absorbance was measured at 405 nm at a time interval of 1 minute for a total duration of 3 minutes.

The ALP activity was calculated using the formula ALP activity (U/L) = (ΔOD/ min) x 2750.

3.2.14.4 Total Bilirubin

According to the method previously proposed by Walters & Gerarde (1970) the total bilirubin was estimated.

Principle

This diazotized sulfanilic acid, which reacts with bilirubin, is formed by the reaction of sulfanilic acid and sodium nitrite. Bilirubin in the presence of TAB (diazo reagent) reacts with diazotized sulfanilic acid to form azobilirubin, a pink coloured complex that is measured at 546 nm.

Procedure

The kit for bilirubin analysis was purchased from Agappe Diagnostics Ltd. 50 µl of serum, 20 µl of total bilirubin activator and 1 ml bilirubin reagent (28.9 mmol/l of sulfanilic acid and 9 mmol/l of TAB) was mixed thoroughly and incubated at room temperature for 5 minutes. The solution mentioned above without serum was kept as blank. After the incubation period, absorbance was measured at 546 nm.

The total bilirubin in the sample was estimated using the formula:

Total bilirubin concentration (mg/dl) =OD of the test – OD of blank × 25

3.2.14.5 Total Protein (TP)

The method previously proposed by Lowry et al., 1951 was used to determine the total protein present by the colorimetric method.

Principle

In an alkaline medium, reduction of components like phosphomolybdate and phosphotungstate, present in the Folin-Ciocalteau reagent, is done by the amino acids such as tyrosine and tryptophan present in proteins. This produces a bluish

purple colour, and the absorbance can be measured spectrophotometrically at 660 nm.

Procedure

10 µl of blood was diluted by adding 990 µl of distilled water to make it upto 1 ml. 5 m of alkaline copper sulfate (50 ml of 2% sodium carbonate (Na_2CO_3) in 0.1N NaOH and 1 ml of 0.5% CuSO4 in 1% sodium potassium tartarate) was added to it. Further, it was mixed well and was incubated for 10 minutes at room temperature. Following this, Folin-Ciocalteu reagent (0.5 ml) in water (1:1) was added. A thorough mixing was done and the sample was incubated at room temperature for a duration of 30 minutes. The absorbance was measured at 660 nm.

The serum total protein calculated using the formula:

$$\text{Total protein (g/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \text{Conc. of Standard}$$

3.2.14.6 Albumin (ALB)

The method previously proposed by Doumasa et al., 1971 was used to determine the albumin present by the colorimetric method.

Principle

The reaction between albumin in the serum and bromocresol green produces a change in colour that is proportional to the albumin concentration. The absorbance can be measured spectrophotometrically at 620 nm.

Procedure

BCG (Bromocresol Green) Albumin Assay Kit was purchased from Beacon Diagnostics Ltd, for the determination of albumin in the serum. The kit consists of two reagents, reagent 1 (BCG reagent) and reagent 2 (Albumin standard 4 gm/dl). Clean dry test tubes labelled as blank (B), standard (S) and test (T). To B, 1 ml of reagent 1 was added. To S, 10 µl of reagent 2 and 1 ml of reagent 1 was added. 10 µl of the sample and 1 ml of reagent 1 was added to test tube marked T. They were mixed thoroughly and incubated for 60 seconds at room temperature. The absorbance of the standard and sample against the reagent blank at 630 nm (600 - 650 nm) was calculated using the formula:

$$\text{Albumin Conc. (gm/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 4$$

3.2.15 Renal function

The levels of urea and creatinine in the serum was analyzed to test the renal function, using commercial diagnostic kits.

3.2.15.1 Estimation of Serum urea

According to the method proposed by Young et al. (1975), the serum urea levels were determined.

Principle

Urea is converted to carbon dioxide and ammonia with the help of urease enzymes. This ammonia reacts with α-ketoglutarate and NADH to form glutamate and NAD. The decrease in absorbance, resulting from the oxidation of NADH to NAD is proportional to the amount of urea present in the sample and is measured at 340 nm.

Procedure

For urea estimation, a diagnostic kit was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. Initially, 4 volumes of BUN (blood urea nitrogen) reagent and 1 volume of urease was mixed to prepare the working solution. 1 ml of this working reagent was mixed well with 10 µl of serum and was incubated at 37ºC. The absorbance was then measured at 340 nm. After 30 s (A1) the initial absorbance was recorded, and the final absorbance after 60 s (A2). The change in absorbance (ΔA) was obtained by subtracting A1 from A2. The same experiment was repeated with the standard received with the assay kit.

The concentration of urea present in the sample was calculated using the formula:
Urea (mg/dl) =(ΔATΔAS) x 50

Where, ΔAT and ΔAS represent change in the absorbance of test and standard respectively. 50 was the concentration of standard.

3.2.15.2 Estimation of Creatinine

The creatinine level in the serum was determined using Jaffe's kinetic method with slight modification (Bones & Tausky, 1945; Toro & Ackermann, 1975).

Principle

In alkaline condition, the picric acid gets converted to sodium picrate. This sodium picrate reacts with creatinine in the sample to form an orange-coloured creatinine

picrate complex. This complex shows maximum absorbance at the wavelength 520 nm.

Procedure

For creatinine estimation, a diagnostic was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. The kit had two reagents; R1; picric acid reagent and R2; alkaline buffer. Equal volumes of R1 and R2 were mixed to prepare the working solution. 1 ml of this working reagent and 50 µl of serum was mixed and absorbance was measured at 520 nm. The initial absorbance was recorded after 30 s (A0), and the final absorbance after 90 s (A1). The change in absorbance (ΔA) was obtained by subtracting A0 from A1. The same experiment was repeated with creatinine aqueous standard received with the assay kit.

The concentration of creatinine present in the sample was estimated using the formula: Creatinine (mg/dl) =(ΔATΔAS) x 2

Where, ΔAT and ΔAS represent change in the absorbance of test and standard respectively. 2 represents the concentration of standard.

3.2.16 Lipid profile

3.2.16.1 Estimation of total cholesterol

The total cholesterol in the serum was determined by the CHOD-POD method (cholesterol oxidase phenol 4-aminoantipyrine peroxidase method) (Young, 1997).

Principle

Cholesterol esterase catalysis the hydrolysis of cholesterol esters to free cholesterol and fatty acids. This free cholesterol was oxidized to H_2O_2 and 4-cholesterol-3-one with the help of the enzyme, cholesterol oxidase. These byproducts in the presence of peroxidase, couples with 4-aminoantipyridine and phenol to yield a red quinone. This was measured at 505 nm.

Procedure

The cholesterol reagent kit includes, phenol (26 mmol/L), cholesterol oxidase (300 U/L), pipes (90 mmol/L, pH 6.9), cholesterol esterase (100 U/L), 4-aminophenazone (0.4 mmol/L) and peroxidase (650 U/L). 10 µL of serum was mixed thoroughly with 1 ml of the reagent and incubated for 5 minutes at 37ºC. Next, the absorbance of the

solution was measured at 500 nm. The experiment was repeated with the standard received with the assay kit.

The total cholesterol in the sample was estimated using the formula:

Total cholesterol (mg/dl) = (Absorbance of sample / Absorbance of standard) x 200.

3.2.16.2 Estimation of Triglycerides (TG)

A diagnostic kit purchased from Euro diagnostic systems, Chennai, India was used to estimate the amount of triglycerides present in the blood and the glycerol phosphate dehydrogenase-peroxidase (GPO-POD) method was followed.

Principle

Glycerol and free fatty acids are formed upon the incubation of lipoprotein lipase with serum triglycerides. In the next step, the glycerol undergoes phosphorylation to form glycerol-3-phosphate. This glycerol-3-phosphate gets converted into dihydroxyacetone phosphate (DHAP) and H_2O_2 with the help of glycerol phosphate dehydrogenase enzyme. A red coloured compound quinone is formed when H_2O_2 reacts with 4-aminophenazone (4-AP) and p-chlorophenol, mediated by a peroxidase enzyme. The absorbance of this compound is measured at 505 nm. The intensity of quinone and the amount of triglycerides in the sample are directly proportional.

Procedure

To 10 µL of serum, approximately 1 ml of reagent in the diagnostic kit was mixed and incubated for 5 minutes at 20ºC. The absorbance was then measured at 505 nm. The experiment was repeated with the standard received with the assay kit. (The reagent includes; 2 mmol/L p-chlorophenol, 50 mmol/L Good's buffer (pH 6.3), 0.1 mmol/L 4-aminophenazone, 150000 U/L lipoprotein lipase, 3500 U/L glycerol 3-kinase, 500 U/L glycerol kinase and 0.1 mmol/L ATP).

The total triglycerides in the sample were estimated using the formula:

Total triglycerides (mg/dl) = (Absorbance of sample / Absorbance of standard) x 200.

3.2.16.3 HDL cholesterol (HDLc)

The enzymatic photometric method proposed by Naito (1985) was used for the estimation of HDL cholesterol in serum. Commercial HDL kit was purchased from Euro Diagnostic Systems Pvt. Ltd., Chennai, India for the assay.

Principle

The HDLc in the serum is solubilized by the detergent present in the kit. This gets released and reacts with the enzymes such as cholesterol esterase and cholesterol oxidase. Finally, the HDLc reacts with chromogens to develop a colour that is measured using a colourimetric method. The inhibition by non-HDL lipoproteins and chylomicrons are overruled as they get absorbed by the detergent. The amount of HDLc in the sample is proportional to the intensity of colour developed.

Procedure

450 µL of R1 (<1000 U/L cholesterol oxidase and <1 mMDSBmT) solution was added to three test tubes labeled as blank, sample and calibrator. To the tube labeled as sample, 10 µl of serum was added and to the calibrator tube, lyophilized human serum (39.2 mg/dl conc) was added. Thorough mixing of the solution was followed by its incubation at 37°C for 5 minutes. Post incubation, to all the test tubes, R2 solution (150 µl) <1 mM 4-aminoantipyrine, <1500 U/L cholesterol esterase, <1300 U/L peroxidase <3000 U/L ascorbic oxidase and <2% detergent) was added and mixed thoroughly. It was then incubated at 37°C for 5 minutes. Against the blank, absorbance was measured at 600 nm. The levels of HDL cholesterol in the sample was estimated using the formula: HDLc (mg/dl) = (ΔA sample / ΔA calibrator) x concentration of calibrator.

3.2.16.4 LDL cholesterol (LDLc)

The LDL cholesterol in the serum was determined by using Friedewald's formula (Friedewald et al. 1972)

$$\text{LDLc (mg/dl)} = \text{Total Cholesterol} - \text{HDLc} - (\text{Triglycerides}/5)$$

3.2.16.5 VLDL cholesterol (VLDLc)

VLDL cholesterol in the serum was also determined using the formula given below:

$$\text{VLDLc (mg/dl)} = \text{Triglycerides (mg/dl)}/5$$

3.2.17 Serum Electrolyte Analysis

3.2.17.1 Estimation of sodium and potassium in the blood

Principle

For the estimation of sodium and potassium, Flame photometry method was used (Deal, 1954). The photometer equipped with a photodetector, captures a specific frequency of light that is emitted upon aspiration of an alkali metal in solution into a low temperature flame in an aerosol form. The light produced during this procedure is directly proportional to the concentration of alkali.

Procedure

Stock solutions containing sodium (1000 mEq/l) and potassium (100 mEq/l) were prepared. Mixed working standards such as S1 (120/2 mEq/l), S2 (140/4 mEq/l) and S3 (160/6 mEq/l) were prepared from the stock solution. Next, 100 µl of serum (test) and working standards (S1, S2 and S3) were added to various tubes. To all the tubes approximately 10 ml of distilled water was also added and after thorough mixing, it was subjected for flame photometry. The concentration of the sample was determined from the calibration curve.

3.2.17.2 Determination of chloride ion

An electrolyte diagnostic reagent kit and a photometer equipped system was used for determining the chloride ion content in the serum (Schoenfeld & Lewellen, 1964; Levinson, 1976).

Principle

Red coloured ferric thiocyanate and mercuric chloride is formed when chloride ions react with a solution containing mercuric thiocyanate and ferric nitrate. The amount of chloride ion present in the serum is directly proportional to the intensity of the red colour formed in the solution.

Procedure

The serum and the reagent in the diagnostic kit were mixed in the ratio 10 µl:1 ml and the absorbance was measured at 505 nm against the blank using a Photometer 4010 system. Using chloride provided with the diagnostic kit, a standard solution was also prepared.

The estimation of chloride ions in the serum was done using the following formula:

Chloride (mmol/l) =Absorbance of sample/ Absorbance of standard x100

3.2.18 Histopathological Analysis of vital organs and tumor sections

Post experimental duration, the animals were sacrificed using a CO2 chamber. After that, nine organs, such as heart, brain, liver, stomach, intestine, lungs, kidney, spleen, testes and ovary from test and control groups were excised using a sterile blade and washed with normal saline (0.9% NaCl) for the removal of unwanted debris. Tissues were then fixed in 10% formalin solution and dehydrated using increasing grades of alcohol and were cleared in xylene. Paraffin-embedded 99 tissue samples were subjected for microtome sectioning to obtain 3-4 μm thick sections. The sections after deparaffinization were stained in haemotoxylin and eosin (H & E) stains. Post staining the sections was examined under a microscope (Leica DM 500) and microscopic images were obtained using the camera mounted on it. In the anti-tumor study, post the experimental duration, the animals were sacrificed in a CO_2 chamber and the tumor mass was excised using a sterilized blade. To remove traces of blood and debris, the tumor mass was washed with normal saline (0.9% NaCl). 10% neutral buffered formalin was used to fix the tissues excised. Ascending alcohol grades were used to dehydrate the tissue. It was then cleared in xylene. The samples were then embedded in paraffin, and this paraffin embedded blocks were used for microtome sectioning. 3-4 μm thick sections were taken. Xylene was used to remove excess paraffin from the section and rehydration was done. For rehydration, the sections were passed through descending alcohol grades. Hematoxylin and eosin staining was done in the rehydrated sections. Hematoxylin is a nuclear stain with potassium or ammonium (alum) - 100 g, mercuric oxide- 2.5 g, 10% alcoholic hematoxylin-50 ml, glacial acetic acid-20 ml and distilled water-1000 ml. Eosin on the other hand is a cytoplasmic stain consisting of Eosin Y- 1 g, 70% ethanol -100 ml and glacial acetic acid- 5 mL. A stereo microscope at 40× magnification was used to observe the slides. Small portions of the tumor mass were used for histological examination (Feldman & Wolfe, 2014) some portions were used for apoptosis analysis using Tunel assay (Loo, 2011).

3.2.19 Evaluation of antitumor activity using DLA cell induced solid tumor model

Male Swiss albino mice (6-8 weeks old) weighing 25-30 g were grouped into seven groups (n=6). Group 1: Control (untreated tumor induced mice) Group 2: Vehicle control (1% propylene glycol), Group 3: Standard (cyclophosphamide drug 10 mg/kg b.wt), Group 4: DME low dose (200 mg/kg b.wt), Group 5: DME high dose (400 mg/kg b.wt), Group 6 and 7: TME low dose (200 mg/kg b.wt), and high dose (400 mg/kg b.wt) respectively. Approximately 1×10^6 of DLA cells in 100 μl of cell suspensions were intramuscularly injected into the right hind limb of all animals. The cell suspension was prepared by aspirating DLA cells from the peritoneal cavity of tumor bearing mice and washing with PBS. The treatment (p.o.) began after 24 h post tumor cell inoculation and continued for 10 consecutive days. A vernier caliper was used to measure the diameter of the tumor growth in two perpendicular planes. The readings were taken for a period of 30 days with an interval of three days between each reading. The tumor volume was calculated using the formula;

$V = 4/3\pi\ r^2_1\ r_2$; where r_1 is the minor radius and r_2 is the major radius.

3.2.20 TUNEL Assay

The tumor mass extracted from animals was subjected to TUNEL analysis. The slides prepared from these tumor masses were fixed in 4% paraformaldehyde and were deparaffinized followed by rehydration steps. The TUNEL staining was executed using TUNEL enzyme and TUNEL label mix with fluorescein dUTPs and dNTPs (Roche-SigmaAldrich). The slides were immersed in a mixture of 0.1 M Tris HCl, 3% BSA and 20% normal bovine serum for a duration of 30 minutes at room temperature at pH 7.5. This was followed by rinsing the slides twice in 1×PBS. Further, 50 µl of TUNEL mixture (45 µl TUNEL label + 5 µl TUNEL enzyme) was added to these slides and incubated at 37°C for 60 minutes in the dark. Each slide was then rinsed thrice in PBS for 5 minutes and 10 µl of PI was added to each slide for obtaining red fluorescence with green illumination. The samples were further rinsed in PBS and were observed under a Fluorescent Cell Imager (BIO RAD, Singapore) after adding a drop of PBS. The images were captured at a magnification of 100x.

3.2.21 The *in silico* studies

3.2.21.1 Docking Studies Using AutoDockVina

AutoDock Vina an open source program designed and developed by Dr. Oleg Trott (Trott & Olson, 2010) was used for conducting docking studies. The docking software called Vina can be run with the help of AutoDock tools (ADT), PyMol visualisation software and Discovery studio.

3.2.21.2 Protein and ligand preparation for docking

The receptor and ligand used for docking studies need to have their chemical structures in certain formats. RSCB Protein Data Bank was used to download three dimensional structure of proteins in .pdb format derived from crystallographic techniques. Similarly, the ligands were downloaded in .sdf or. mol formats from PubChem database or ZINC database.

The crystal structure of alpha-beta tubulin of *H. contortus* was not available and hence was predicted by homology modelling. For molecular docking PyMol visualization software was used to prepare the protein/receptor molecules. Several changes like removal of water molecules, unwanted bound ligands and co-factors were done to the protein using command prompt of PyMol. Addition of hydrogen atoms (for balancing valancies) and selection of polypeptide chains functionally relevant to the receptor-ligand interaction as in the case of a protein complex were also done. Ligand .sdf file or. mol file that was downloaded from PubChem was converted into .pdb format using Open Babel software. The .pdb format of proteins and ligand were converted to. pdbqt format and saved using AutoDock Tools 1.5.6 (ADT MGL tools) to perform molecular docking using AutoDock Vina.

3.2.21.3 Finding the active site of the protein

The receptor .pdb file was uploaded in Biovia's Discovery Studio Visualizer, and the center x-y-z coordinates of the ‘active site’ of the receptor were obtained. The active site of the receptor comprises of amino acid residues that form temporary bonds with the substrate (binding site) and also catalyze a reaction of that substrate (catalytic site). Prior knowledge about the binding site significantly increases the docking efficiency. The binding sites of the receptor was defined with the aid of Biovia’s Discovery Studio. Here, a receptor needs to be defined and then its largest

binding site will be displayed. It shows all possible sites, however, the first one shown is usually the most favourable one for docking.

3.2.21.4 Generation of vina config file

Pre calculated grid maps are required for AutoDock. The potential energy arising from the interactions with the receptor is stored in these grids and it must also surround the active site of the receptor (Forli et al., 2016). Coordinates of the center of box (x-y-z) were considered the grid box parameters, as obtained through Discovery Studio. The input file with details such as the receptor. pdbqt, ligand. pdbqt, output. pdbqt, grid coordinates of center and a proper value of size x-y-z were composed.

3.2.21.5 Docking

AutoDockVina was used to perform the molecular docking studies between the receptor and ligand. Results obtained known as 'affinity' or 'the docking score' was expressed in kcal/mol. In this approach, the ligand is flexible while the receptor is kept rigid. Orientation of the ligand inside the binding site is known as 'POSE'. The preferred orientation is chosen after several algorithm searches are done to look through different poses. Finally, to predict the strength of association between the ligand and the receptor, scoring functions were employed. The results are shown as binding energy/affinity for each pose in Kcal/mol with rmsd values (root mean square deviation) at the end of docking. The first pose with the least binding energy and zero rmsd values are generally taken into account.

3.2.21.6 Analysis of Docking output using Discovery Studio Visualiser

Biovia's Discovery Studio was used to analyze the docking outputs. 2D and 3D images of receptor ligand interaction was visualized and recorded. Redocking the ligands found co crystallized with the receptor structures in the RCSB-PDB website may be done for validation.

3.2.22 Statistical Analysis

The values are expressed as Mean±SD. The level of significance was calculated by analyzing the p-value using Student's t-test. The IC_{50} was calculated using SPSS software. Results were considered statistically significant when $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

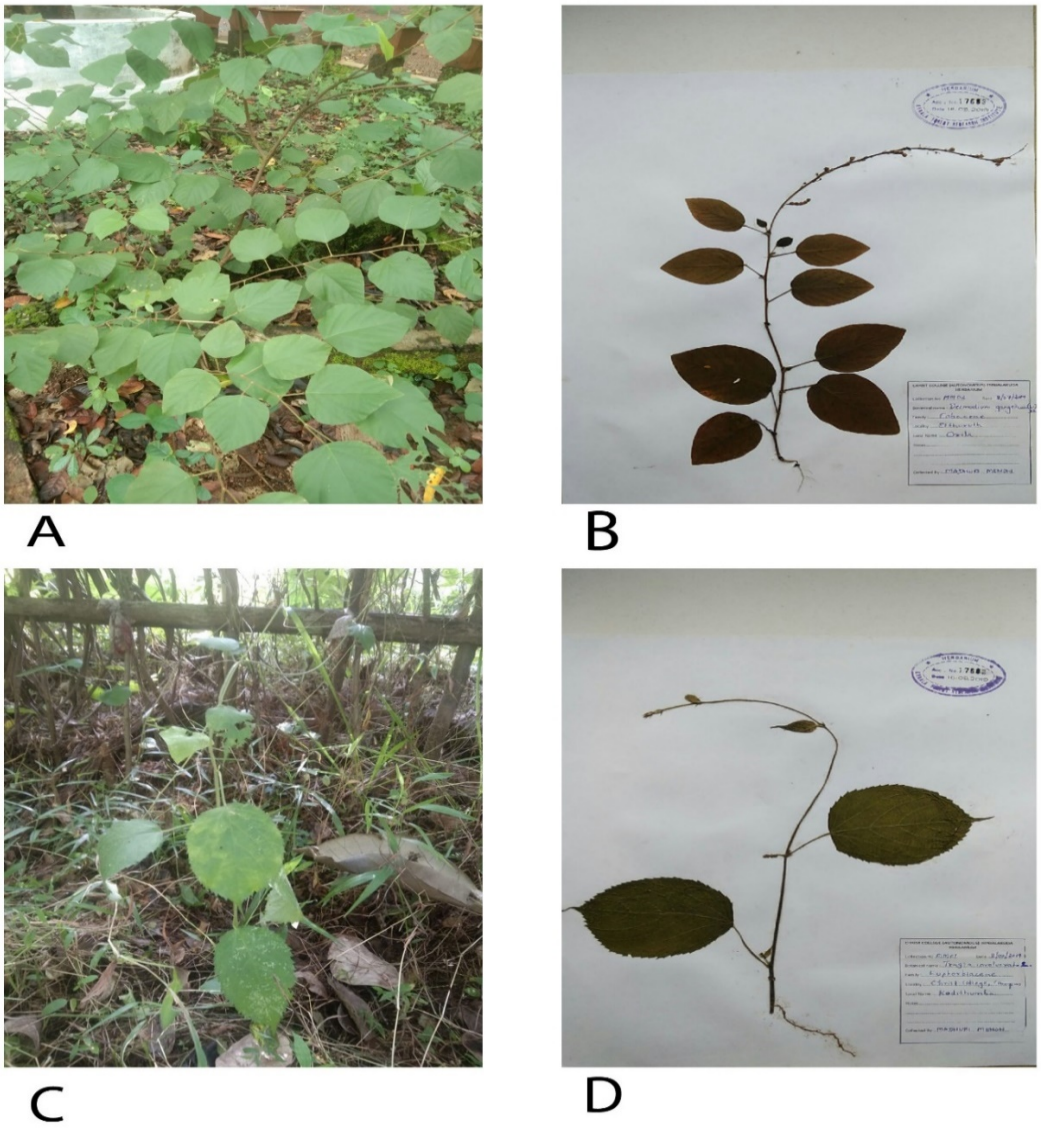

Figure 3.1: Images of Plants and its herbarium. A and B: shows the plant *Pleurolobus gangeticus* and its herbarium respectively; C and D: shows the plant *Tragia involucrata* and its herbarium respectively.

CHAPTER: 4

EXTRACTION AND PHYTOCHEMICAL PROFILING OF *P. GANGETICUS* AND *T. INVOLUCRATA*

4.1 Introduction

The initial synthesis of secondary metabolites often takes place in a single organ like the roots, fruits or leaves. It is then transported throughout the plant and stored in different tissues. The polarity of the compounds decides the site of its storage. Alkaloids, glucosides and tannins are hydrophilic in nature and hence are stored in the vacuoles or idioblasts. On the other hand, terpene-based compounds which are lipophilic are stored in trichomes, glandular hairs, resin ducts, thylakoids membranes or cuticles (Acamovic & Brooker, 2005). In this chapter, we have discussed the extraction procedure of phytochemicals from *P. gangeticus* (Stem and root), and *T. involucrata* (whole plant) in three different solvents namely; ethyl acetate, ethanol and water. The chapter also deals with qualitative and quantitative phytochemical analysis of the extracts, along with chromatographic identification of phytocompounds in the bioactive extracts.

4.2 Methodology

4.2.1 Preparation of the plants for extraction

P. gangeticus were collected from 'Thrissur' district (10.5270° N, 76.3608° E), India, during the month of November, 2019 and *T. involucrata*, was procured from nearby area (10.3469° N, 76.2074° E) during the month of September, 2019. Voucher specimens of both the plants were submitted at Kerala Forest Research (KFRI), Kerala, India and authenticated with accession number 17683 and 17682 respectively. Parts of *P. gangeticus* except leaves, and the whole plant of *T. involucrata* used for further study were cleaned thoroughly, dried at 55^{o}C using a hot air oven, and ground to a fine powder for extraction.

4.2.2 Accelerated solvent Extraction

The extraction was carried out using ethyl acetate, ethanol and water in an accelerated solvent extractor as mentioned in Chapter 3 section 3.2.1.

4.2.3 Phytochemical screening of plant extracts

Phytochemical screening of various extracts was carried out according to standard procedures (Harborne, 1973; Evans & Trease, 1989; Sofowora, 1993; Harborne, 1998) as mentioned in Chapter 3 section 3.2.2.

4.2.4 Quantitative estimation of total phenols and flavonoids

The Folin-Ciocalteu spectrophotometric method was used to estimate the Total Phenolic Content (TPC) of the extracts, as proposed by Singleton & Rossi (1965). The Total Flavonoid Content of the extracts were estimated using an aluminum chloride colorimetric method as per Chang et al., 2002 with minor modifications. The details of the methodology are given in Chapter 3 section 3.2.3.3 and 3.2.3.4.

4.2.5 GC-MS analysis

The ethyl acetate and ethanol extracts of *P. gangeticum* and *T. involucrata were* examined using Shimadzu GC-MS (model no: QP2010S). The aqueous extract did not show any activity on cytotoxicity and hence was not analyzed further. The GC was performed as per the procedure explained in Chapter 3 section 3.2.3.1.

4.2.6 LC-MS analysis of the bioactive extracts

Since the ethanol extracts of both the plants showed higher biological activity compared to others, non-volatile analytes of these extracts were further examined using LC-MS. Ultra Performance Liquid chromatography coupled to UPLC-Q-ToF-MS (Quadrupole time-of-flight mass spectrometry) was carried out for chromatographic separation and detection of non-volatile analytes as mentioned in Chapter 3 section 3.2.3.2.

4.3 Results

4.3.1 The yield of extraction

The percentage yield of *P. gangeticus* extracts prepared using solvents of increasing polarity is given in Table 4.1. The yield of DEE (ethyl acetate), DME (ethanol) and DWE (water) (w/w) were found to be 2±1.13, 10.5±1.13 and 5±1.13% respectively. In the case of *T. involucrata* the percentage yield of TEE (ethyl acetate), TME

(ethanol) and TWE (water) (w/w) were 2±1.2, 10.4±0.03 and 5±0.05 % respectively. The maximum yield was found to be in the ethanol extraction for both plants.

4.3.2 Qualitative phytochemical analysis

P. gangeticus ethyl acetate extract (DEE) revealed the presence of flavonoids, polyphenols and tannins. Other phytochemicals such as alkaloids, coumarins, glycosides, saponins, steroids and terpenoids were not detected in it. DWE showed the presence of alkaloids, flavonoids, polyphenols and tannins. In DME, except saponins and coumarins all the other tested phytochemicals were present (Table 4.2). Similarly, TME showed the presence of all phytochemicals except coumarins. Flavonoids, tannins and terpenoids were detected both in TEE and TWE. However, in addition to them TWE also showed the presence of polyphenols and saponins (Table 4.3).

4.3.3 Estimation of total phenol and flavonoid content

The total phenol and flavonoid contents were high in the ethanol extracts of *P. gangeticus* when compared to ethyl acetate and aqueous extracts. The phenolic content in DME was 40.44±0.58 mg GAE/g, followed by DEE (34.8±0.51 mg GAE/g) and DWE (18±1 mg GAE/g). The flavonoid content in DME, DEE and DWE were 36.3±0.20, 31.5±0.77 and 25.14±0.22 mg QCE/g respectively. TME and TWE showed a phenolic content of 33.2 ± 0.77 and 16.0±1.71 mg GAE/g respectively. However only trace amounts of phenol content were observed in TEE (0.05±0.03 mg GAE/g). TME showed the highest flavonoid (38.2±0.3 mg QCE/g) content followed by TEE (29.6±0.81 mg QCE/g) and TWE (17±1 mg QCE/g) (Table 4.4).

4.3.4 GC-MS analysis

The ethanol extracts of both the plant were further analysed for their volatile compounds using gas chromatography coupled with mass spectrometric method. The compounds were identified by comparing the reference spectra in the NIST and WILEY libraries. Compounds present in each extract are arranged in the decreasing order of their abundance (area %) in Table 4.5 and 4.6. The GC-MS chromatograms are shown in Figure 4.1 and 4.2. In DME, 14 compounds were identified in which

Mome inositol (20.87%) was the most abundant one. TME had 14 volatile analytes and clionasterol was found to have the highest area % of 21.05.

4.3.5 LC-MS analysis

The LC-MS analysis of the most bioactive ethanol extracts revealed the presence of several secondary metabolites in both plants. Out of nine compounds identified in DME some of the pharmacologically potent molecules like chavicine, schaftoside, glucotropaeolin, rubone and okanin were detected for the first time in *P. gangeticus*. In TME, out of 10 compounds identified, 7 of them viz; corilagin, cynaroside, apigetrin, leufolin A, loquatoside, agathisflavone and echinacin have not been reported earlier in *T. involucrata* (Table 4.7 and 4.8). Chavicine and Corilagin were the most abundant one among other analytes detected in DME and TME respectively.

4.4 Discussion

For phytochemical extraction, multiple solvent systems are commonly used. Dried and powdered plant parts are preferred for the extraction of bioactive compounds from them, as the water content may interfere with the procedure (Altemimi et al., 2017). The Accelerated solvent extraction method chosen for our study, as compared to the conventional soxhlet method, is less time consuming and at the same time gives a better yield. Elevated temperature and pressure are utilized by this automated technique for extraction, which is capable of extracting 10 g of sample in less than 15 minutes using just 15 ml of the solvent. High temperature reduces the viscosity of the solvent, and thus increases its ability to solubilize the target analytes. Since many of the organic solvents used in the extraction boil at low temperatures, elevated temperature alone is not enough to accomplish a proper extraction. Elevated pressure provided aids in the pumping of solvent through a packed bed. For the ease in downstream processing like solvent exchange and bioassays, the initial step of solvent selection must be done very carefully. The solvents must be volatile and should not leave any residue when dried (Yan et al., 2008). According to Kumar & Baskar (2015), ethanol and methanol were the most efficient solvents for phytochemical extraction and our results also show that ethanol extraction yield was much higher as compared to ethyl acetate and water.

The preliminary phytochemical screening revealed presence of flavonoids, tannins and polyphenols in all the extracts except TEE. In the GC-MS analysis of the DME, Mome inositol was the most abundant one (20.87%), with several other pharmacologically important molecules such as Lupeol (2.70%), Stigmasterin (3.39%) and 3-P-Cymenol/thymol (3.32%). Antiproliferative, anti-cirrhotic, anti-alopecic and anti-neuropathic are some of the potentials attributed to mome inositol (Mathi et al., 2015). Lupeol was proved to have *in vitro* growth inhibitory effects on *Plasmodium falciparum* and also on *Plasmodium berghei*, showing its antiprotozoal potentials. It has also shown antitumor activities in various cancer cell lines. Lupeol had proven cytotoxic potentials and was shown to bring about this by inhibiting Topoisomerase Ⅱ. In cancer types where Ras oncogene plays a vital role, lupeol was found to inhibit the farnesyltransferase enzyme (Gallo & Sarachine, 2009). Stigmasterin, also known as stigmasterol was known to have several biological potentials such as anti-osteoarthritic, anti- hypercholestrolemic, anti-oxidant, anti-mutagenic, anti-inflammatory and analgesic properties. It was also known to induce cytotoxicity and had anti-tumor potentials by inhibiting the lyase activity of DNA polymerase β. It was observed to increase the insulin levels thereby being hypoglycemic in nature (Kaur et al., 2011). Thymol detected in these extracts are also a well-known compound with several pharmacological potentials. The nematicidal effect of thymol was observed in the egg, larval and adult stages of *Haemonchus contortus,* a gastro-intestinal parasite seen in goats and sheep (Ferreira et al., 2016). Thymol is also known to have several other properties with antioxidant and anticancer potentials being the notable ones. It is known to induce cell death via apoptosis and shows anti-apoptotic and anti-genotoxic effects in normal cells (Islam et al., 2019a). The presence of gamma-sitosterol detected in the study has also been previously reported to be present in the roots of *P. gangeticus* (Vasani et al., 2022).

DME was further analysed for the presence of non-volatile analytes via LC-MS. Several secondary metabolites with important pharmacological and therapeutic potentials were detected. It showed the presence of nine compounds and none of them have been reported in *P. gangeticus* earlier. Chavicine showed the highest retention time (5.058) and relative abundance. Rubone, Okanin and Glucotropaeolin are highly potent bioactive molecules detected in the extract. Rubone had tumor inhibition potentials as they were found to inhibit human hepatocellular carcinoma

tumors. They did not have any inhibitory effect on non-tumorigenic human hepatocytes (Xiao et al., 2014). Okanin and glucotropaeolin are known to cause cell death by inhibiting the NF-κB signaling and inducing apoptosis respectively (Hou et al., 2017; Arumugam & Razis, 2018).

In the GC-MS analysis of TME, fourteen compounds were detected. The most abundant one was clianosterol with an area % of 21.05 and the least abundant one was phenylacetaldehyde diethylacetal with 1.06% area. Several pharmacologically important compounds, such as squalene (13.91%), vitamin E (11.21%), phytols (8.18%), neophytadiene (6.61%) and viminalol/α-amyrin (4.41%) were also present in the extract.

Further analysis of the TME for its non-volatile analytes by LC-MS revealed the presence of ten secondary metabolites. Quercetin (rt 3.813) has been detected in the extract and several derivatives of quercetin such as dihexosyl quercetin, quercetin-3-O-rutinoside and rhamnosyl hexosyl methyl quercetin have been previously detected in the 80% aqueous methanol extracts of the whole plant of *T. involucrata* (Sulaiman & Balachandran, 2016). The newly identified analytes in *T. involucrata* ethanol extracts were agathisflavone, laquatoside, leufolin A, echinacin, apigetrin, cynaroside and corilagin. Corilagin was the most abundant compound in TME and is known to be present in several plants belonging to different genus, however it has not been previously reported to be present in *Tragia involucrata*. Corilagin has been known to inhibit the multiplication of different kinds of microorganisms including bacteria, fungi and viruses. It is known to have free radical scavenging properties thus highlighting its antioxidant potentials. It has also been known to affect insulin resistance thereby being a potential herbal source for managing type 2 diabetes. In hemorrhagic-shock induced liver, it showed hepatoprotective activity and also antiinflammatory potentials were revealed as it was able to reduce the levels of proinflammatory cytokines in LPS-stimulated mouse macrophage cell line (RAW 264.7), by blocking the NF-κB pathway. Most importantly corilagin had anti-tumor potentials as they induced apoptosis and also blocked the cell cycle thereby putting a check on cell proliferation (Li et al., 2018). Agathisflavone another molecule identified in TME is known to have several biological activities like antioxidant effect, anti-inflammatory, antiviral, neuroprotective, hepatoprotective, antiparasitic and cytotoxic effects (Islam et al., 2019b). Apigetrin was known to induce apoptosis

in human gastric cancer cells by elevating Bax and Caspase 9 and 3 activations and reducing Bcl2 expression (Sun et al., 2018). Apigetrin is also known to inhibit neuroinflammation in BV-2 microglia thereby protecting the HT22 hippocampal cells (Lim et al., 2016). Cynaroside, a luteolin derivative also has several biological activities including anti-inflammatory, antiallergic, antileishmanial, apoptosis induction and tumorigenesis inhibition properties (Szekalska et al., 2020; Tabrez et al., 2021; Ji et al., 2021).

Table 4.1. The yield of extraction of *P. gangeticus* and *T. involucrata* in different solvent systems

Name of the plant	Solvent	Code	Yield (%)
Pleurolobus gangeticus	Ethyl acetate	DEE	2 ± 1.13
	Ethanol	DME	10.5 ± 1.13
	Water	DWE	5 ± 1.13
Tragia involucrata	Ethyl acetate	TEE	2 ± 1.2
	Ethanol	TME	10.4 ± 0.03
	Water	TEE	5 ± 0.05

Values are expressed as Mean ± SD

Table 4.2: **Phytochemical screening of ethyl acetate (DEE), ethanol (DME) and aqueous (DWE) extracts of the stem and root parts of *P. gangeticus***

Phytochemicals	DEE	DME	DWE
Alkaloids	-	+	+
Coumarins	-	-	-
Flavanoids	+	+	+
Glycosides	-	+	-
Polyphenols	+	+	+
Saponins	-	-	-
Steroids	-	+	-
Tannins	+	+	+
Terpenoids	-	+	-

(+) – indicate presence, (-) – indicate absence

Table 4.3: **Phytochemical screening of ethyl acetate (TEE), ethanol (TME) and aqueous (TWE) extracts of the whole plant of *T. involucrata***

Phytochemicals	TEE	TME	TWE
Alkaloids	-	+	-
Coumarins	-	-	-
Flavanoids	+	+	+
Glycosides	-	+	-
Polyphenols	-	+	+
Saponins	-	-	+
Steroids	-	+	-
Tannins	+	+	+
Terpenoids	+	+	+

(+) - indicate presence, (-) - indicate absence

Table 4.4: Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Name of the Plant	Extracts	TPC (mg GAE/g)	TFC (mg QCE/g)
Pleurolobus gangeticus	DEE	34.8±0.51	31.5±0.77
	DME	40.44±0.58	36.03±0.20
	DWE	18±1	25.14±0.22
Tragia involucrata	TEE	0.05±0.03	29.6±0.81
	TME	33.2±0.77	38.2±0.3
	TWE	16.0±1.71	17±1

Values are expressed as Mean ± SD

Table 4.5: The phytochemical constituents DME identified by GC-MS analysis

SI No	Peak No	RT	Area	Area %	Height	Height %	Name	Base m/z
1	4	15.341	13442835	20.87	935306	7.42	Mome inositol	87.00
2	7	23.468	8633852	13.40	2308432	18.32	Ethyl oleate	55.05
3	6	23.354	4275636	6.64	1315759	10.44	Ethyl linoleate	67.05
4	9	30.736	3722488	5.78	1289992	10.24	Iso-octyl phthalate	148.95
5	13	44.733	3321887	5.16	448174	3.56	.gamma.-Sitosterol	55.05
6	3	14.217	3206007	4.98	1191003	9.45	Cis-isoapiole	221.95
7	11	43.159	2183749	3.39	343981	2.73	Stigmasterin	55.05
8	1	9.929	2136929	3.32	449370	3.57	3-P-Cymenol	135.05
9	12	43.560	2072664	3.22	351729	2.79	Scandenone-4'-methyl ether	403.10
10	10	31.015	1834445	2.85	646021	5.13	Ethyl elaidate	55.05
11	5	19.449	1838695	2.85	604009	4.79	Ethyl palmitate	88.05
12	15	46.494	1740750	2.70	271909	2.16	Lupeol	55.05
13	8	27.503	810605	1.26	295745	2.35	Ethyl 9-hexadecenoate	55.05
14	2	11.734	522583	0.81	346915	2.75	(E)-Caryophyllene	91.00

Table 4.6: The phytochemical constituents of TME identified by GC-MS analysis

Peak No	RT	Area	Area %	Height	Height %	Name	Base m/z
13	44.720	8735569	21.05	1194877	10.24	Clionasterol	57.10
11	34.974	5770780	13.91	2083089	17.86	Squalene	69.05
10	30.732	5426104	13.08	1933068	16.57	2-Ethylhexyl phthalate	149.00
12	40.139	4652911	11.21	1077618	9.24	Vitamin E	165.05
5	22.297	3394734	8.18	946928	8.12	Phytol	71.05
4	19.447	3052529	7.36	986452	8.46	Ethyl palmitate	88.05
6	23.358	2047835	4.94	669657	5.74	Ethyl linoleate	67.05
2	16.641	1901598	4.58	770970	6.61	Neophytadiene	68.05
14	47.183	1831069	4.41	272999	2.34	Viminalol	218.10
8	23.517	1575776	3.80	502728	4.31	Linolenic acid	79.05
7	23.468	1317704	3.18	531644	4.56	Ethyl elaidate	55.05
9	24.010	807835	1.95	263079	2.26	Ethyl octadecanoate	88.05
3	17.313	534681	1.29	194239	1.66	(E)-Phytol	81.10
1	10.308	440224	1.06	238884	2.05	Phenylacetaldehyde diethylacetal	103.05

Table 4.7: Compounds of DME extract identified by LC-MS

Sl. No	Retention time	Name	Molecular Formula	m/z value	Relative abundance
1	5.058	Chavicine	$C_{17}H_{19}NO_3$	373.126	14.99
2	2.751	Schaftoside	$C_{26}H_{28}O_{14}$	563.139	4.24
3	2.135	Glucotropaeolin	$C_{14}H_{19}NO_9S$	408.042	0.75
4	5.049	(5S,6E,8Z)-5,18-Dihydroxy-6,8-octadecadienoic acid	$C_{18}H_{32}O_4$	311.221	0.73
5	5.024	2',3,4,4',6'-Pentamethoxy chalcone	$C_{20}H_{20}O_6$	355.118	0.56
6	2.072	PhenylthioAceto - hydroxamic acid	C_8H_9NOS	168.049	0.39
7	4.344	3,6-Dihydroxy-9-oxo -9H-xanthene-4, 5-dicarbaldehyde	$C_{15}H_8O_6$	285.039	0.13
8	3.927	Rubone	$C_{20}H_{22}O_7$	373.126	0.12
9	3.728	Okanin	$C_{15}H_{12}O_6$	287.054	0.11

Table 4.8: Compounds of TME extract identified by LC-MS

Sl. No	Retention time	Name	Molecular Formula	m/z value	Relative abundance
1	2.674	Corilagin	$C_{27}H_{22}O_{18}$	633.072	0.44
2	3.057	Cynaroside	$C_{21}H_{20}O_{11}$	447.092	0.28
3	2.883	Isoquercetin	$C_{21}H_{20}O_{12}$	465.104	0.19
4	3.257	Apigetrin	$C_{21}H_{20}O_{10}$	431.097	0.13
5	3.970	Leufolin A	$C_{30}H_{28}O_{12}$	579.150	0.12
6	2.962	1,2,36-tetrakis-O-galloyl-B-D-glucose	$C_{34}H_{28}O_{22}$	787.095	0.12
7	4.153	Loquatoside	$C_{20}H_{22}O_{11}$	437.107	0.09
8	4.310	Agathisflavone	$C_{30}H_{18}O_{10}$	537.082	0.089
9	3.813	Quercetin	$C_{15}H_{10}O_{7}$	303.049	0.087
10	3.805	Echinacin	$C_{30}H_{26}O_{12}$	577.133	0.020

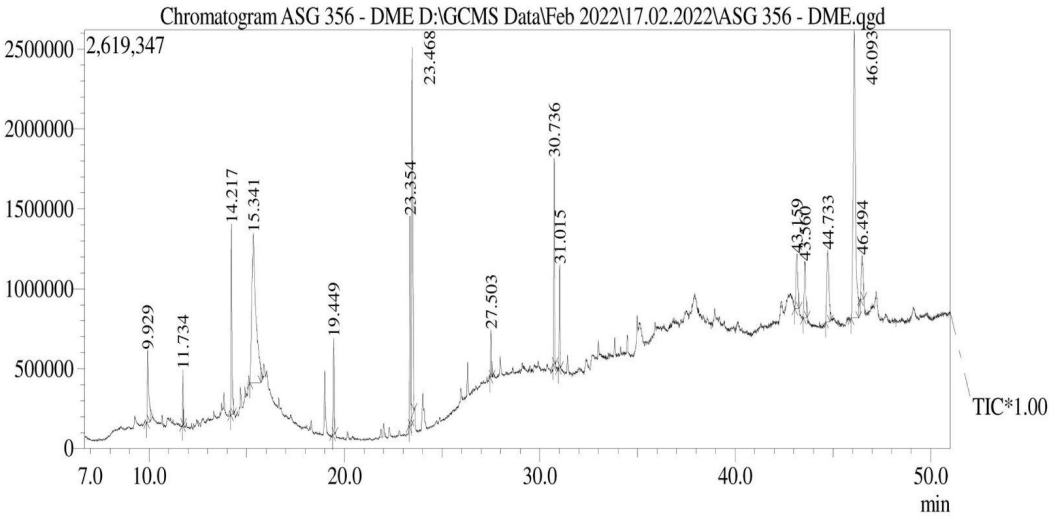

Figure 4.1: **GC-MS chromatogram DME**

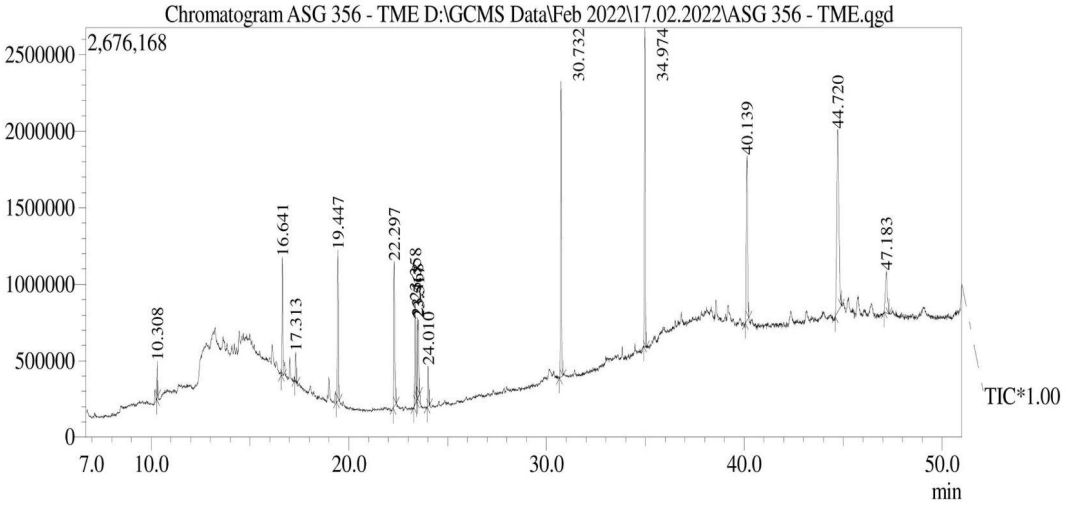

Figure 4.2: GC-MS chromatogram of TME

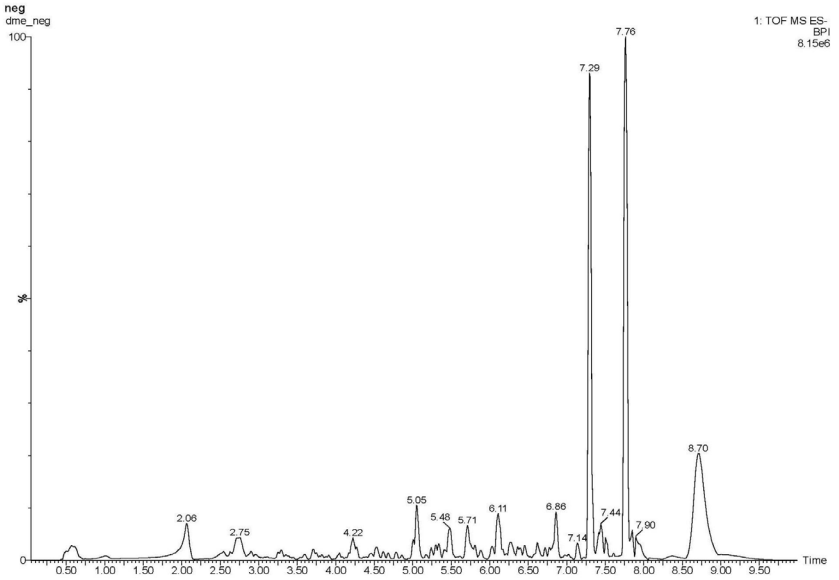

Figure 4.3: Total ion chromatogram of DME in negative ion mode

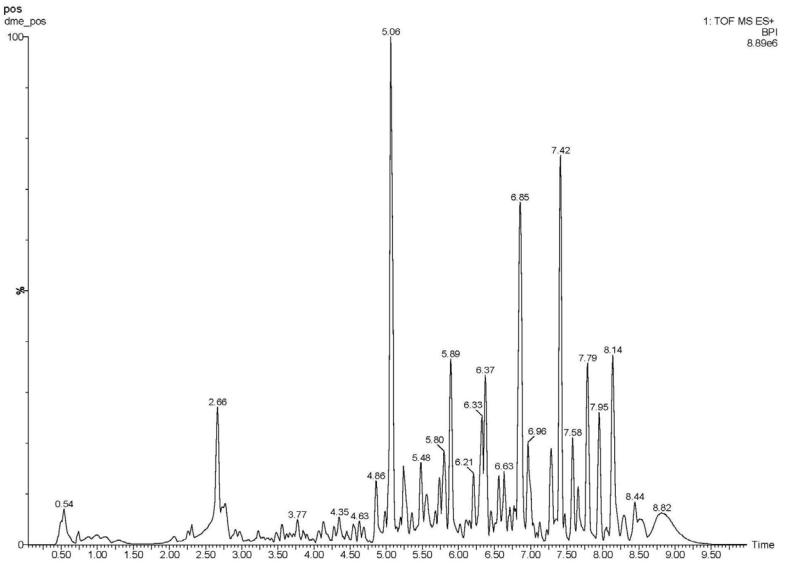

Figure 4.4: Total ion chromatogram of DME in positive ion mode

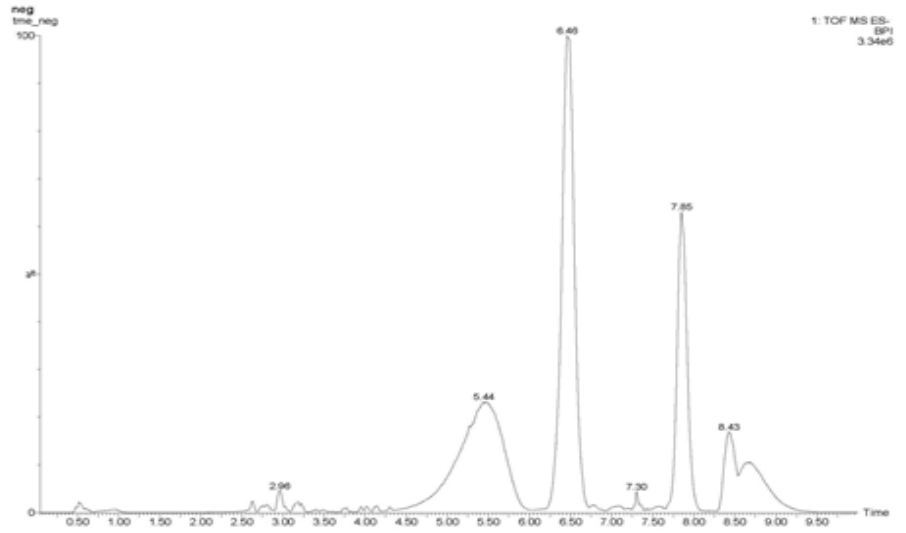

Figure 4.5: Total ion chromatogram of TME in negative ion mode

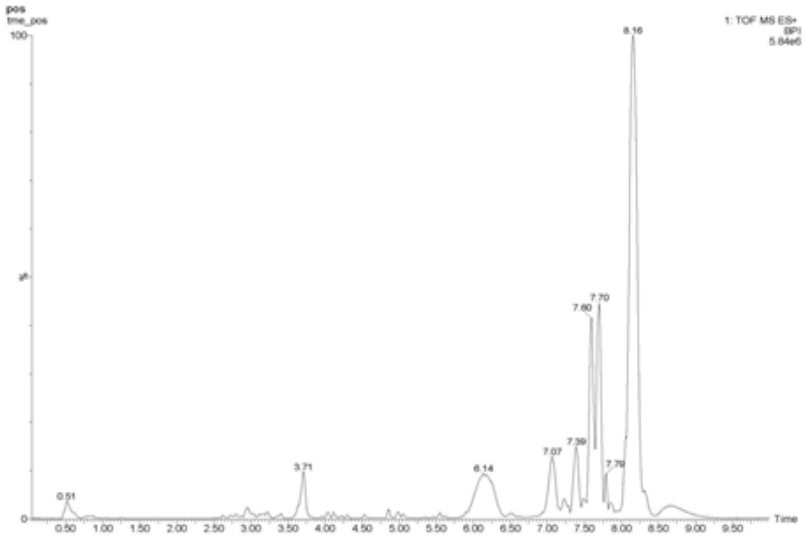

Figure 4.6: Total ion chromatogram of TME in positive ion mode

CHAPTER: 5

ANTICANCER POTENTIALS OF *P. GANGETICUS* AND *T. INVOLUCRATA* IN THE *IN VITRO* SYSTEMS

5.1 Introduction

The U.S National Cancer Institute (NCI) has recognized the anticancer potential of natural agents since 1950s and further research has attributed the discovery of several natural anticancer agents since then (Fouché et al., 2008). Vinca alkaloids namely; vinblastine and vincristine from *Catharanthus roseus* were the first plant-derived anticancer agents introduced in clinical use (Prakash et al., 2013). Etoposide and teniposide are semisynthetic derivatives of podophyllotoxin from *Podophyllum peltatum*. They are clinically effective agents and are used to treat lymphoma, bronchial and testicular cancer (Agarwal et al., 2012). For studying various biological mechanisms involved in cancer, cancer cell lines have been widely used as an efficient tool for research purposes. In order to study the genetic, epigenetic and cellular pathway, appropriate *in vitro* models are vital in cancer research. It also enables the study of various other factors like cell proliferation, apoptosis, cancer progression, defining potential molecular markers and screening of cancer therapeutics (Ferreira et al., 2013). In the present chapter we discuss the anticancer potentials of *P. gangeticus* and *T. involcrata* using *in vitro* systems.

5.2 Methodology

5.2.1 Plant authentication and Extraction

The detailed procedure of plant collection and extraction have been given in chapter 3 section 3.2.1. The ethyl acetate, ethanol and aqueous extracts of both plants were used for cytotoxicity analysis. Further *in vitro* assays were performed with the most potent extracts which had the lowest IC_{50} values.

5.2.2 Cell lines

YAC-1 and DLA cell lines used for the study were procured from National Centre for Cell Sciences (NCCS), Pune and Amala Cancer Research Centre, Thrissur respectively. The former was cultured in RPMI medium supplemented with 10 % FBS, Streptomycin (100 µg/ml), Penicillin (100 U/ml) and incubated at 37°C in a CO_2 incubator (Heracell 150i MD) which provided 5 % CO_2 and 95% relative humidity; DLA cells were maintained in the peritoneal cavity of mice and the procedure is detailed in chapter 3 section 3.1.5.

5.2.3 Trypan blue exclusion assay

The plant extract contains diverse group of chemicals, that may give false positive results for cell viability by reducing the activity of MTT compounds, hence MTT assay was avoided in our study (Karakas et al., 2017). Short term cytotoxicity was conducted according to Strober et al., 1997 with minor modifications. The tumor cells aspirated from the peritoneal cavity of tumor bearing mice were washed thrice with PBS. Viable cell suspension (1×10^5 cells in 0.1 ml) was added to tubes containing various concentrations of the extracts, and the volume was made to 1 ml using PBS. Control tube contained only the cell suspension. The procedure is detailed in chapter 3 section 3.2.4.

The percentage cytotoxicity was calculated by using the formula:

$$\% \text{ cytotoxicity} = \frac{\text{No.of dead cells}}{\text{No.of live + dead cells}} \times 100$$

5.2.4 Peripheral Blood Lymphocyte culture for mitotic index analysis

Peripheral blood lymphocyte culture was done according to Moorhead et al. (1960), with modifications. Triplicates were maintained for each sample. The detailed procedure is given in chapter 3 section 3.2.5. GTG banding was done according to SeaBright (1971) with minor modifications. The mitotic index was analyzed by metaphase counting. The value was calculated using the formula (Bulla et al., 2014).

$$\text{Mitotic index} = \frac{\text{No.of Metaphases}}{\text{Total No: of cells counted}} \times 100$$

5.2.5 Annexin V/PI staining for Apoptosis analysis

The apoptosis analysis using Annexin V/PI (propidium iodide) was done according to Crowely et al., 2016 with minor modifications. Approximately 1×10^6 YAC-1 cells were seeded in a T 25 cell culture flask (triplicates) and treated with the extracts at three different concentrations (5, 10 and 15µg/ml). Details of the procedure are given in chapter 3 section 3.2.6. The cells were then analyzed using a flow cytometer.

5.2.6. Statistical Analysis

All the experiments were done in triplicates and the values are expressed as Mean±SD. The level of significance was calculated by analyzing the p-value using Student's t-test. The IC_{50} was calculated using SPSS software.

5.3 Results

5.3.1 Cytotoxic and anti-proliferative effect of the extracts

Considerably high toxicity was observed in DLA cells treated with organic solvent extracts, while the aqueous extracts showed no signs of toxicity even at 100 µg/ml concentration in the three-hour treatment (Table 5.1 and 5.2). The IC_{50} of DME was calculated to be 17.5 µg/ml and that for DEE was 21.6 µg/ml. Similarly, the IC_{50} values for TME was calculated to be 16.092 µg/ml which was the lowest among the others. Considering the lower IC_{50} values, further studies were focused only on DME and TME. Inorder to look for the growth inhibition exerted by DME and TME, a cell proliferation assay was conducted in YAC-1 cells. The cells were treated with lower concentration (5, 10 and 15 µg/ml) but for longer time periods *viz*. 12 h, 24 h and 48 h. The results revealed that the number of live cells decreased considerably with longer duration of treatment indicating the stability of DME and TME to induce cell death for a prolonged time at physiological temperature (Figure 5.1 and 5.2).

5.3.2 Mitotic indexing using PBLC

Due to the degree of chromosome condensation, metaphase is the most visible phase of cell division and mitotic index (MI) aims at metaphase counting (Bulla et al., 2014). The MI% of control were much less (3.5%) compared to the colchicine (6.8%), DME (7.5%) or TME (8%) treated cells (Figure 5.3). The number of cells in metaphase stage was 5.33±0.57 in control, while in DME and TME treated cells it was 11.33±1.15 and 12.0±1 respectively. The colchicine (10.33±0.57) treated cells also showed higher metaphase stages than control as expected (Table 5.3).

5.3.3 Apoptosis induction by the extract treatment *in vitro*

Most of the anticancer drugs induce apoptosis in tumor cells and the expression of an intact cell death pathway is vital in this strategy. Mitotic blockade caused due to

interference of the drug with the organization of the mitotic spindle and microtubule dynamics, finally lead to apoptosis (Pathania & Rawal, 2018). Considering the cytotoxicity nature and their effect on mitotic index, an assay was designed to see if the extracts could induce apoptosis. Flow cytometry studies were performed on YAC-1 cells, with DME and TME using the same concentrations (5, 10 and 15 µg/ml) of cell proliferation assay (Figure 5.4 and 5.5). The percentage of apoptotic cells after 24 h of incubation with DME showed a dose dependent increase. The early apoptosis was 22.8% in 5 µg/ml, 34.7% in 10 µg/ml and 44.7% in 15 µg/ml concentration (Table 5.4). Similarly, a dose dependent increase in the percentage of apoptotic cells were also observed in TME treated cells. The early apoptosis was 11.5% in 5µg/ml, 26.1% in 10 µg/ml and 32.2% in 15 µg/ml concentration (Table 5.5).

5.4 Discussion

Several compounds isolated from plants are being rigorously evaluated for their anticancer potentials. However, a synergistic effect of composite mixture of several compounds present in the plant have been known to make the plant more beneficial rather than single constituent agents (Solowey et al., 2014). The crude ethanol extracts of both *T. involucrata* and *P. gangeticus* showed good anticancer potentials *in vitro* against lymphoma cell lines. In the short term cytotoxicity study, DME showed 93.1% cell death at 200µg/ml. The IC_{50} value of DME was calculated to be 17.5µg/ml, which was low when compared to the IC_{50} (21.6 µg/ml) of DEE. Similarly, TME showed an IC_{50} value of 16.092 µg/ml which was much lower when compared to the IC_{50} value (154.7 µg/ml) of TEE. TME induced 87.6% cell death at 200 µg/ml within 3 hours. Therefore, DME and TME were chosen for further studies owing to their lowest IC_{50} values. The effect on cell proliferation was analysed using trypan blue staining method. Both time and dose dependent inhibitory effects were observed on cell proliferation of YAC-1 lymphoma cell lines for DME and TME treatment.

The antiproliferative abilities of DME and TME correlates well with its potential to act on the cellular microtubule network. Microtubules are composed of α-β tubulin heterodimers and are very dynamic structures that take part in several important aspects like maintaining the shape of the cell, cellular movements, segregation of

condensed chromosomes during mitosis etc among others (Laisne et al., 2021). During mitotic phases of the cell cycle, microtubule binding agents halt the microtubule assembly/disassembly dynamics (Diab et al., 2021). Any error or imbalance in tubulin-microtubule equilibrium disrupts tubulin dynamics which ultimately leads to cell cycle arrest and apoptosis (Khwaja et al., 2018). The assays using PBLC showed that there was a considerable increase in the mitotic index in colchicine (6.8%) (reference drug), DME (7.5%) and TME (8%) treated cells when compared to controls (3.5%). This points out the potential of DME and TME to interact with the microtubule complex, thereby causing metaphase arrest.

The human vascular anticoagulant annexin V is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has high affinity for the anionic phospholipid phosphatidylserine (PS). PS is located on the cytoplasmic surface of the plasma membrane in healthy cells. The structural changes of the plasma membrane during apoptosis, causes the translocation of PS from inner to the extracellular side of the plasma membrane (Demchenko, 2013). FITC fluorochrome is attached to the annexin V and hence the FITC alone positive cells show early apoptosis. The propidium iodide (PI) is a fluorochrome that binds to the DNA and is also membrane impermeable. Hence only when the nuclear membrane degenerates and the DNA is exposed it will bind to PI. Therefore, when both FITC and PI are positive it shows late apoptosis. 24 h of treatment could induce considerable amount of cells to apoptosis. It was interesting to note that both early and late apoptotic cells were found to be increased in number by the treatment of DME as well as TME.

GC-MS analysis of DME could detect at least fourteen molecules. Though most of them were primary metabolites and are detected in several plant species, some of them (γ-Sitosterol, Stigmasterin, Lupeol, 3-P-Cymenol and (E)-Caryophyllene) were secondary metabolites and were attributed strong anticancer properties to them (Sundarraj et al., 2012; Rauth et al., 2016; Fidyt et al., 2016; Islam et al., 2019a; Zhang et al., 2022). Lupeol present in DME is known to bring about a G2/M phase cell cycle arrest thereby leading to apoptosis. It was also known to modulate microtubule functioning by interfering with and lowering the levels of α & β tubulin proteins in DU145 and LNCaP cells. (Saleem et al., 2009). γ-sitosterol in DME and TME is known to inhibit cell cycle at the G2/M phase (Tobungan et al., 2022). Phytols present in TME is known to induce apoptosis in lung cancer cells (Sakthivel

et al., 2018). LC-MS analysis was also done to identify the relevant non-volatile compounds present in DME and TME. The results revealed several important compounds out of which okanin and glucotropaeolin may be of particular interests as they are reported to have cell death inducing capacity (Hou et al., 2017; Arumugam & Razis, 2018). Corilagin present in TME is known to inhibit cell proliferation in U251 glioblastoma cells and also assumed to have a close relation with the mechanism of cell cycle as it was found to increase the frequency of cells in G2/M phase (Yang et al., 2016).

The observed anticancer abilities may be attributed to these molecules present in the extracts that may be acting synergistically. Cell death started as early as 3 h after treatment and was persistant during the 48 h period of treatment. Diverse death inducing machinery might be used by different molecules of these extracts including but not limited to ferroptosis, apoptosis, cell cycle arrest and mitotic arrest. A detailed mechanistic study using isolated/purified compounds will be necessary to make more conclusive findings and to promote these molecules for further preclinical studies.

Table 5.1: Short term cytotoxicity assay of the crude extracts of *P. gangeticus* against DLA cells

Concentrations of extract (µg/ml)	Percentage (%) cell death after 3 h treatment		
	DEE	DME	DWE
10	30.2±0.57	34.5±1.52	0
20	52.5±1.15	58.0±1	0
50	68.4±1.15	70.9±1.15	0
100	80.9±1	84.2±0.57	0
200	83.0±1	93.1±1.7	6
IC_{50} value	**21.60**	**17.5**	**-**

Table 5.2: Short term cytotoxicity assay of the crude extracts of *T. involucrata* against DLA cells

Concentrations of extract (µg/ml)	Percentage (%) cell death after 3 h treatment		
	TEE	TME	TWE
10	12.33±0.57	35.0±1	0
20	27.0±1.73	59.6±2.08	0
50	34.6±1.15	74.6±2.08	0
100	40.3±2.08	82.3±0.57	0
200	54.6±0.57	87.66±1.15	0
IC_{50} value	**154.7**	**16.092**	**-**

Table 5.3: The effect of DME and TME on Peripheral blood lymphocytes

Concentration	Total No: of cells counted	No: of Metaphases	Mitotic index (MI%)
Control	100	5.33±0.57	3.5
Colchicine (0.016 mg ml-1)	100	10.33±0.57	6.8
DME (15 µg/ml)	100	11.33±1.15	7.5
TME (15 µg/ml)	100	12±1	8

Table 5.4: Apoptosis assay using DME treated (24 h) YAC-1 cells

Type of cell population	Percentage (%) of cells at various stages of apoptosis			
	Control	5 µg/ml	10 µg/ml	15 µg/ml
Late apoptotic	0.6	4.2	4.6	4.2
Early apoptotic	1.6	18.6	30.1	40.5
total	2.2	22.8	34.7	44.7

Table 5.5: Apoptosis assay using TME treated (24 h) YAC-1 cells

Type of cell population	Percentage (%) of cells at various stages of apoptosis			
	Control	5 µg/ml	10 µg/ml	15 µg/ml
Late apoptotic	0.6	2.7	6.9	9.3
Early apoptotic	1.6	11.5	26.1	32.2
total	2.2	14.2	33	41.5

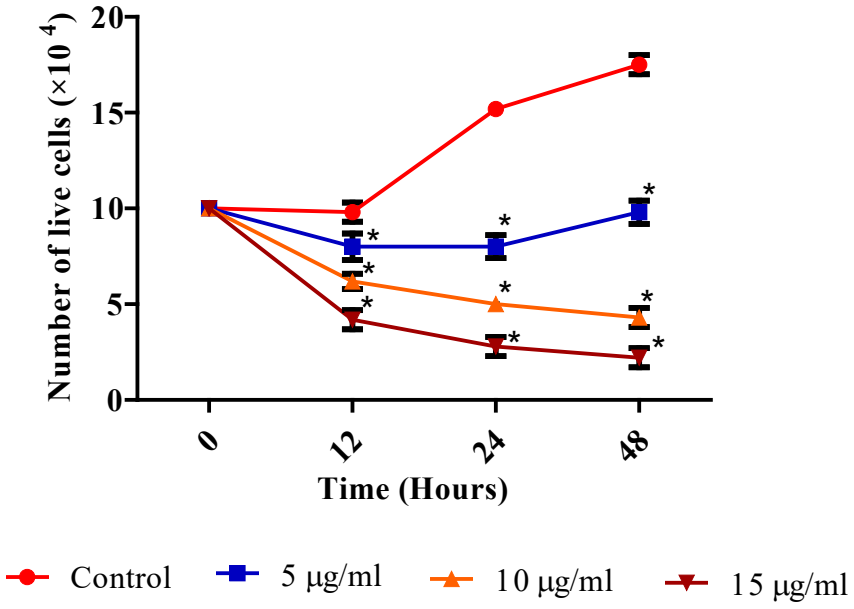

Figure 5.1: Graph showing the number of live cells (YAC-1) at three different time points (12, 24 and 48 h) after treatment with DME (5, 10 and 15 µg/ml). Comparisons were made between vehicle controls with the treated groups separately at each time point. The symbol (*) represents statistical significance at p≤0.05.

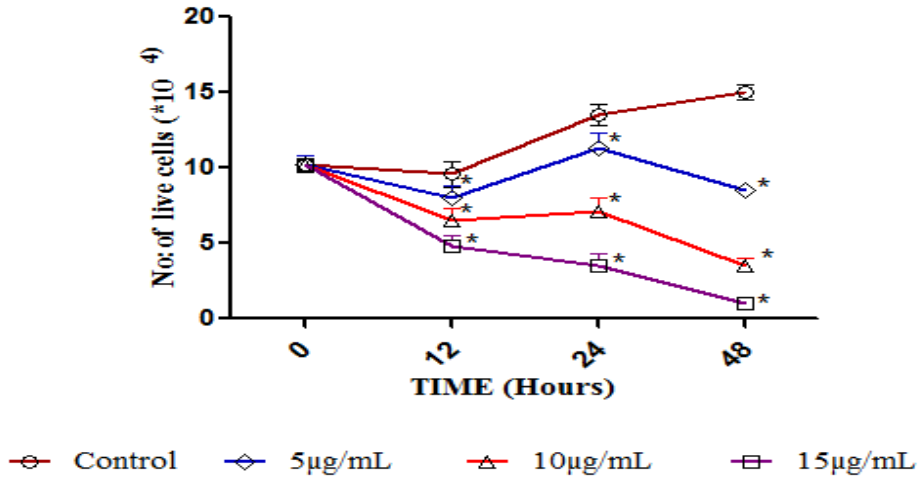

Figure 5.2: Graph showing the number of live cells (YAC-1) at three different time points (12, 24 and 48 h) after treatment with TME (5, 10 and 15 µg/ml). Comparisons were made between vehicle controls with the treated groups separately at each time point. The symbol (*) represents statistical significance at p≤0.05.

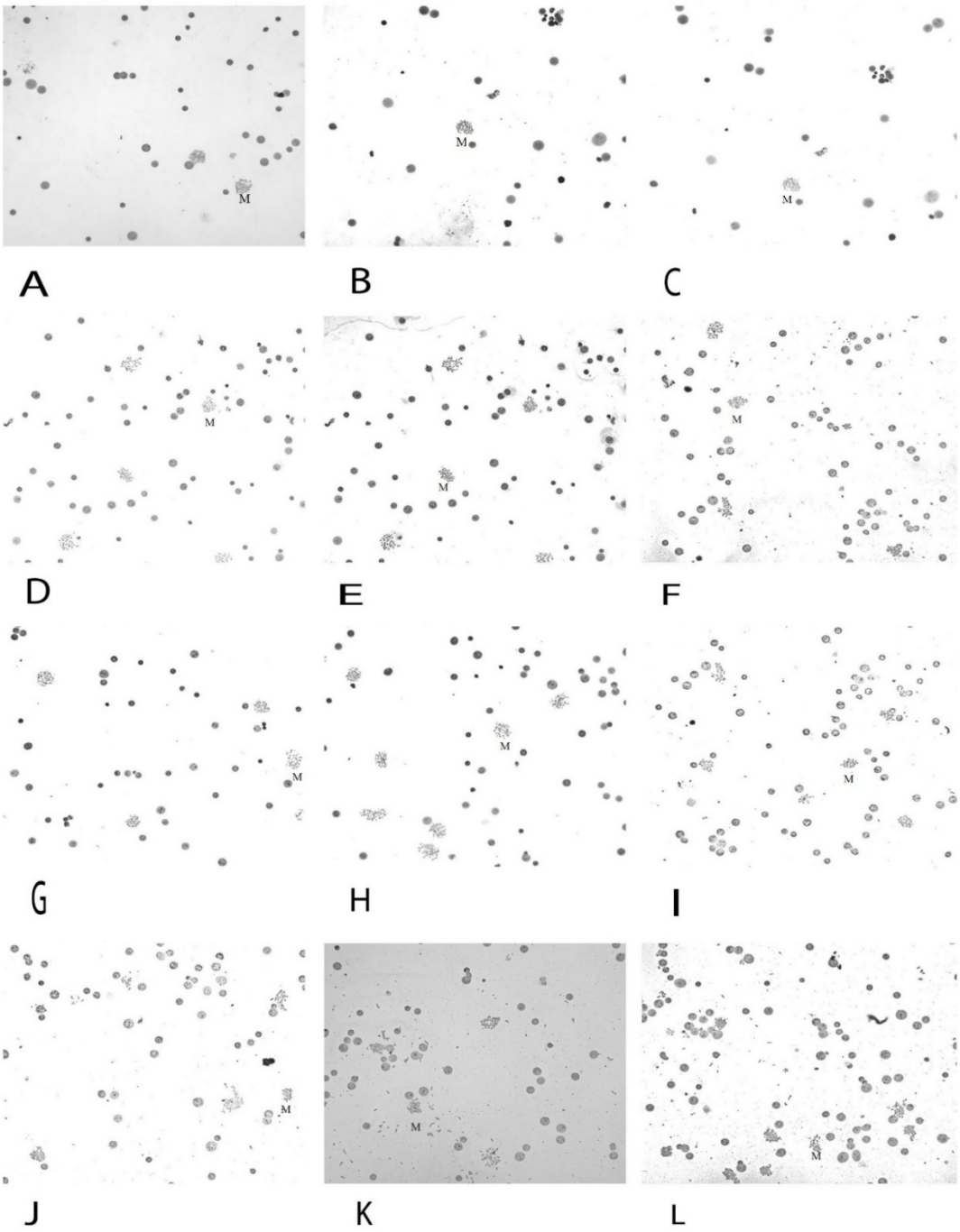

Figure 5.3: Image (10x magnification) shows peripheral human lymphocytes arrested at metaphase stage (M). A, B and C; untreated cells. D, E and F; shows colchicine treated cells. G. H, I; shows the DME treated cells and J, K, L; shows TME treated cells

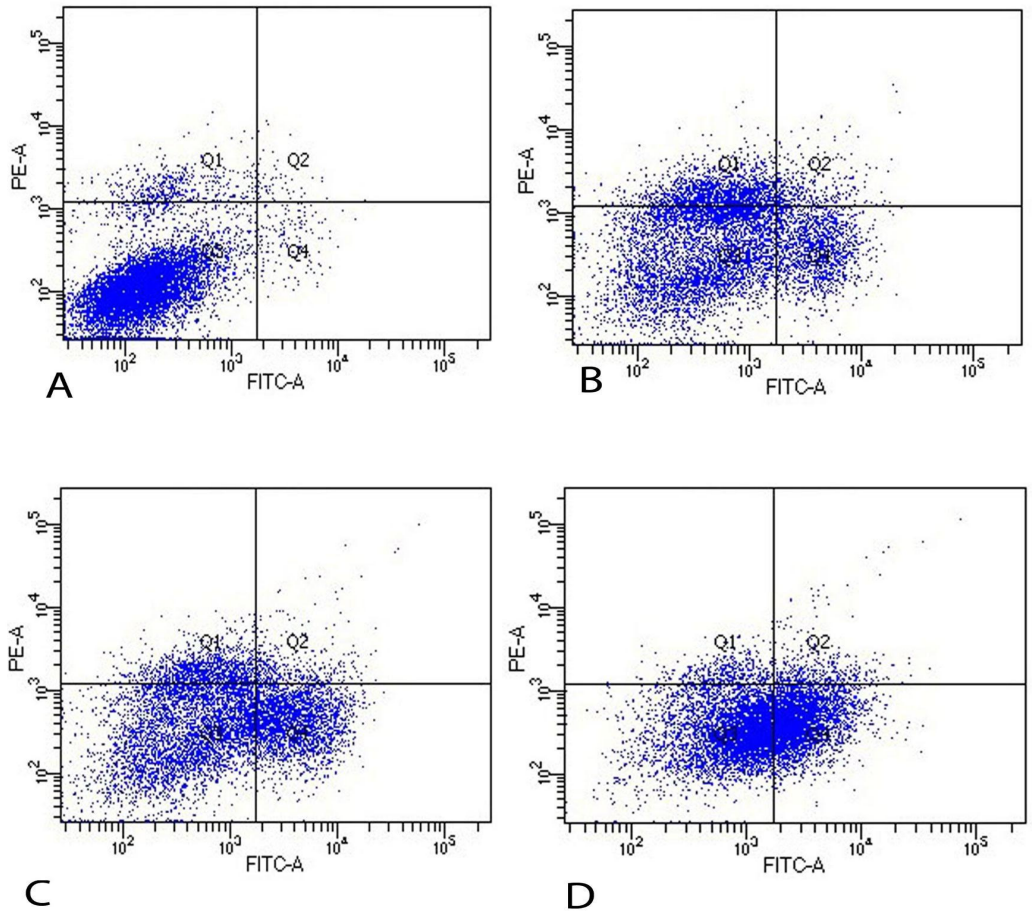

Figure 5.4: The flow cytometric analysis of DME treated YAC-1 cells using annexin V - FITC and PI staining. A: Control YAC-1 cells without any treatment; B (5 µg/ml), C (10 µg/ml) and D (15 µg/ml) are YAC-1 cells treated with different concentrations of DME as mentioned. Each diagram is divided into four regions that are defined as follows: Q1- necrotic cells (PI/FITC. +/−); Q2 late apoptotic cells (PI/FITC +/+); Q3 viable cells (PI/FITC −/−) and Q4 early apoptotic cells (PI/FITC −/+).

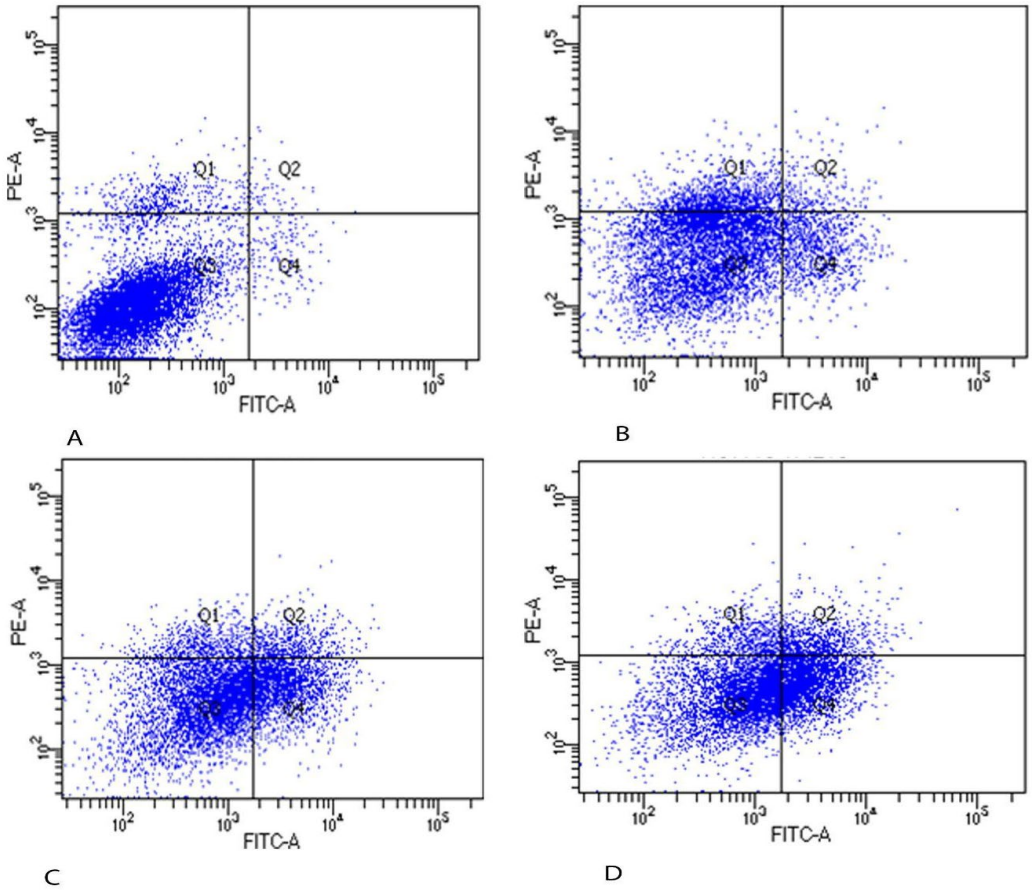

Figure 5.5: The flow cytometric analysis of TME treated YAC-1 cells using annexin V - FITC and PI staining. A: Control YAC-1 cells without any treatment; B (5µg/ml), C (10 µg/ml) and D (15µg/ml) are YAC-1 cells treated with different concentrations of TME as mentioned. Each diagram is divided into four regions that are defined as follows: Q1- necrotic cells (PI/FITC. +/−); Q2 late apoptotic cells (PI/FITC +/+); Q3 viable cells (PI/FITC −/−) and Q4 early apoptotic cells (PI/FITC −/+).

CHAPTER: 6

TOXICITY PROFILING AND ANTITUMOR STUDIES OF DME AND TME IN MICE

6.1 Introduction

With advances in areas of science like genomics and molecular biology, chemotherapy and immunotherapy are promising in combating cancer, however multidrug resistance and drug induced toxicity remains a major cause of concern. Plant-derived drugs, an alternative treatment to fight cancer is the need of the hour as it has the potential to deliver highly efficient and non-toxic treatment (Zou et al., 2021). The efficacy of anticancer agents depends on its ability to increase apoptosis, decrease cell proliferation, induce cell differentiation, modulate intracellular pathways and inhibit activity of DNA topoisomerase and angiogenesis (Gezici et al., 2019).

Even Though the plant- derived natural products are much safer compared to several synthetic drugs available in the market, some of them, due to their underlying toxicity, are unsuitable for direct administration. Therefore, it is mandatory to determine safe doses of any plant-derived product by conducting appropriate toxicity studies as specified by the OECD guidelines to decide the safe dose for administration prior to pre-clinical studies. For the selection of pharmacological doses of solvent extracts, safety evaluation using acute and subacute toxicity models is mandatory.

The present chapter deals with detailed acute and sub-acute toxicity studies executed to evaluate the safety of DME and TME and further its antitumor potential was evaluated using DLA induced solid tumor model. All the studies were conducted according to the OECD guidelines and the acute toxicity study was conducted using both the sexes of Swiss albino mice for a duration of 14 days. The IC_{50} value was calculated for each extract, and based on this the doses for sub-acute toxicity study was decided. This study was conducted for a duration of 28 days. Further the antitumor study with both the extracts were conducted in male Swiss albino mice.

6.2 Methodology

6.2.1 Preparation of Extracts

The plant extracts were prepared according to the method mentioned in Chapter 3, section 3.2.1 1% propylene glycol was used to dissolve the extracts for oral

administration in mice. A vehicle control group was maintained throughout the experiment in which the mice were given 1% propylene glycol (PG) alone orally.

6.2.2 Animals

Male and Female Swiss albino mice (25-30 gm) procured from Small Animal Breeding Station (SABS), Kerala Veterinary and Animal Science University, Mannuthy, Thrissur was used as an animal model for *in vivo* studies. The animals were maintained in the animal house facility of Amala Cancer Research Centre (24 - 28°C, 60-70% humidity, 12 h dark/light cycle) following standard conditions, and were fed with standard rat feed from Sai Durga Feeds, Bangalore, India and water *ad libitum.* All the animal experiments were conducted after getting approval from the Institutional Animal Ethics Committee (IAEC) (Approval No: ACRC/IAEC/20(1)-P06) and also by strictly abiding the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by Ministry of Environment and Forest, Government of India.

6.2.3 Acute toxicity studies of the extracts

Female Swiss albino mice were used for acute toxicity studies. Three groups were maintained with six animals in each group. Group 1 animals were provided 1% propylene glycol (PG) orally, and groups 2 and 3 animals were orally administered with DME and TME dissolved in 1% PG, at a single dose of 2000 mg/kg b.wt and closely observed for 14 consecutive days for any toxic symptoms like weakness, diarrhoea, aggressiveness, eye or ear discharges, noisy breathing and mortality. The food and water consumption as well as body weight of the mice were monitored regularly. At the end of the study period mice were sacrificed and the visceral organs were observed for any toxic changes.

6.2.4 Sub-acute toxicity studies of the extracts

Sub-acute toxicity was conducted in both sexes of Swiss albino mice. The doses for subacute toxicity were fixed as 1/5th (high) and 1/10th (low) of 2000 mg/kg b.wt. of mice. The mice (n=6) were sought into twelve groups. Group 1: males untreated control, Group 2: males vehicle control (1% propylene glycol), Group 3: males received low dose DME (200 mg/kg b.wt.) and Group 4: males received high dose DME (400 mg/kg b.wt.). Group 5: males received low dose TME (200 mg/kg b.wt.)

and Group 6: males received high dose TME (400 mg/kg b.wt.). Group 7: females untreated control, Group 8: females served as vehicle control (1% propylene glycol), Group 9: females received low dose DME (200 mg/kg b.wt.) and Group 10: females received high dose DME (400 mg/kg b.wt. of mice). Group 11: females received low dose TME (200 mg/kg b.wt.) and Group 12: females received high dose TME (400 mg/kg b.wt.). The treatment started on day 0 and continued for 28 days at 24 h interval. During the experiment food and water consumption were monitored every third day along with weekly recordings of the body weight of each mouse. At the end of the experimental period animals were fasted overnight and sacrificed on the following day using a CO_2 chamber. Blood was collected in EDTA coated tubes by cardiac puncture for analyzing hematological parameters as mentioned in chapter 2, section 3.2.13. For serum parameters, a small quantity of blood was kept aside in a test tube and allowed to clot. It was then subjected to centrifugation at 3000 rpm for 10 minutes. The serum thus collected was used for liver function analysis. Several parameters like SGOT, SGPT, ALP, GLOB, ALB, TB and TP were analyzed. Checking parameters like urea and creatinine aided in kidney function analysis. The lipid profiling was done to determine the levels of CHO, TGL, HDL, LDL and VLDL. These parameters were analyzed using diagnostic kits (mentioned in chapter 2, sections 3.2.14, 3.2.15 and 3.2.16). The serum electrolytes like sodium, potassium, chloride and bicarbonate ions were also analyzed (Chapter 2, section 3.2.17). The vital organs like brain, liver, stomach, kidney, intestine, heart, lungs, spleen and ovary/testes were excised and a small portion of the organs were taken and fixed in 10% neutral buffered formalin. Tissue sections of 4µ thickness were taken using a microtome, and after processing was stained with hematoxylin and eosin for histopathological analysis (Chapter 2, section 3.2.18). The observations were made at 40× magnification using Leica 500M microscope and images were captured using the LAS EZ software.

6.2.5 Antitumor studies of the extracts

Male Swiss albino mice (6-8 weeks old) weighing 25-30g were grouped into seven groups (n=6). Group 1: Control (untreated tumor induced mice) Group 2: Vehicle control (1% propylene glycol), Group 3: Standard (cyclophosphamide drug 10 mg/kg b.wt), Group 4: DME low dose (200 mg/kg b.wt), Group 5: DME high

dose (400 mg/kg b.wt) Group 6 and 7: TME low dose (200 mg/kg b.wt), and high dose (400 mg/kg b.wt) respectively Approximately 1×10^6 of DLA cells in 100 μl of cell suspensions were intramuscularly injected into the right hind limb of all animals. The cell suspension was prepared by aspirating DLA cells from the peritoneal cavity of tumor bearing mice and washing with PBS. All the treatment (p.o) began 24 h post tumor cell inoculation and continued for 10 consecutive days. A Vernier caliper was used to measure the diameter of the tumor growth in two perpendicular planes. The readings were taken for a period of 30 days with an interval of three days between each reading. The tumor volume was calculated using the formula;

$V = 4/3\pi\ r_1^2\ r_2$: where r_1 is the minor radius and r_2 is the major radius.

6.2.6 Statistical Analysis

The values are expressed as Mean ± SD. The level of significance was calculated between vehicle control and extract treated groups by calculating the p-value using Student's t-test. The IC_{50} was calculated using SPSS software. A value $p\leq0.05$ was considered significant.

6.3 Results

6.3.1 Acute toxicity profiling

The single dose of 2000 mg/kg b.wt of DME and TME extracts administered orally showed no signs of toxicity in female Swiss albino mice selected for the study. The general appearance and behavioral patterns remained unchanged. No drug related alterations were seen in the treated groups when compared the untreated control groups (Table 6.1) during the 14 days observation period. Weekly monitoring of body weight showed no signs of adverse effects in treated groups when compared to the vehicle control group (Table 6.2) (Figure 6.1).

6.3.2 Sub-acute toxicity studies

The OECD guideline 407 (OECD 2008) was followed for conducting the sub-acute toxicity studies.

6.3.2.1 Behavioral patterns

During the experimental period of 28 days, no signs of toxicity or mortality was observed in any of the animals treated with DME or TME when compared to

untreated control groups. Adverse behavioral patterns like convulsions, vigorous rolling, tilting of head, salivation, tachycardia, abnormal breathing pattern, restlessness, loss of grip, strength and biting were not observed.

6.3.2.2 Body weight

No significant change in the body weight was observed for both the sexes upon oral administration of DME and TME (Figure 6.2, 6.3, 6.4 and 6.5). The body weight was calculated thrice (alternate days) every week, and then a weekly average was calculated as Mean ± SD.

6.3.2.3 Food consumption

No significant change was observed in DME treated animals when compared to vehicle control groups in both the sexes. Similar results were found in the case of TME administered animals (Figure 6.6 and 6.7). Small fluctuations were observed in both the cases, but gradually around the end of the study period food consumption was at par with the initial stage (pre-treatment) or slightly higher than that.

6.3.2.4 Water consumption

No significant change was observed in DME treated animals when compared to vehicle control groups in both the sexes. Similar results were found in the case of TME administered animals (Figure 6.8 and 6.9). Small fluctuations were observed in both the cases, but gradually around the end of the study period water consumption was at par with the initial stage (pre-treatment) or slightly higher than that.

6.3.2.5 Relative organ weight

Tables 6.3 and 6.4 show relative organ weight of mice of both sexes. No significant changes were observed in the weight of liver, kidney, spleen, stomach, intestine, heart, lungs, brain and ovary/testes with respect to body mass index of DME and TME administered animals when compared with the untreated group.

6.3.2.6 Hematological parameters

Depending on the toxicity of the extract, the interaction of its phytochemicals with the cellular components may show significant changes in the hematological parameters. In this study the extract did not have any toxic effects on RBC as well as on the hemoglobin levels in the blood. Tables 6.5 and 6.6 show several hematological parameters that were analyzed in both DME and TME treated groups

of animals. Though some of the parameters (RBC, WBC and PLT) showed significant variations, all the values were within the normal range as prescribed by the Guidelines of the Ministry of Ayush, Government of India.

6.3.2.7 Serum biochemical parameters

Electrolytes play a vital role in basic body functioning. Sodium, Potassium, chloride and bicarbonates are some of the important electrolytes that have several functions in the body. No significant changes were seen in the serum parameters of DME (Table 6.7) and TME (Table 6.8) treated animals when compared to the vehicle controls confirming the nontoxic nature of the extracts.

6.3.2.8 Analysis of liver function markers

Several immunological functions are regulated by the liver. Its reticuloendothelial capacity plays a vital role in phagocytosis and clearance of endotoxins from the blood (Hoekestra et al., 2013). Parameters like SGOT, SGPT, ALP, TB, TP, ALB and GLOB are liver function markers, which determine the health status of the liver. Variations in these parameters beyond the normal range are considered hepatotoxic. DME did not show any significant changes in these parameters in male mice, whereas in females some of them (SGOT, SGPT and ALP) showed significant variations as shown in Table 6.9. However, these variations were not beyond the normal ranges and hence the extract was considered non-toxic. The TME treatment showed no significant changes in both the sexes as shown in Table 6.10

6.3.2.9 Lipid profile

The lipid profiling revealed that both the extracts had the ability to reduce the levels of triglycerides significantly (Tables 6.11 and 6.12). Hypertriglyceridemia is a precursor of atherosclerotic diseases. The reduction in TGL and VLDL levels are seen in the treated animals, which is an indication of the hypocholesterolemic effects of these extracts. The HDL levels also showed a significant reduction, whereas no significant changes were seen in CHO and LDL levels.

6.3.2.10 Creatinine and urea levels

The blood urea and creatinine levels are indicators of renal functioning. Though in the high dose groups of DME and TME treated male mice, creatinine levels showed

significant changes, the values were within the normal range (Tables 6.13 and 6.14). Hence our study showed that both the extracts did not have any toxic renal effects.

6.3.2.11 Histopathological analysis

At the end of the 28-day long study after euthanisation the body wall was cut open and the main organs of animals were macroscopically examined and were then subjected to histopathological analysis. (Figures 6.10, 6.11, 6.12 and 6.13). No characteristic changes were observed in the organs of both the extract treated and vehicle control groups when compared to the normal ones. The liver tissue showed normal portal triads and hepatic veins. Kupffer cells appeared normal. Normal astrocytes and glial cells were seen in the sections of the brain. The kidney section showed normal glomeruli and the section of spleen also showed normal architecture. Normal mucosa, submucosa and muscle layers were observed in the sections of the stomach and the lungs showed normal bronchiole and alveoli. Sections of the intestine showed a normal serosal layer and also a normal mucosa and villi. Sections of heart showed normal endocardium, pericardium and myocardium. The ovary appeared normal with normal stroma and the testis also featured a normal architecture. No pathologically significant organ lesions were observed in the major organs of the mice treated with the plant extracts for a period of 28 days.

6.3.3 Tumor volume reduction

The tumor volume reduced significantly in both DME and TME treated group of animals, when compared to the vehicle control group. At the final day (30^{th}) day of the study, the tumor volume in the vehicle control group was 4.91 ± 0.7 cm^3 and that in DME low dose and high dose treated groups were 2.29 ± 1.26 cm^3 and 1.77 ± 0.74 cm^3 respectively. Similarly, in the TME low dose and high dose treated groups, the tumor volume was 2.30 ± 1.24 cm^3 and 1.33 ± 0.78 cm^3 respectively at the 30^{th} day. The Figures 6.14 and 6.15 show the tumor volume reduction over a period of 30 days for the treated, vehicle and untreated groups. The significant reduction ($p\leq0.05$) is shown as (*) in the graph.

6.3.4 Histopathological and TUNEL analysis of treated tumor tissue

Several changes were seen in tumor tissue of treated groups when compared to the vehicle control. Pleomorphic cells with hyperchromatic nuclei, mononucleated and multinucleated giant cells were observed in the sections of untreated and vehicle

controls while sections from DME (Figure 6.16) and TME (Figure 6.17) treated animals showed fewer numbers of mitotic cells and degenerating cells with extensive areas of necrosis. Clear evidence for apoptotic changes were seen in tissues extracted from treated (DME and TME 400 mg/kg b.wt) groups of animals when compared to untreated control groups (Figure 6.18 and 6.19). In the TUNEL mix the FITC-tagged dUTPs bind to free 3-OH' of nicked DNA strands. Hence green fluorescence (FITC), indicates the early to middle stage of apoptosis. The late apoptosis/necrosis is characterized by disrupted cell membrane integrity and hence red fluorescence (PI) is an indication of late apoptosis/necrosis as the PI easily traverses across the disrupted cell membrane.

6.4 Discussion

The acute and subacute toxicity studies was performed in Swiss albino mice by abiding to the OECD guidelines. In the acute toxicity studies (single oral dose of 2000 mg/kg b.wt) both DME and TME did not show any mortality in the animals. Several other signs of toxicity such as behavioral changes, weight loss, decreased appetite, hair loss etc. were also ruled out in these animals. Therefore, it was inferred that a dose of 2000 mg/kg b.wt of DME and TME extracts are tolerable to the mice, and the lethal dose to induce 50% mortality (LD_{50}) would be higher. The sub-acute toxicity study of DME and TME were conducted by taking $1/10^{th}$ and $1/5^{th}$ of 2000 mg/kg b.wt as low and high doses respectively. Several parameters like the changes in body weight, food and water intake were monitored and it appeared normal throughout the study. The relative organ weight of vital organs in the tested group of animals of both the sexes appeared normal and no significant changes were observed when compared with the vehicle controls. The hematological and biochemical parameters like levels of Hb, RBC, WBC, platelets, packed cell volume, MCV, MCH, MCHC, neutrophils, eosinophils, lymphocytes, serum electrolyte levels, lipid profile, renal and liver function tests etc. were also analyzed. In most of the cases no significant changes were observed in the test group of animals when compared to the vehicle controls. Though some changes were statistically significant in some of the parameters the observed average values were well within the permissible limit. The histopathological examination of the vital organs also revealed no signs of toxicity. Conclusively, post-acute and subacute toxicity studies it was clear that the

aforementioned doses are relatively safe for further *in vivo* studies and therefore used for the antitumor studies.

In an earlier study, salicin was isolated from methanol extracts of *P. gangeticus* leaves which is a strong COX-2 (Cyclooxygenase) inhibitor (Srivastava et al., 2013). They further found that the leaf extract reduced the tumor burden significantly and also increased the life span of EAC (Ehrlich Ascites Carcinoma) tumor bearing mice and this effect was attributed to salicin present in extract (Srivastava et al., 2015). Here we excluded the leaves of *P. gangeticus* to make the solvent extracts and evaluated it's potential to inhibit lymphoma. It was interesting to note that salicin was not detected in our extract by chromatographic techniques (Chapter 4, sections 4.3.4 and 4.3.5), indicating the possible role of other molecules in the tumor reduction potential of DME.

Since aerial parts of *T. involucrata* were known to be used traditionally in villages of Kerala for treating tumors, a study was conducted earlier in ascites tumor models using its ethyl acetate and hexane extracts and a notable decrease in tumor burden and considerable increase in lifespan was observed (Joshi et al., 2011). The ethyl acetate and chloroform extracts of leaves of *T. involucrata* showed potent antiproliferative effect when tested against K562 cell lines (Thomas et al., 2021). Further exploration of this activity was not conducted and hence here we used the ethanol extract of *T. involucrata* to conduct an elaborate antitumor study.

The histopathological analysis of the tumor tissue treated in DME and TME revealed areas of apoptosis and also necrosis, as there were extensive areas of degenerating cells, with fewer number of mitotic cells when compared to the control and untreated tissue sections. The TUNEL analysis of the tumor tissue extracted from mice, re confirmed that the tumor reduction was due to apoptosis. Both early and late stages of apoptosis were visible in the TUNEL analysis of the tumor tissues as green and red fluorescence respectively, highlighting the apoptosis inducing ability of the extracts (Figure 6.3 and 6.4).

The tumor reduction observed *in vivo* may be due to the interference of the extract components with the rapidly dividing tumor cells, disrupting its mitotic spindle and thereby leading to apoptotic cell death (Refer section 5.4). The evidence for apoptosis *in vitro* and *in vivo* strongly supports the potential of the extract as an

antitumor herbal source and proposes further investigation to locate the molecules that impart this potential. The multicomponent extracts may have multiple target sites and hence further studies on each compound may be required to understand their mechanism of action.

Table 6.1 General appearance and behavioral observations of animals

Observations	**Vehicle**	**DME**	**TME**
Food intake	Normal	Normal	Normal
Water intake	Normal	Normal	Normal
Body weight	No change	No change	No change
Drowsiness	Not present	Not present	Not present
Change in skin	No change	No change	No change
Diarrhea	Not present	Not present	Not present
Sedation	No effect	No effect	No effect
General physique	Normal	Normal	Normal
Alive	Alive	Alive	Alive

Table 6.2: Change in weight (g) of female Swiss albino mice following single oral dosage of DME and TME

BODY WEIGHT (g)			
Groups	Day 1	Day7	Day 14
Untreated control	28.1±2.4	28.36±2.35	28.93±1.98
Vehicle control (1% propylene glycol	27.46±3.16	28.16±2.75	28.2±3.24
DME (2g/kg b.wt)	27.43±1.94	27.73±2.36	27±1.4
TME (2g/kg b.wt)	27.3±2.07	26.53±5.0	27.73±3.10

Values are expressed as Mean ± SD

Table 6.3: Relative organ weights of liver, kidney, spleen, stomach, intestine, heart, lung, brain and ovary of female Swiss albino mice following administration of DME and TME for a period of 28 days.

Treatment group	Liver	Kidney	Spleen	Stomach	Intestine	Heart	Lung	Brain	Ovary
Untreated control	4.60±2.12	1.49±0.79	0.34±0.16	0.69±0.35	4.83±2.26	0.48±0.23	0.62±0.28	1.09±0.51	0.39±0.33
Vehicle control	4.55±0.38	1.05±0.06	0.29±0.10	0.55±0.02	5.50±0.32	0.39±0.03	0.62±0.03	0.93±0.06	0.10±0.04
DME (200mg/kg b.wt)	4.19±0.31	1.14±0.08	0.30±0.13	0.84±0.17	5.03±0.78	0.42±0.02	0.65±0.10	1.13±0.22	0.14±0.06
DME (400mg/kg b.wt)	5.21±0.59	1.57±0.11	0.34±0.08	1.05±0.36	6.26±1.55	0.58±0.16	0.76±0.16	1.37±0.30	0.11±0.06
TME (200mg/kg b.wt)	5.36±0.80	1.38±0.05	0.62±0.44	2.25±2.39	6.63±2.17	0.51±0.05	0.72±0.14	1.40±0.49	0.15±0.10
TME (200mg/kg b.wt)	4.9±0.09	1.33±0.20	0.39±0.04	0.95±0.26	5.89±1.20	0.46±0.10	0.54±0.29	1.23±0.16	0.14±0.07

Data are expressed as Mean ± SD

Table 6.4: **Relative organ weights of liver, kidney, spleen, stomach, intestine, heart, lung, brain and testis of male Swiss albino mice following administration of DME and TME for a period of 28 days.**

Treatment group	Liver	Kidney	Spleen	Stomach	Intestine	Heart	Lung	Brain	Testis
Untreated control	4.67±2.17	1.71±0.80	0.44±0.20	0.85±0.38	3.91±1.78	0.53±0.24	0.63±0.24	1.18±0.53	0.68±0.30
Vehicle control	4.20±0.67	1.44±0.16	0.45±0.17	0.85±0.11	4.41±1.28	0.48±0.06	0.64±0.10	1.35±0.24	0.63±0.10
DME (200mg/kg b.wt)	4.07±1.42	1.53±0.18	0.34±0.09	0.77±0.16	4.96±1.10	0.52±0.06	0.65±0.11	1.21±0.11	0.60±0.09
DME (400mg/kg b.wt)	5.02±0.60	1.47±0.14	0.58±0.29	1±0.16	4.26±0.33	0.48±0.12	0.70±0.24	1.32±0.17	0.66±0.11
TME (200mg/kg b.wt)	4.57±0.70	1.62±0.23	0.49±0.22	0.77±0.12	3.93±1.07	0.51±0.04	0.60±0.08	1.10±0.26	0.58±0.12
TME (200mg/kg b.wt)	4.28±0.61	1.47±0.16	0.44±0.29	0.81±0.05	4.03±1.17	0.49±0.05	0.56±0.08	1.09±0.14	0.61±0.10

Data are expressed as Mean ± SD

Table 6.5: Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of DME

Treatment	**Hb** (g/dl)	**RBC** ($\times 10^6$/mm^3)	**PLT** ($\times 10^5$/mm)	**PCV** (%)	**WBC** ($\times 10^3$/mm^3)	**MCV** (fl)	**MCH** (pg)	**MCHC** (g/dl)	**P** (%)	**L** (%)	**E** (%)
Female mice											
Untreated Control	13.03± 0.15	8.2± 0.2	10.13± 0.90	46.33± 0.57	12.59±0	88.33± 0.5	28.33± 0.57	30.66± 0.57	12±2	85±3	2.6± 0.57
Vehicle Control	13.27± 0.21	7.37± 0.06	9.33± 0.15	42.33± 1.53	9.5± 0.14	88.33± 0.58	27.67± 0.58	31.33± 1.53	11±1	86.3± 1.5	2±1
DME (200mg/kg b.wt)	13.60± 0.20	8.07± 0.21$^{\#}$	11.13± 0.80$^{\#}$	43.67± 0.58	6.9± 1.02$^{\#}$	89±1	28.33± 0.58	31±1	12±1	84.67± 1.5	2±1
DME (400 mg/kg b.wt)	13.43± 0.15	7.36± 0.15	10.8± 0.91*	42±1	7.5± 1.26*	86± 1.73	28.33± 0.57	31.66± 0.57	10.56± 2.57	85±1	3.33± 1.52
Male mice											
Untreated Control	14.93± 0.35	8.1± 0.5	11.26± 1.22	48±1	7.6± 0.09	88±1	27±1	31±1	21± 3.6	70.3± 0.57	4±1
Vehicle Control	14.4± 0.26	8.46± 0.05	10.43± 0.11	48.66± 3.75	9.48±0	88.3± 0.57	27.6± 0.57	29.3± 1.52	18±1	66± 13.5	4±1
DME (200 mg/kg b.wt)	14.6± 0.10	7.4± 0.10	10.50± 0.30	48±1	4.11± 1.0$^{\$}$	89±1	28.3± 0.57	27.6± 0.57	17.3± 2.08	66.6± 0.5	3.3± 1.52
DME (400 mg/kg b.wt)	13.1± 1.57	7.2±0.20$^{\$}$	10.80±0.11	47±1	4.91± 0.03$^{\$}$	87.6±0.57	27.6± 0.57	31±1	16.3± 2.51	69.3± 3.51	3±1

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$, ($^{\#}$) $p \leq 0.01$ ($^{\$}$) $p \leq 0.001$

Table 6.6: Hematological parameters of male and female Swiss albino mice following sub-acute oral administration of TME

Treatment	**Hb** (g/dl)	**RBC** ($\times 10^6$/mm^3)	**PLT** ($\times 10^5$/mm^3)	**PCV** (%)	**WBC** ($\times 10^3$/mm^3)	**MCV** (fl)	**MCH** (pg)	**MCHC** (g/dl)	**P** (%)	**L** (%)	**E** (%)
Female mice											
Untreated Control	13.03± 0.15	8.2±0.2	10.13± 0.90	46.33± 0.57	12.59±0	88.33± 0.57	28.33± 0.57	30.66± 0.57	12.0 ±2	85 ±3	2.66± 0.57
Vehicle Control	13.27± 0.21	7.37± 0.06	9.33± 0.15	42.33± 1.53	9.5± 0.14	88.33± 0.58	27.67± 0.57	31.33± 1.53	11.0±1	86.33± 1.53	2.0± 1.0
TME (200mg/kg b.wt)	12.83± 0.05	7.13± 1.25	7.86± 2.70	38.33± 6.50	11.77 ±0$^{\$}$	88.66± 0.57	27.66± 0.57	29.0± 1.73	11.26 ±0.6	86±6	2.66± 0.5
TME (400 mg/kg b.wt)	12.06± 0.51	7.53± 0. 45	9.03± 0.85	41.66± 0.57	9.05± 0.10 $^{\#}$	88.66± 0.57	28.66± 0.57	31.0±1	12.1± 0.36	89± 3.6	2.66± 0.57
Male mice											
Untreated Control	14.93± 0.35	8.1±0.5	11.26±1.22	48.0±1	7.6±0.09	88±1	27±1	31.0±1	21.0± 3.6	70.33± 0.57	4±1
Vehicle Control	14.4± 0.26	8.46± 0.05	10.43±0.11	48.66± 3.05	9.48±0	88.33± 0.57	27.66± 0.57	29.33± 1.52	18.0±1	66± 13.52	4±1
TME (200 mg/kg b.wt)	14.76± 0.05*	8.42±0.25	11.13± 0.15$^{\$}$	50.0±0	9.6±0.3	88.33± 0.57	28.66± 0.57	29.0±1	18.0±0	67±1	3±1
TME (400 mg/kg b.wt)	14.90± 0.7	8.2±0.10	13.36± 0.32$^{\$}$	49±1	10.45± 0.1$^{\$}$	88.66± 0.57	27.66± 0.57	30.66± 0.57	19.33± 1.52	70.33± 4.5	4.6±1.15

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$, ($^{\#}$) $p \leq 0.01$ ($^{\$}$) $p \leq 0.001$

Table 6.7: Serum electrolyte levels of male and female Swiss albino mice post oral administration of DME for a period of 28 days

Treatment group	**Na** (mmol/L)	**K** (mmol/L)	**Cl** (mmol/L)	**Bicarbonate** (mmol/L)
Female mice				
Untreated control	150.33±1.52	4.66±0.57	106.3±1.15	26±2
Vehicle control	149.3±0.58	4.67± 0.58	105.33±0.58	25.33±0.58
DME (200 mg/kg b.wt)	149±1	4.67±0.57	105±1	25±3
DME (400 mg/kg b.wt)	150±2	5±0	105±1	24.66±0.57
Male mice				
Untreated control	147.33±0.57	5±0	104.6±2.3	27±2.6
Vehicle control	146.33±1.52	4.3±0.57	104.5±0.5	24±1
DME (200 mg/kg b.wt)	147.46±0.5	5±0	103.66±1.52	25.33±0.57
DME (400 mg/kg b.wt)	146±1	4.66±0.57	103.66±2.08	24.66±1.15

Data are expressed as Mean ± SD

Table 6.8: Serum electrolyte levels of male and female Swiss albino mice post oral administration of TME for a period of 28 days

Treatment group	**Na** (mmol/L)	**K** (mmol/L)	**Cl** (mmol/L)	**Bicarbonate** (mmol/L)
Female mice				
Untreated control	150.3±1.52	4.66±0.57	106.3±1.17	26±2
Vehicle control	149.33±0.58	4.67±0 .58	105.3±0.58	25.3±0.58
TME (200 mg/kg b.wt)	150.33±1.52	4±0	105±3	26±2
TME (400 mg/kg b.wt)	150±1	4.66±0.57	104±1	25.33±0.57
Male mice				
Untreated control	147.33±0.57	5±0	104.6±2.3	27±2.6
Vehicle control	146.33±1.52	4.33±0.57	104.5±0.5	24±=1
TME (200 mg/kg b.wt)	146±1	4.33±0.57	105.5±0.5*	25.66±2.08
TME (400 mg/kg b.wt)	147.33±1.52	4.66±0.57	105.33±1.52	26.33±1.52*

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$

Table 6.9: Liver function markers following oral administration of DME for 28 days

Treatment group	SGOT (IU/L)	SGPT (IU/L)	ALP (U/L)	TB	TP (g/dl)	Albumin	Globulin
Female mice							
Untreated control	288.66±8.14	83.0±4.50	185.66±4.50	0.3±0.1	6.6±0.1	3.3±0.1	3.3±0.1
Vehicle control	296.67±4.16	74±4.58	174.67±0.58	0.24±0.05	6.60±0.10	3.50±0.10	3.03±0.06
DME (200 mg/kg b.wt)	289.0±1.0*	66.33±1.15*	154±1.0$^{\$}$	0.25±0.05	6.53±0.25	3.46±0.05	3.0±0.26
DME (400 mg/kg b.wt)	297.66±2.51	75±5.00	130.33±8.08$^{\$}$	0.25±0.05	6.5±0.2	3.36±0.05	3.4±0.36
Male mice							
Untreated control	251.67±30.13	51.33±4.93	105.5±2.51	0.2±0.1	6.6±1.04	3.46±0.47	3.7±0.6
Vehicle control	205.33±39.52	62.66±5.13	120±44.19	0.3±0	7.83±0.05	3.5±0	4.3±0.1
DME (200 mg/kg b.wt)	252.67.0±52.08	77.66±17.03	119.33±32.08	0.3±0.1	7.3±0.41	3.4±0.2	3.8±0.7
DME (400 mg/kg b.wt)	230.67±19	75.66±15.55	131.67±25.10	0.3±0.1	7.6±0.2	3.36±0.2	3.6±0.57

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$ and ($^{\$}$) $p \leq 0.001$

Table 6.10: Liver function markers following oral administration of TME for 28 days

Treatment group	SGOT (IU/L)	SGPT (IU/L)	ALP (U/L)	TB	TP (g/dl)	Albumin	Globulin
Female mice							
Untreated control	288.66±8.14	83.0±4.50	185.66±4.50	0.3±0.1	6.6±0.1	3.3±0.1	3.3±0.1
Vehicle control	296.67±4.16	74±4.58	174.67±0.58	0.24±0.05	6.60±0.10	3.50±0.10	3.03±0.06
TME (200 mg/kg b.wt)	292.0±3.46	70.0±5	169.3±0.07	0.3±0.1	6.23±0.35	3.2±0.1	2.6±0.4
TME (400 mg/kg b.wt)	291.3±3.51	72.33±2.51	171.00±3.6	0.3±0.1	6.46±0.15	3.4±0.1	3.0±0
Male mice							
Untreated control	251.67±30.13	51.33±4.93	105.5±2.51	0.2±0.1	6.6±1.04	3.46±0.47	3.7±0.6
Vehicle control	205.33±39.52	62.66±5.13	120±44.19	0.3±0	7.83±0.05	3.5±0	4.3±0.1
TME (200 mg/kg b.wt)	194.0±50.46	77.66±17.03	119.33±32.08	0.3±0.1	7.43±0.4	3.4±0.1	4.06±0.9
TME (400 mg/kg b.wt)	198.33±29.02	64±11.0	118.0±9.55	0.3±0.1	7.3±0.43	3.36±0.15	3.8±1.03

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$

Table 6.11: Blood lipid profile of Swiss albino mice of both the sexes after oral administration of DME for a period of 28 days

Treatment group	**CHO** (mg/dl)	**TDL** (mg/dl)	**HDL** (mg/dl)	**LDL** (mg/dl)	**VLDL** (mg/dl)
Female mice					
Untreated control	80.66±0.57	223±15.5	42±1	20±1.0	23±4.35
Vehicle control	80.0±1.0	231.0±27	41±1	19.33±0.58	28.0±2.0
DME (200 mg/kg b.wt)	81±1	103.6±6.35$^{\$}$	35.33±0.57$^{\$}$	18.3±0.57	17.3±0.57$^{\$}$
DME (400 mg/kg b.wt)	81.3±1.15	114±0.57$^{\$}$	35.3±0.57$^{\$}$	19.66±0.57	18±1$^{\$}$
Male mice					
Untreated control	77.66±0.57	174.67±26.0	40.33±1.52	19.66±0.57	18±3.6
Vehicle control	78.66±0.57	156.33±5.13	43±1	19.33±0.57	11±1
DME (200 mg/kg b.wt)	79.3±1.15	141.3±3.51$^{\#}$	38±1$^{\$}$	18.66±0.57	11.6±0.57
DME (400 mg/kg b.wt)	79.6±1.52	128±14.10$^{\#}$	39.3±1.52$^{\#}$	20.6±1.15	11.6±1.52

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$, ($^{\#}$) $p \leq 0.01$ ($^{\$}$) $p \leq 0.001$

Table 6.12: Blood lipid profile of Swiss albino mice of both the sexes after oral administration of TME for a period of 28 days

Treatment group	**CHO** (mg/dl)	**TDL** (mg/dl)	**HDL** (mg/dl)	**LDL** (mg/dl)	**VLDL** (mg/dl)
Female mice					
Untreated control	80.66±0.57	223±15.5	42±1	20±1.0	23±4.35
Vehicle control	80.0±1.0	231.0±27	41±1	19.33±0.58	28.0±2.0
TME (200 mg/kg b.wt)	80.33±0.57	135±17$^{\$}$	36.6±0.5$^{\$}$	19.66±0.57	19±3$^{\#}$
TME (400 mg/kg b.wt)	79.66±0.57	127.33±2.51$^{\$}$	36.33±1.52$^{\#}$	20.33±57	19.33±1.52$^{\$}$
Male mice					
Untreated control	77.66±0.57	174.67±26.0	40.33±1.52	19.66±0.57	18±3.6
Vehicle control	78.66±0.57	156.33±5.13	43±1	19.33±0.57	11±1
TME (200 mg/kg b.wt)	78.33±0.57	132.67±4.04$^{\$}$	40.33±0.57$^{\#}$	18.66±0.57	12±3
TME (400 mg/kg b.wt)	79.33±0.57	105.33±1.52$^{\$}$	41±1*	19.33±0.57	13±1.7

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$, ($^{\#}$) $p \leq 0.01$ ($^{\$}$) $p \leq 0.001$

Table 6.13: Creatinine and urea levels in the blood following oral administration of DME

Treatment group	Urea (mg/dl)	Creatinine (mg/dl)
Female mice		
Untreated control	46.66±9.01	0.55±0.02
Vehicle control	41±1.0	0.53±0.04
DME (200 mg/kg b.wt)	42.0±1.0	0.53±0.45
DME (400 mg/kg b.wt)	42.6±3.05	0.51±0.06
Male mice		
Untreated control	47.33±6.50	0.55±0.01
Vehicle control	42.33±11.50	0.64±0.08
DME (200 mg/kg b.wt)	42.33±5.50	0.54±0.03
DME (400 mg/kg b.wt)	37.66±0.57	0.53±0.01*

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$

Table 6.14: Creatinine and urea levels in the blood following oral administration of TME

Treatment group	Urea (mg/dl)	Creatinine (mg/dl)
Female mice		
Untreated control	46.66±9.01	0.55±0.02
Vehicle control	41±1.0	0.53±0.04
TME (200 mg/kg b.wt)	41.0±1.41	0.53±0.04
TME (400 mg/kg b.wt)	41.0±1.41	0.5±0.007
Male mice		
Untreated control	47.33±6.50	0.55±0.01
Vehicle control	42.33±11.50	0.64±0.08
TME (200 mg/kg b.wt)	43.33±2.51	0.52±0*
TME (400 mg/kg b.wt)	50.0±1	0.54±0

Data are expressed as Mean ± SD; Significant in relation to vehicle control at (*) $p \leq 0.05$

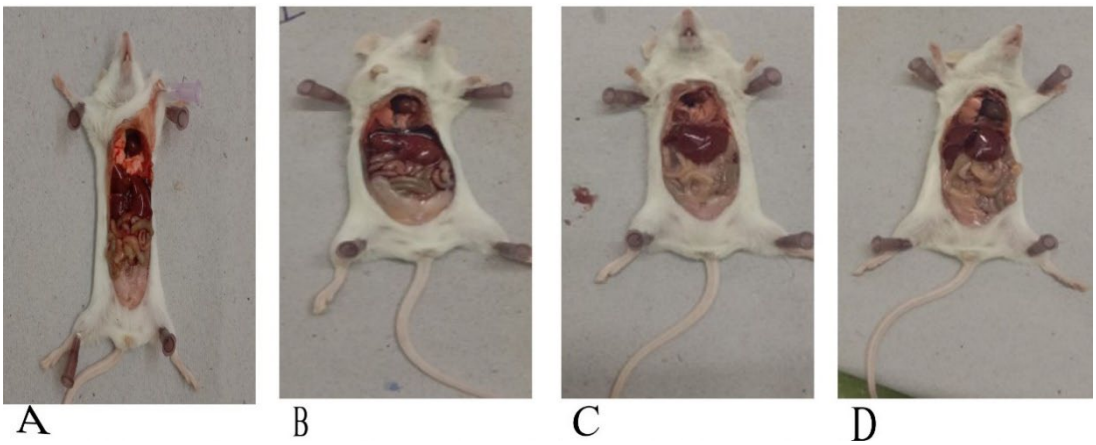

Figure 6.1: Visceral organs of Swiss albino (female) mice post 14-day acute toxicity study. A; B; C and D; shows mice of untreated control, vehicle control and treated groups of DME (2000 mg/kg b.wt) and TME (2000 mg/kg b.wt) respectively.

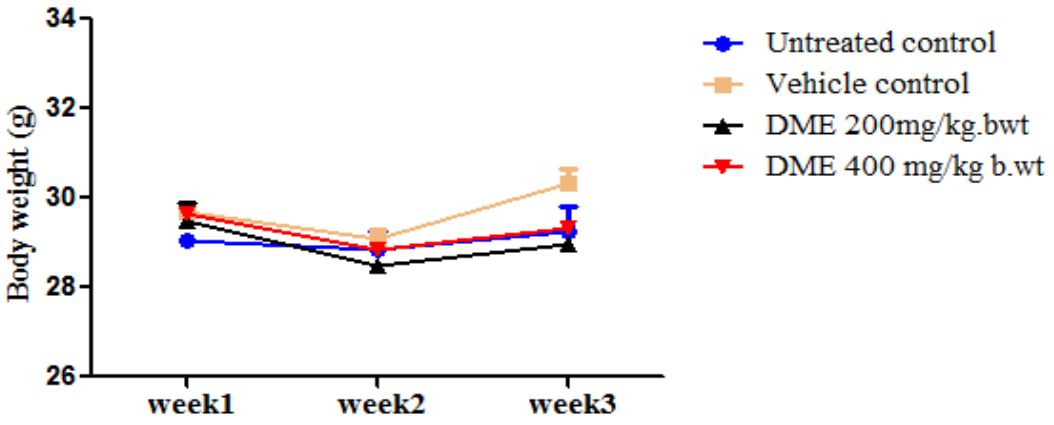

Figure 6.2: Body weight of female Swiss albino mice on the subacute oral administration of DME (200 and 400 mg/kg b.wt). Extracts were administered orally on a daily basis for 28 days. The body weight was monitored at an interval of 3 days every week and the weekly average was calculated as Mean ± SD.

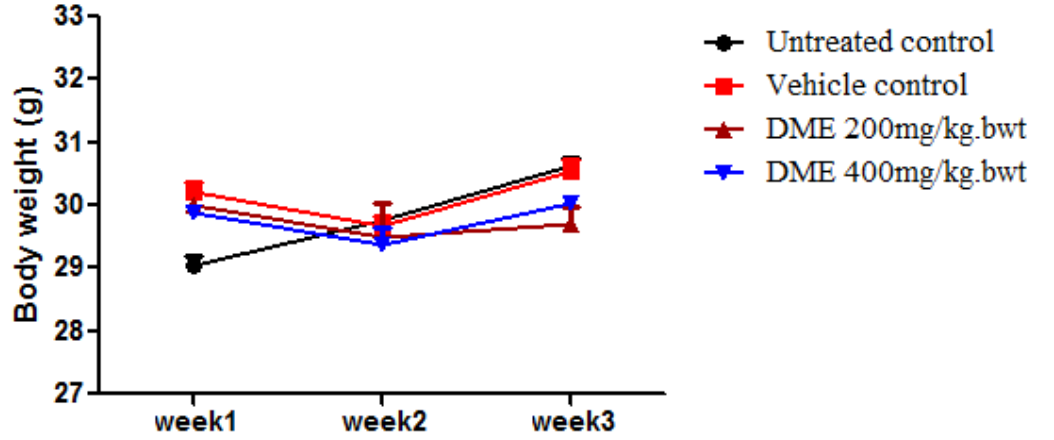

Figure 6.3: Body weight of male Swiss albino mice on the subacute oral administration of DME (200 and 400 mg/kg b.wt). Extract was administered orally on a daily basis for 28 days. The body weight was monitored at an interval of 3 days every week and the weekly average was calculated as Mean ± SD.

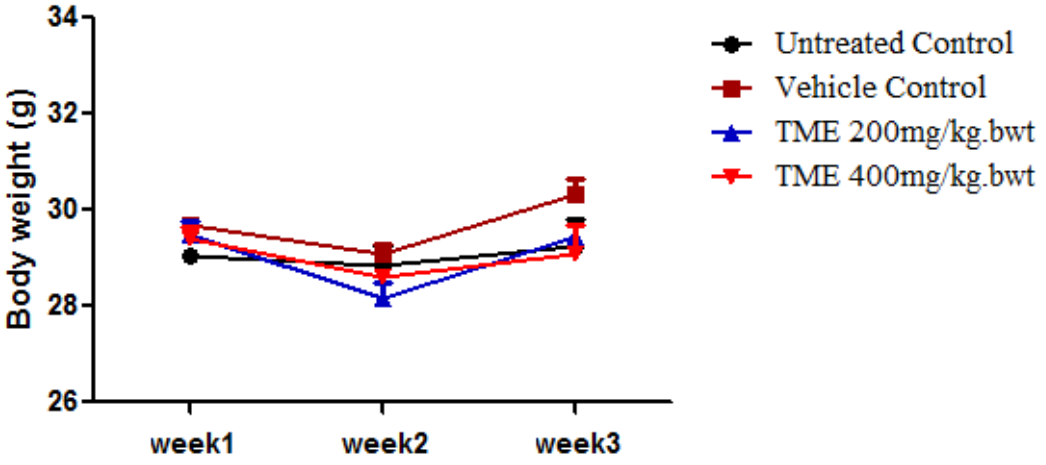

Figure 6.4: Body weight of female Swiss albino mice on the subacute oral administration of 200 and 400 mg/kg b.wt TME extract. Extract was administered orally on a daily basis for 28 days. The body weight was calculated with an interval of 3 days every week and the weekly average was calculated as Mean ± SD.

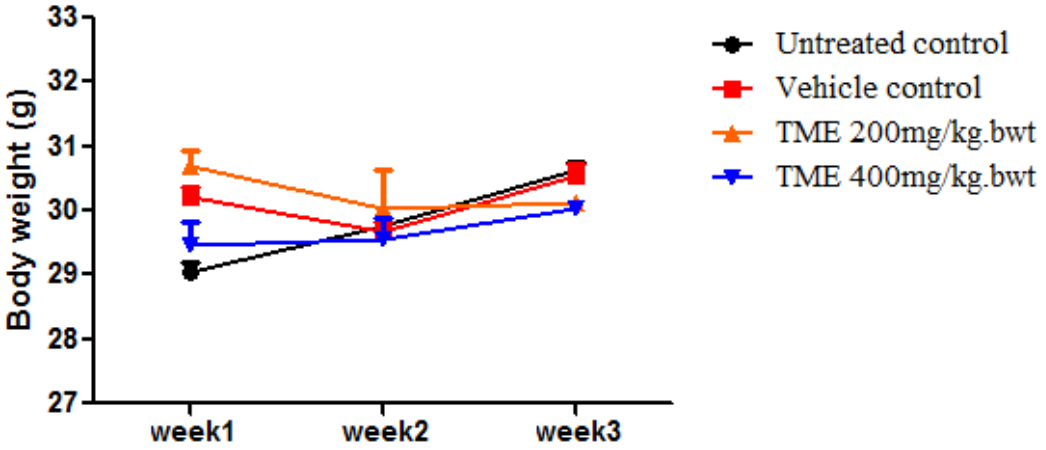

Figure 6.5: Body weight of male Swiss albino mice on the subacute oral administration of TME (200 and 400 mg/kg b.wt). Extract was administered orally on a daily basis for 28 days. The body weight was monitored at an interval of 3 days every week and the weekly average was calculated as Mean ± SD.

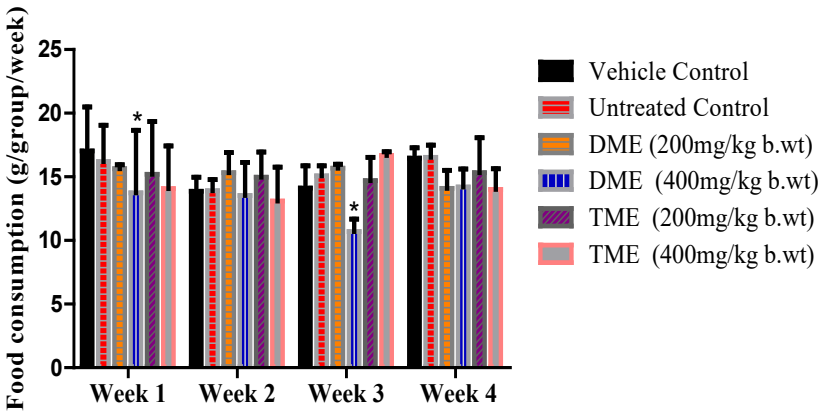

Figure 6.6: The pattern of food consumption of female Swiss albino mice following sub-acute administration of the DME and TME. Both the extracts were administered in low (200 mg/kg b.wt) or high (400 mg/kg b.wt) doses orally for 28 days consecutively. The food consumption /gram/per group was recorded each week for every four weeks during the course of the experiment. Each base represents Mean and standard deviation and the p value ≤ 0.05 (*) was considered significant

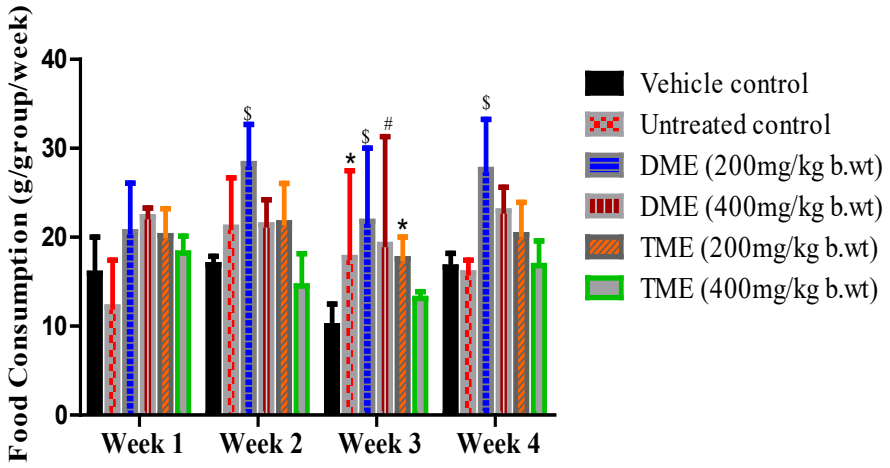

Figure 6.7: The pattern of food consumption of male Swiss albino mice following sub-acute administration of the DME and TME. Both the extracts were administered in low (200 mg/kg b.wt) or high (400 mg/kg b.wt) doses orally for 28 days consecutively. The food consumption /gram/per group was recorded each week for every four weeks during the course of the experiment. Each base represents Mean and standard deviation and the p value ≤ 0.05 (*) p ≤ 0.01 (#) and p ≤ 0.001($), were considered significant

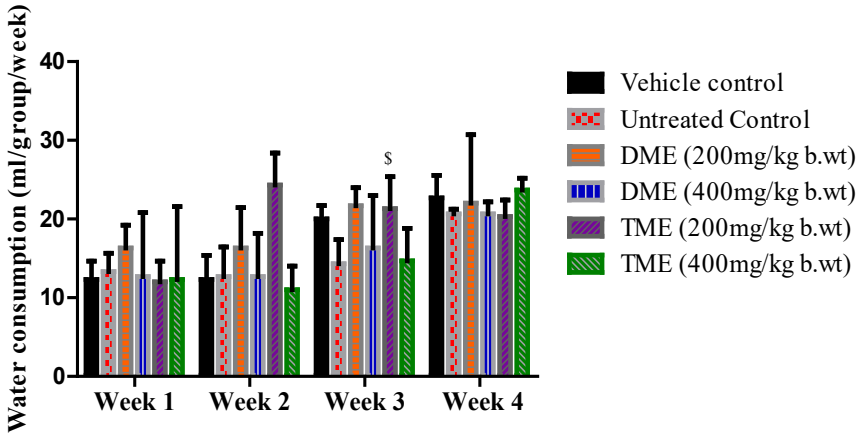

Figure 6.8: The pattern of water consumption of female Swiss albino mice following sub-acute administration of the DME and TME. Both the extracts were administered in low (200 mg/kg b.wt) or high (400 mg/kg b.wt) doses orally for 28 days consecutively. The water consumption /ml/ per group was recorded each week for every four weeks during the course of the experiment. Each base represents Mean and standard deviation and the p value ≤ 0.001 ($) was considered significant.

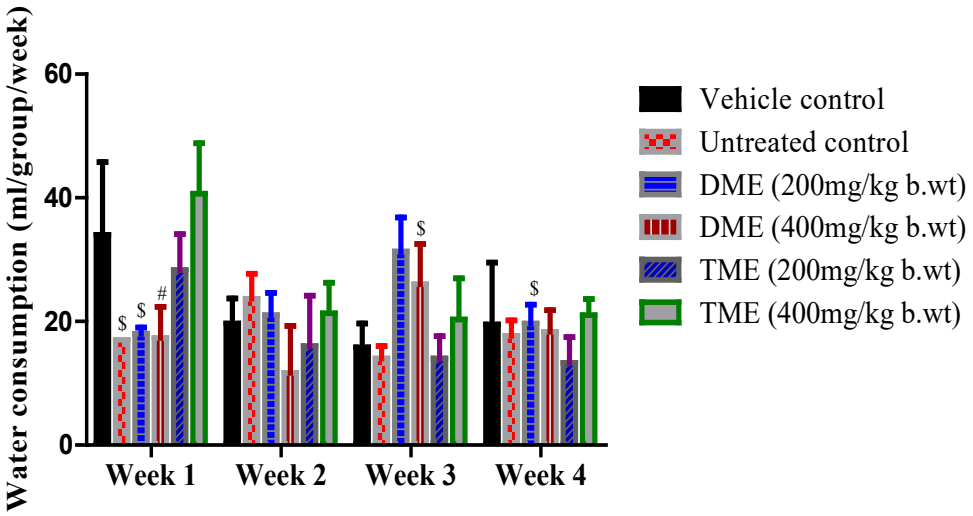

Figure 6.9: The pattern of water consumption of male Swiss albino mice following sub-acute administration of the DME and TME. Both the extracts were administered in low (200 mg/kg b.wt) or high (400 mg/kg b.wt) doses orally for 28 days consecutively. The water consumption /ml/ per group was recorded each week for every four weeks during the course of the experiment. Each base represents Mean and standard deviation and the p value ≤ 0.01 (#), p value ≤ 0.001 ($) was considered significant.

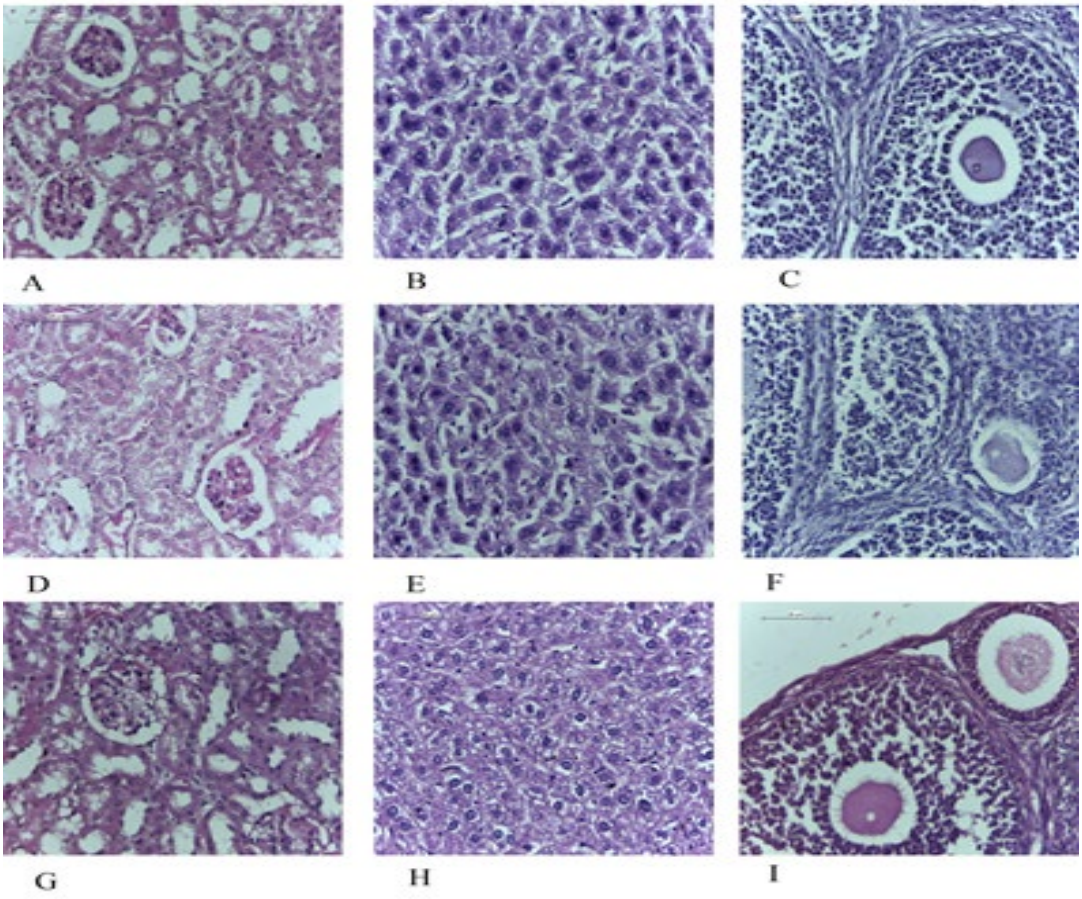

Figure 6.10: Histopathological analysis (40x magnification) of vital organs of female Swiss albino mice after subacute toxicity study. Representative microscopic images: A, D and G shows sections of kidney from untreated control, vehicle control and DME (400 mg/kg b.wt) group respectively, B, E and H shows sections of liver from untreated control, vehicle control and DME (400 mg/kg b. wt) group respectively and C, F and I show sections of ovary from untreated control, vehicle control and DME (400 mg/kg b.wt) group respectively.

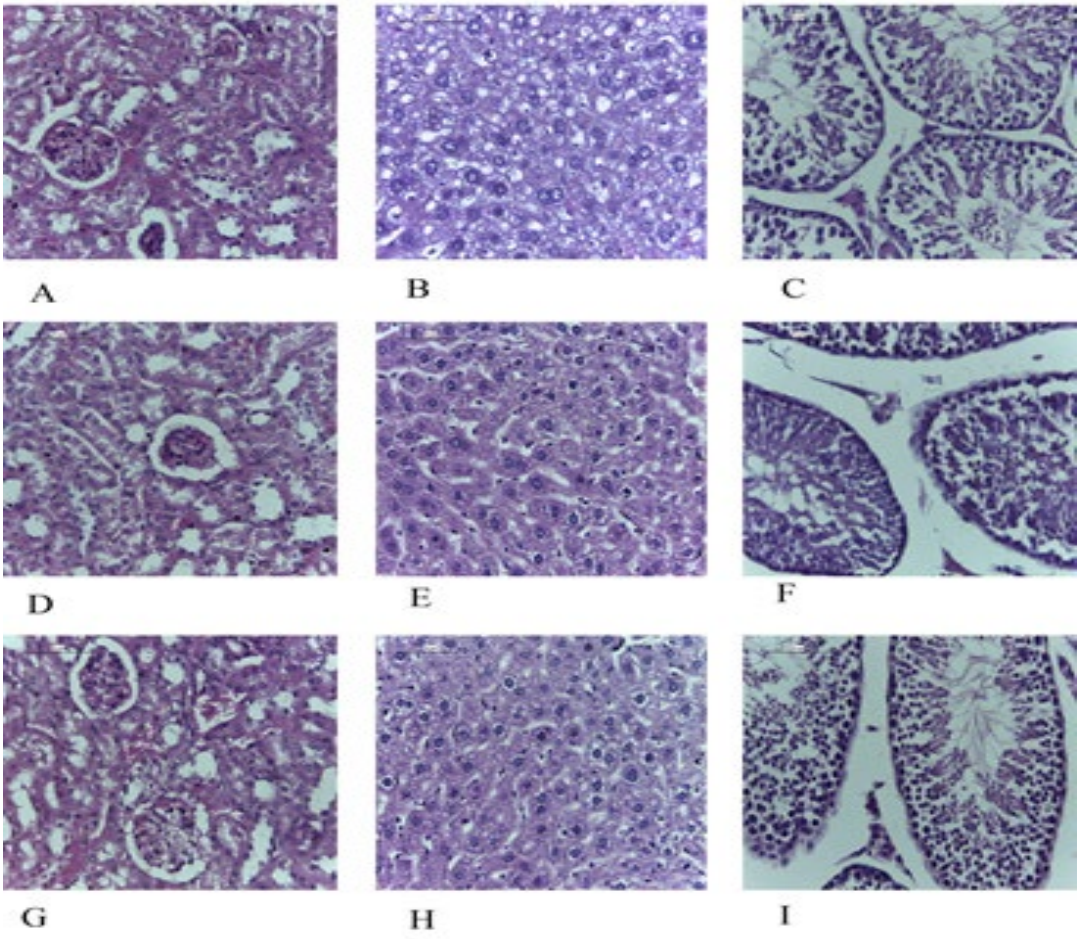

Figure 6.11: Histopathological analysis (40x magnification) of vital organs of male Swiss albino mice from subacute toxicity study. Representative microscopic images: A, D and G; sections of kidney from untreated control, vehicle control and DME (400 mg/kg b.wt) group respectively, B, E and H; sections of liver from untreated control, vehicle control and DME (400mg/kg b.wt) group respectively and C, F and I; sections of testis from untreated control, vehicle control and DME (400mg/kg b.wt) group respectively.

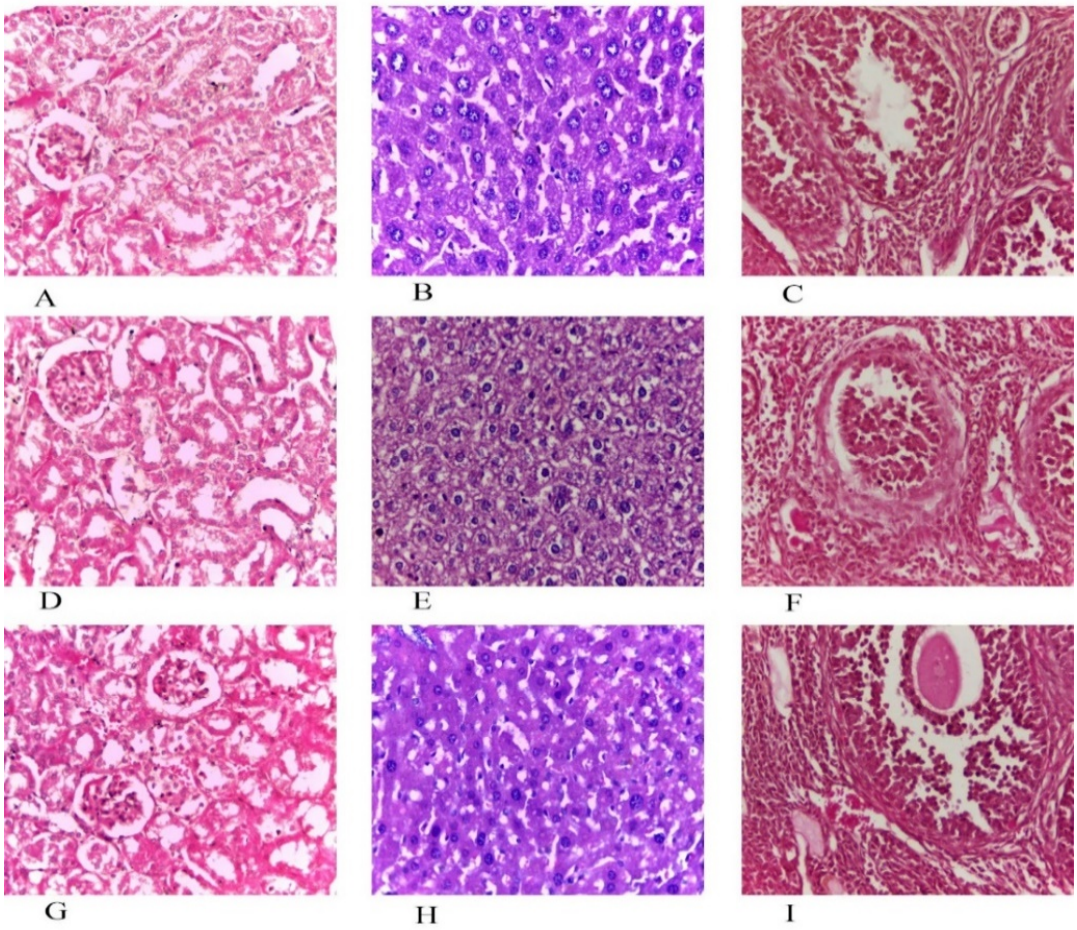

Figure 6.12: Histopathological analysis (40x magnification) of vital organs of female Swiss albino mice from subacute toxicity study. Representative microscopic images A, D and G; sections of kidney from untreated control, vehicle control and TME (400 mg/kg b.wt) group respectively, B, E and H; sections of liver from untreated control, vehicle control and TME (400 mg/kg b.wt) group respectively and C, F and I; sections of ovary from untreated control, vehicle control and TME (400 mg/kg b.wt) group respectively.

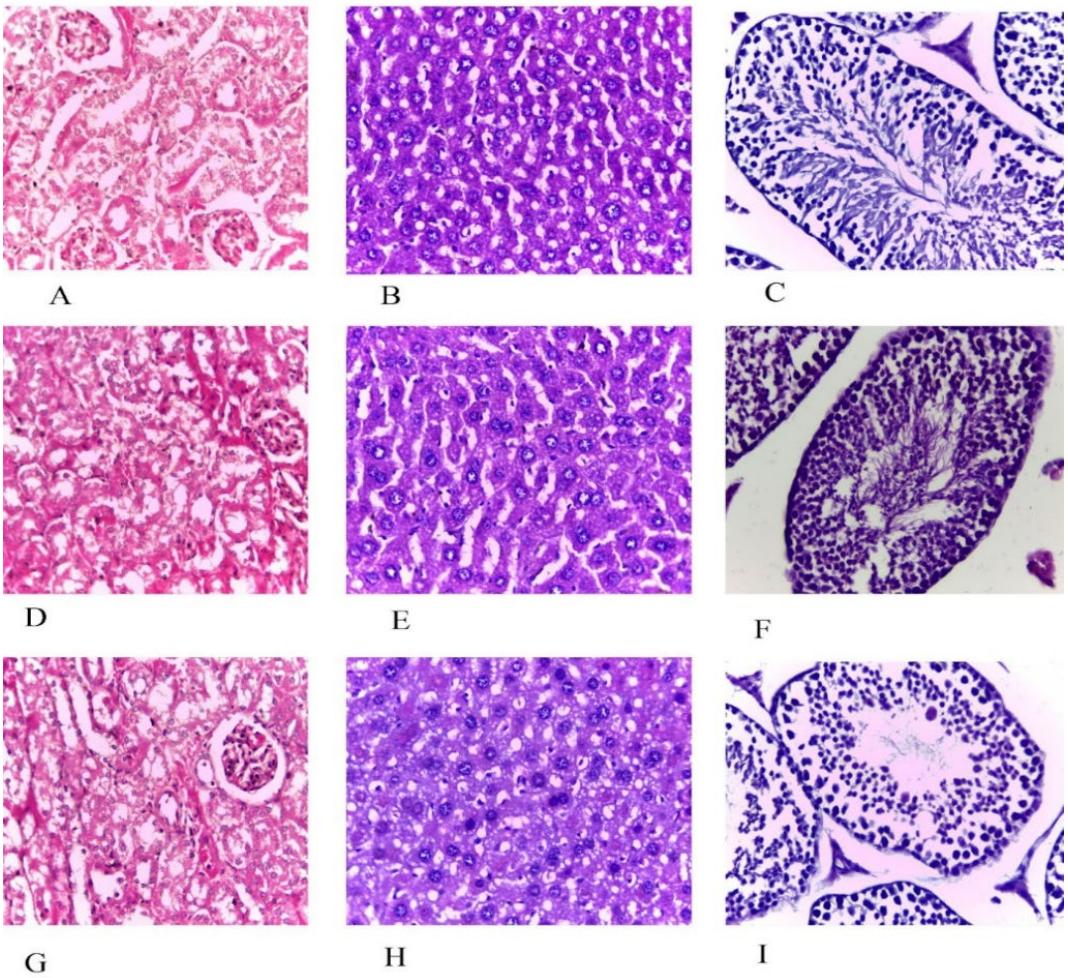

Figure 6.13: Histopathological analysis (40x magnification) of vital organs of male Swiss albino mice from subacute toxicity study. Representative microscopic images: A, D and G; sections of kidney from untreated control, vehicle control and TME (400 mg/kg b.wt) group respectively, B, E and H; sections of liver from untreated control, vehicle control and TME (40 0mg/kg b.wt) group respectively and C, F and I; sections of testis from untreated control, vehicle control and TME (400 mg/kg b.wt) group respectively.

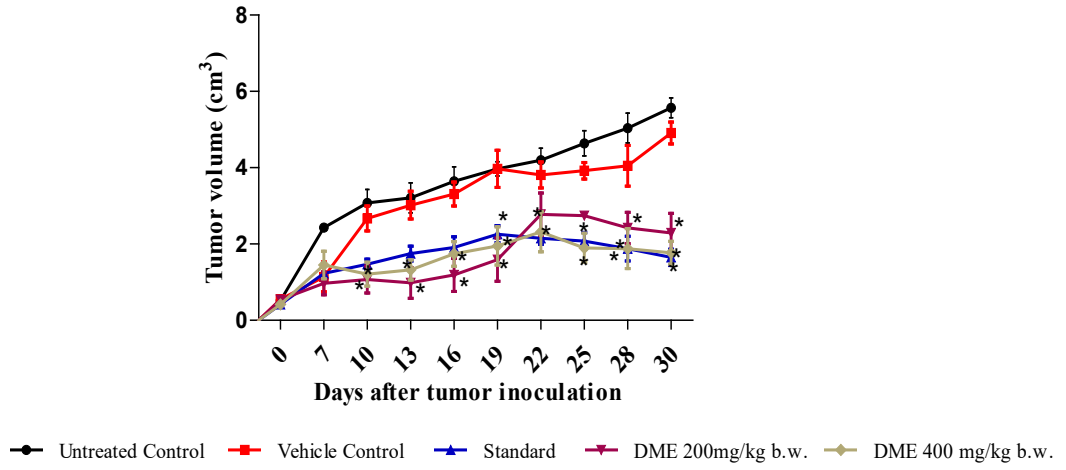

Figure 6.14: Graph showing the tumor volume of DLA solid tumors in controls as well as DME treated Swiss albino mice over a period of 30 days. Untreated control, vehicle control and standard (Cyclophosphamide) groups are included and the comparisons were made between vehicle control with the extract treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$.

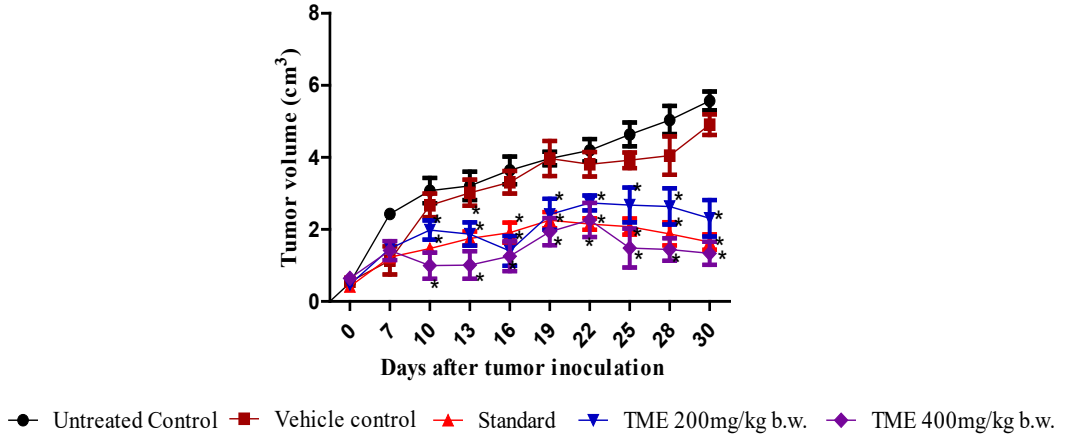

Figure 6.15: Graph showing the tumor volume of DLA solid tumors in controls as well as TME treated Swiss albino mice over a period of 30 days. Untreated control, vehicle control and standard (Cyclophosphamide) groups are included. Comparisons were made between vehicle control with the extract treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$.

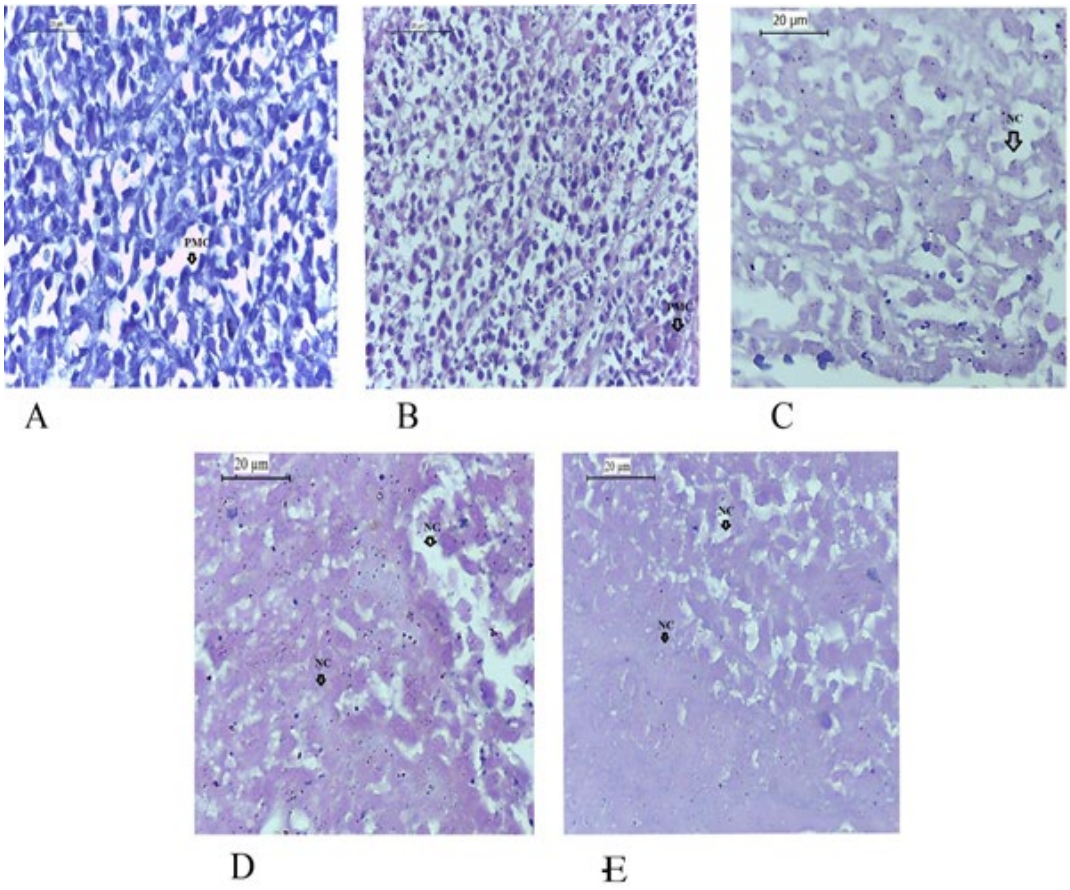

Figure 6.16: Figure showing the histopathological analysis (H&E) of untreated, vehicle or DME treated DLA solid tumor. Tumor extracted from A; untreated control, B; vehicle control, C; cyclophosphamide treated, D; DME 200 mg/kg b.wt treated and E; DME 400 mg/kg b.wt treated. The pleomorphic cells (PMC) and giant cells (GC) with hyperchromatic nuclei in the tumor tissues are seen in both untreated and vehicle treated animals as shown. In the tissue sections of extract treated animals as well as in the cyclophosphamide treated animals, the areas of necrosis are seen which is marked as NC

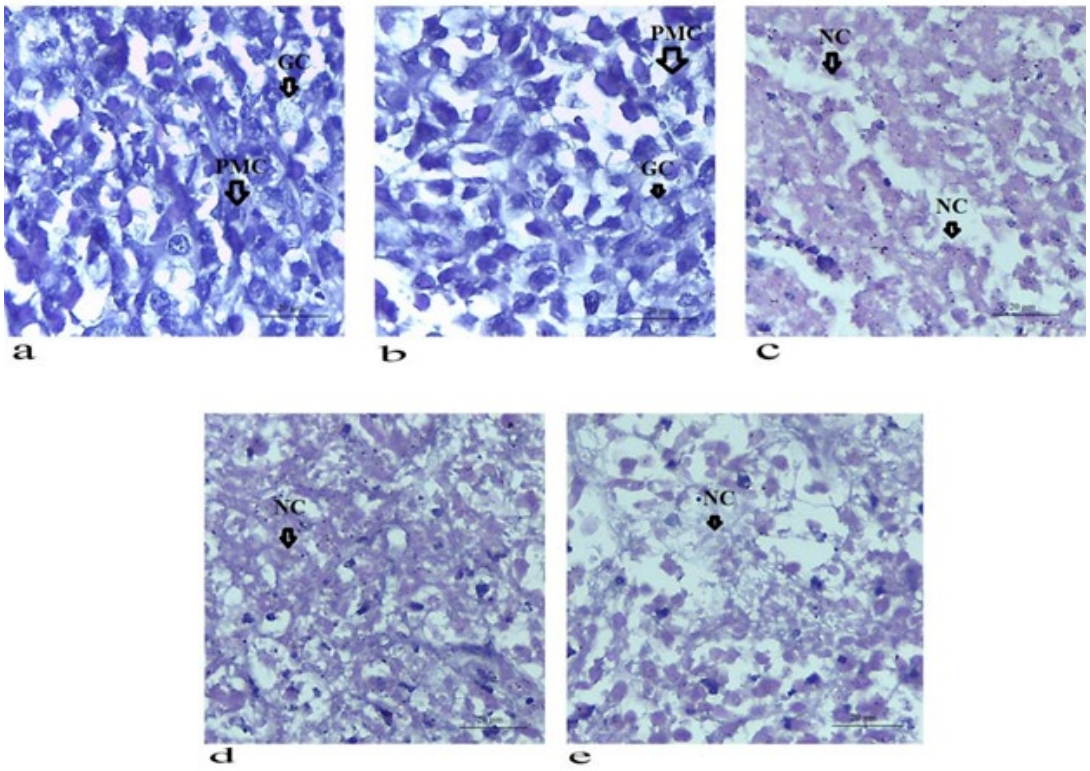

Figure 6.17: Figure showing the histopathological analysis (H&E) of, untreated vehicle or TME treated DLA solid tumor. Tumor extracted from a; untreated control, b; vehicle control, c; cyclophosphamide treated, d; TME 200 mg/kg b.wt treated and e; TME 400 mg/kg b.wt treated. The untreated and vehicle-treated animals showed pleomorphic cells (PMC) and giant cells (GC) with hyperchromatic nuclei in the tumor tissues. The areas of necrosis marked as NC are shown in the extract treated as well as in the cyclophosphamide treated animal tissues.

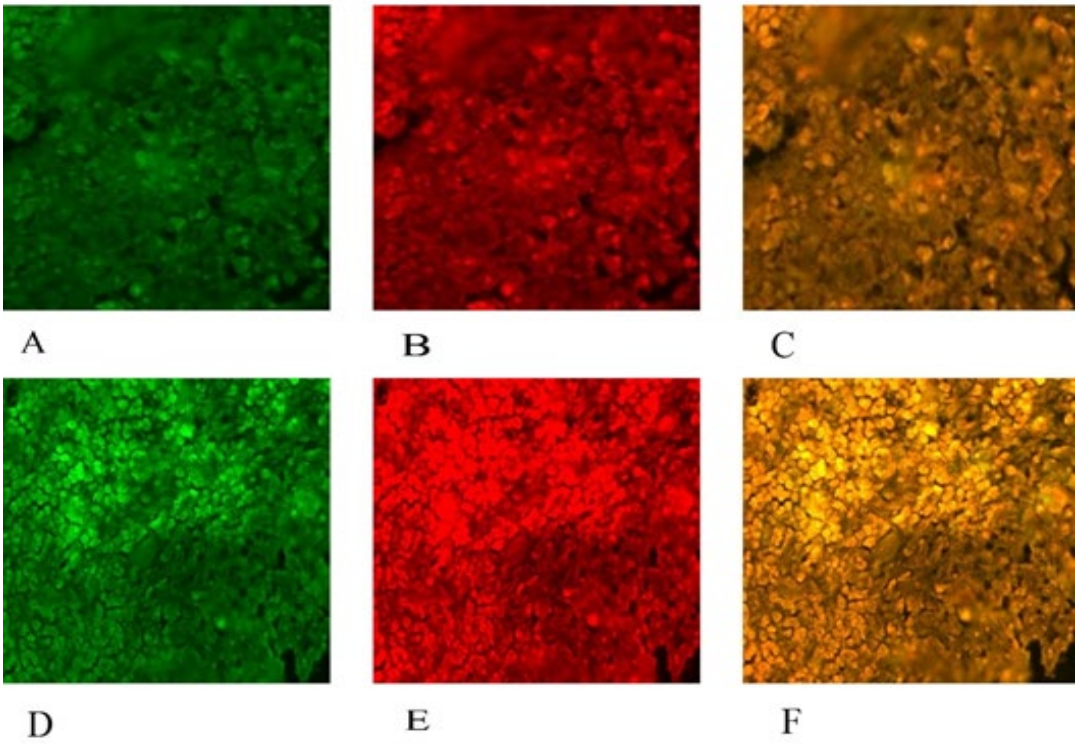

Figure 6.18: TUNEL assay analysis of DLA solid tumor tissue. A, B and C; untreated control and D, E and F treated with DME 400 mg/kg b.wt. No fluorescence was observed in the untreated group, which shows that apoptosis has not taken place in them. Green fluorescence indicates early to middle stage of apoptosis with dUTP-FITC-positive cells. The terminal stage of apoptotic/necrotic cells with PI-positive fragmented nuclei is indicated by the red fluorescence. Figure C and F are merged. In the treated groups, both green and red fluorescence was observed and it is an indication of early and terminal stages of apoptosis respectively.

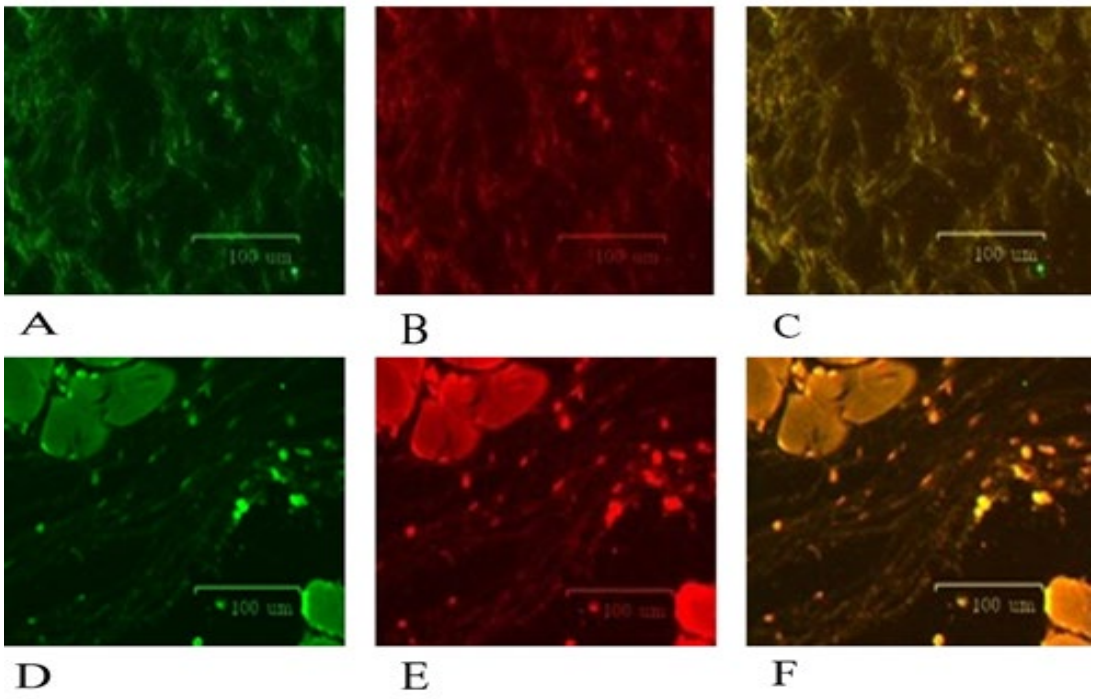

Figure 6.19: TUNEL analysis of DLA solid tumor tissue: A, B and C; untreated control and D, E and F treated with TME 400 mg/kg b.wt. In the untreated control group, no fluorescence was observed which shows that apoptosis has not taken place in them. Green fluorescence indicates early to middle stage of apoptosis with dUTP-FITC-positive cells. Red fluorescence indicates terminal stage apoptotic/necrotic cells with PI-positive fragmented nuclei. Figure C and F are merged. In the treated groups, both green and red fluorescence was observed and it is an indication of early and late stages of apoptosis respectively.

CHAPTER: 7

NEMATICIDAL AND TREMATOCIDAL POTENTIALS OF *P. GANGETICUS* AND *T. INVOLUCRATA*

7.1 Introduction

7.1.1 Gastrointestinal nematodes

The grazing ruminants are usually affected by one or more nematodes belonging to the order Strongylida and cause parasitic gastroenteritis. One of the most pathogenic strongylid nematodes seen in small ruminants is *Haemonchus contortus* (Barber's pole worm). It leads to haemonchosis by rapid larval contamination (Roeber et al., 2013). Gastrointestinal nematode parasites take a toll on economic status, as they affect the farm animals, thereby causing heavy infections and mortality among them. It is believed that all the economically vital gastrointestinal parasites of ruminants belong to the Order: Strongylida (Balic et al., 2000). Adult worms feed on the blood capillaries in the abomasal mucosa (Schwarz et al., 2013). Several advantages of *H. contortus* like short pre-patent period (8-21 days), convenient maintenance of individual host animals for several months after infecting it with L 3 larvae, high fecundity of the parasite makes them one of the best models for anthelmintic studies (Gilleard, 2013).

7.1.2 Trematodes

Trematode diseases and infections are a widespread and hidden affliction on humanity. The term 'digenetic trematodes' is given to trematodes infecting man and other mammalian hosts. They belong to the Phylum Platyhelminthes, Class Trematoda and Subclass Diginea. These endoparasites with an indirect life cycle usually show an oral and ventral sucker as the holdfast organs. The other subclass of trematode, Monogenea, usually includes ectoparasites of fishes and infrequently endoparasites of frogs and turtle. The third sub class Aspigogastrea, includes endoparasites of fishes, turtles and molluscs (Kumar, 1998). Infections to humans are due to the consumption of improperly cooked food like fresh water fish and aquatic invertebrates containing cysts (metacercariae) of various digenetic trematodes (Fried & Abruzzi, 2010).

Paramphistomosis is caused by rumen flukes that belong to the super family Paramphistomoidea. Chronic ulcerative ruminitis and anaemia are the major

illnesses caused by adult paramphistomes. Several other problems like acute gastroenteritis, dehydration, improper digestion and mortality are observed in young animals attacked by large number of these immature parasites. *Fischoederius cobboldi* belonging to the Family Gastrothylacidae is a digenetic trematode parasite. It takes a toll on the economic wellbeing by decreasing nutritional content and milk production in affected cattle (Anuracpreeda et al., 2016).

7.1.3 Synthetic anthelmintic drugs

Several synthetic anthelmintic drugs are available in the market and have a particular physiological role in controlling the helminths. Some of them are discussed below.

- **Piperazine** - Used since the 1950s is an effective anthelmintic drug and causes reversible paralysis of the body wall muscle, acting as a weak GABA mimetic (Yadav & Singh, 2011).

Benzimidazole - Discovered in 1961, is known to selectively interact with β tubulin, thereby interfering with the cytoskeleton. However, the drug resistant isolates have shown specific alleles for β tubulin (Driscoll et al., 1989).

Levamisole - They cause prolonged activation of the excitatory (nAch) receptors on the body wall muscles, leading to muscle cell depolarization and paralysis. These studies were conducted in *Ascaris suum* and *Oesophagostomum dentanum* using voltage clamp, current clamp and patch clamp techniques (Martin et al., 2005). Levamisole is however known to have nicotine like toxic effects on the central nervous system, causing seizures, insomnia, vomiting, neurological symptoms, hyper activity, thrombopenia and leucopenia (Palcoux et al., 1994)

Paraherquamide - It is known to induce flaccid paralysis in parasitic nematodes. They also block the action of other nicotinic agonists, though not equipotential (Robertson et al., 2002).

Ivermectin - The persistent paralysis of the body walls and pharyngeal muscles is brought about by this drug, also having action on a wide range of ligand- gated ion

channels (Holden-Dye & Walker, 2007). Resistance to ivermectin has been studied in *C. elegans* and is associated with various genetic mutations.

Emodepside- It is found to have anthelmintic effects and is known to mimic the action of inhibitory neuropeptides by triggering inhibitory neuropeptides at nerve terminals (Willson et al., 2003).

7.1.4 Natural source of anthelmintic

The resistance developed by helminths is giving insurmountable problems to the farmers and hence they find it a difficult task to combat these infections. According to Fissiha & Kinde (2021), "Anthelmintic resisitance is a heritable loss of sensitivity of an anthelmintic in a parasite population that was in the past susceptible to the same anthelmintic". The development of herbal remedies in a cost effective manner is important and also a necessity. The bark extracts of *Annona senegalensis* showed promising results for trichostrongyloidea helminth control and can be of immense use to farmers (Alawa et al., 2003). The aqueous and methanolic extracts of roots of *Ficus benghalensis* showed strong anthelmintic activity and were also equipotent to standard anthelmintic drugs (Aswar et al., 2008). The discovery of multi ingredient synergistic formulations has gained renewed interest of researchers rather than focusing on isolation of a single molecule alone (Patwardhan, 2005). The development of such herbal medicines to combat helminthic infections in man and animals is thus the need of the hour.

7.2 METHODOLOGY

7.2.1 Plant collection, extraction and *in vitro* anthelmintic study

The detailed procedure of plant collection and extraction have been given in chapter 3 sections 3.1.6 and 3.2.1. The ethyl acetate, ethanol and aqueous extracts of both the plant were used for the nematicidal and trematocidal studies. The *in vitro* studies to evaluate the anthelmintic potentials of the extract were conducted against the nematode *Haemonchus contortus* and trematode fluke *Fischoederius cobboldi*. Egg hatch and larval paralysis assays were conducted in *H. contortus* and adulticidal assay was conducted in *F. cobboldi*

7.2.2 Egg Hatch Assay (EHA)

The faecal pellets were collected from the rectum of infected donor goats and were subjected to centrifugal floatation. Eggs were isolated from it (Getachew et al., 2012) and the egg-hatch assay was conducted in acquiescence with the World Association for The Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al., 1992). The assay procedure is described in chapter 3 section 3.2.7.2

7.2.3 Larval Paralysis Assay (LPA)

Larval paralysis assay was conducted according to the methodology previously described by Varady & Corba (1999), with minor modifications. Freshly collected goat faecal samples were incubated at room temperature in the dark for 8 to 10 days to get L3 larva of *H. contortus*. The assay procedure is described in chapter 3 section 3.2.7.3

7.2.4 *In vitro* adulticidal assay

From the rumens of infected cattle that was killed for consumption in a local slaughterhouse, adult *F. cobboldi* were collected and transferred to the laboratory and then washed in phosphate buffer saline. The adulticidal assay using the plant extracts were conducted on these worms according to the method proposed by Anuracpreeda et al. (2016) with minor modifications. DMSO (1%) was used for dissolving the test extracts prepared at various concentrations. 25 adult flukes each for test as well as vehicle and positive controls were randomly selected for the study. The detailed procedure of this assay is provided in chapter 3 section 3.2.8.

7.2.5 Staining methods

Two staining methods were used for examination of the adult flukes' histology. Hematoxylin and eosin staining method was used to examine the changes in tissue sections (Anuracpreeda et al., 2015), and Masson's trichrome staining was used to study the changes in connective tissue of the fluke (chapter 3 section 3.2.8.2).

7.2.6 Stereomicroscopic and Light microscopic analysis

Stereo microscopic analysis was done to observe morphological changes to flukes post-treatment. Images were captured by a Leica DMC4500 digital camera attached

to a Leica M205 C stereomicroscope. The Method proposed by Anuracpreeda et al. (2015) was used for preparing the flukes for histological examination. Hematoxylin and Eosin staining was done with the tissue sections, to observe the abnormalities and images were captured using a light microscope (Leica TCM 400). In order to observe the changes in the connective tissue, masson's trichrome staining was also used.

7.2.7 Metaphase indexing in *Allium cepa*

For screening the effect of antimitotic drugs, *Allium cepa* root tip meristematic cells are used extensively. This assay was used to analyze the effect of DME and TME on spindle fiber assembly. The experiment was conducted according to Chakraborty et al., 2021 with minor modifications. The detailed procedure of this assay is given in chapter 3 section 3.2.9.

7.2.8 *In silico* studies

The colchicine binding site (CBS) of alpha-beta tubulin was selected as the target site for docking studies. The reference drugs, albendazole, mebendazole and colchicine were used for molecular docking with this target site of *C. elegans* (PDB ID:6E88) (a closely related nematode to *H. contortus*) and a modelled alpha-beta tubulin of *H. contortus*. Molecular docking studies of certain compounds in DME and TME were performed against the above targets. Using the 3D structure of ligands retrieved from PubChem. Molecular docking studies were carried out using the AutodockVina 1.5.7. The detailed methodology is given in chapter 3 section 3.2.21.

7.2.9 Statistical Analysis

The level of significance ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$) was calculated using Student's t test by comparing each treatment group with control. The survival data was studied using Kaplan-Meier survival analysis in Graph Pad Prism software version 5. To compare the survival curves, the Log-rank (Mantel-Cox) test was used. The SPSS software version 24 was used for probit analysis to calculate the effective concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) inhibition to nematode egg hatch, L3 larval paralysis and fluke mortality.

7.3 RESULTS

7.3.1 Egg Hatch Assay (EHA)

A dose dependent inhibition on egg hatch was observed for all the three extracts of *P. gangeticus* and *T. involucrata* as shown in Table 7.1 and Table 7.2. The lowest activity was observed for the aqueous extracts (DWE and TWE) of both the plants. At the highest tested concentration (10 mg/ml) DEE and DME showed strong egg hatch inhibition of 87.16±1.32% and 90.83±0.98% respectively. Similarly, TEE and TME also showed strong egg hatch inhibition of 86.5±2.34% and 90.5±1.64% respectively. These results were comparable to the egg hatch inhibition potential of the albendazole, the standard drug used against *H. contortus* (Table 7.3). DME showed (Table 7.4) an EC_{50} value of 0.661 mg/ml which was the lowest when compared to DEE (0.771 mg/ml) and DWE (>10 mg/ml). Similarly, with the lowest EC_{50} value of 1.664 mg/ml, TME seemed to be the most potent when compared to TEE (1.827 mg/ml) and TWE (8.760 mg/ml).

7.3.2 Larval Paralysis Assay (LPA)

The paralysis of L3 larvae after treatment with extracts of *P. gangeticus* and *T. involucrata* are shown in Table 7.5 and Table 7.6 respectively. At the highest tested concentration (10 mg/ml) DME and TME induced 65.5±2.25% and 64.5±0.54% of larval paralysis. Furthermore, at the same concentration, DEE and TEE showed 57.5±2.5% and 60±4.42% paralysis respectively. Among others, the aqueous extracts DWE (39.33±0.81%) and TWE (48.83±0.4%) showed the least paralysis of L3 larva at 10 mg/ml. The larval paralysis caused by albendazole are shown in Table 7.7. The lowest EC_{50} values were observed for DME (2.931 mg/ml) and TME (3.876 mg/ml) as consistent with the results of EHA. DEE and TEE had EC_{50} values of 4.370 and 5.166 mg/ml respectively. The EC_{50} values of DWE (>10 mg/ml) and TWE (6.412 mg/ml) were the highest among others (Table 7.8).

7.3.3 *In vitro* Adulticidal assay

A concentration and time dependent decrease in survival of *F. cobboldi* were observed as shown in the Kaplan-Meier survival graphs (Figures 7.1 and 7.2). 54.16% of the flukes could survive the treatment of DEE (25 mg/ml) during the first hour of treatment. In comparison, 58.33% of the DME treated flukes could survive

the initial hour of treatment at this concentration. Interestingly, 100% mortality was observed in DME and DEE post 1.5 h treatment period at a concentration of 12.5 mg/ml. DWE was the least effective, as 50% of flukes were alive even after 2 h of treatment. Albendazole (reference drug) treated flukes showed a gradual decrease in survival with time, and flukes in vehicle control (1% DMSO) were vigorously motile and active throughout the experiment. The significance of the survival curves was compared using the Log-Rank (Mantel-Cox) test. The lowest EC_{50} value was shown by DEE (3.477 mg/ml), followed by DME (4.470 mg/ml) at 2 h treatment period. However, the EC_{50} value for the DWE extract was very high which indicated its low efficacy (Table 7.9). TEE and TME (25 mg/ml), caused 100% mortality after 1.5 h of treatment. However, in TWE, 85.5% of the flukes were motile even after 2 h of treatment. The significance of the survival curves was compared using the Log-Rank (Mantel-Cox) test. The EC_{50} calculation at 2 h treatment period, revealed the high adulticidal activity for TEE (EC_{50} 3.845 mg/ml) followed by TME (EC_{50} 4.280 mg/ml). The high EC_{50} value for TWE indicated that it had very low effect on the survival of these adult flukes (Table 7.9).

7.3.4 Morphological evaluation using stereo zoom microscope

Morphological examinations and image capturing was done using Leica M205 C stereo zoom microscope. In the vehicle treated animals, no changes were observed in the tegumental surface as well as the in the anterior and posterior suckers after 2 h of treatment. Whereas in the albendazole (1 mg/ml), DEE and TEE (25 mg/ml) treated flukes, significant changes were observed after 2 h. The anterior and posterior suckers showed degeneration and tegumental erosion was noticeable. Swollen teguments with blebs and grooves were also observed in tissue of these flukes (Figure 7.3).

7.3.5 Histological studies

The tissue sections of flukes treated in 25 mg/ml of ethyl acetate extracts of both the plants as well as the vehicle controls were studied after staining with Haemotoxylin and Eosin. The surface syncytium was intact, and the tegument had proper corrugations, with undisturbed muscle layers beneath them in the vehicle control (1 % DMSO) treated parasites even after 2 h of treatment. The flukes treated in albendazole (1 mg/ml) and DEE extracts exhibited significant changes to the surface

syncytium, underlying muscle layers and tegument. The basement membrane was detached and the teguments lost its corrugations, giving a smooth appearance. The syncytium had few degenerations and the muscle layers underneath were degraded. The flukes treated with TEE showed visible changes in the tegumental layers with loss of corrugations, when compared to the vehicle control group, which had an intact tegument with corrugations. The muscle layers in the extract treated groups were found to be disrupted, in comparison to intact muscle layers observed in the tissue section of the vehicle treated animals (Figure 7.4).

Masson's trichrome staining particularly stains the collagen fibers and muscles therefore revealed the changes in the connective tissue as shown in Figure 7.5. The bluish-purple colour shows the presence of collagen and intact muscle fibers in the vehicle control section, whereas the loss of this bluish colour in the treated tissues shows the complete disruption of the collagen and muscle fibers

7.3.6 Metaphase indexing in *Allium cepa*

As expected, the colchicine treated root tips showed a higher metaphase index of 32.3 % when compared to vehicle treated samples (22.9%). DME (36.1%) and TME (35.5%) treated root tips also showed higher metaphase indices compared to the controls (Table 7.10 and Figure 7.6).

7.3.7 Molecular Docking Studies

C*aenorhabditis elegans* is a model organism for several fields of research and is a closely related nematode to *H. contortus* and the third larval stage (L_3) is known to be similar in both (Mohandas et al., 2016). Considering the similarity, we used *C. elegans* tubulin (6E88) for homology modelling the structure of *H. contortus* tubulin. The pair wise sequence alignment using protein sequences showed that the alpha and beta tubulin of *C. elegans* and *H. contortus* has a similarity of 87.11% and 93.6% respectively.

Several compounds showed higher binding affinity towards CBS of *C. elegans* and *H. contortus* as compared to the reference drugs albendazole and mebendazole (Table 7.11). The binding affinity of colchicine aginst the CBS of *C. elegans* (-8.6 Kcal/mol) and *H. contortus* (-8.2 Kcal/mol) was even lower than mebendazole. Lupeol, schaftoside, quercetin and thymol are also known to bind with CBS with

high affinity. All these molecules except thymol showed strong binding affinity towards the CBS of *C. elegans* and *H. contortus* that ranged between -8.7/-8.5 – -9.8/-10.9 Kcal/mol. However, thymol had a low binding affinity of -6.0 Kcal/mol and -6.1 Kcal/mol towards the CBS of *C. elegans* and *H. contortus* tubulin respectively.

Apart from the aforementioned molecules several others detected via LC-MS in DME and TME had strong binding affinity towards CBS of *C. elegans* and *H. contortus* tubulin when compared to colchicine (Figure 7.9-7.14). In the case of *C. elegans*, colchicine showed a binding affinity of -8.6 Kcal/mol. However, molecules such as isoquercetin (-9.9 Kcal/mol), corilagin (-9.5 Kcal/mol), loquatoside (-9.5 Kcal/mol), echinacin (-10.9 Kcal/mol), agathisflavone (-9.7 Kcal/mol), apigetrin (-10.7 Kcal/mol), cynaroside (-10.8 Kcal/mol), schaftoside (-9.8 Kcal/mol) and leufolin A (-10 Kcal/mol) showed a much higher binding affinity towrads *C. elegans* tubulin. Colchicine also showed a lower binding affinity (-8.2 Kcal/mol) towards CBS of *H. contortus* tubulin when compared to isoquercetin (-9.6 Kcal/mol), corilagin (-9.7 Kcal/mol), loquatoside (-9.8 Kcal/mol), echinacin (-10.0 Kcal/mol), agathisflavone (-10.2 Kcal/mol), apigetrin (-10.6 Kcal/mol), cynaroside (-10.7 Kcal/mol), schaftoside (-10.9 Kcal/mol), leufolin A (-11.2 Kcal/mol).

7.4 Discussion

Alternative therapeutic methods to combat anthelmintic infections is the need of the hour, as the helminth parasites are increasingly becoming resistant to the synthetic drugs available in the market. Novel herbal molecules are thus in search as they are environment friendly and also relatively less resistant. Several studies have already reported the anthelmintic potential of various plant extracts against the eggs and larvae of *H. contortus* (Hounzangbe et al., 2005; Marie et al., 2009; Kamaraj & Rahman 2011; Kumarasingha et al., 2016; Davuluri et al., 2020). In our study, a dose dependent nematicidal effects were shown by ethyl acetate and ethanol extracts of both plants. However, the ethanol extracts of the plants viz; DME and TME had slightly greater inhibitory effects on the egg hatch of *H. contortus* when compared to the other extracts. The larval paralysis assay also revealed the ethanol extracts to be more effective, as DME and TME showed the lowest EC_{50} values. The ethyl acetate extracts, though not as potent as the ethanol extracts, had commendable anthelmintic

potentials against the egg and larval stages of *H. contortus*. However, the aqueous extracts of both plants viz; DWE and TWE showed very low anthelmintic potentials.

Most of the compounds identified by GC-MS in DME and TME were primary metabolites that are commonly found in most plant species (Refer section 5.4), and hence were not considered further for *in silico* studies. However, lupeol detected in DME have been previously reported to have interactions with mebendazole binding site of β-tubulin proteins of *Bos taurus* and proposes it to be the probable mechanism behind its anthelmintic potentials (Ahmad & Malik, 2022). Similarly, molecular docking studies using thymol showed higher inhibitory effects on β-tubulin isotype-1 protein of *H. contortus* when compared to albendazole. It was also observed that, both thymol and albendazole shared the same 'ligand binding site' though the orientation of interacting amino acid residues were different (Velan & Hoda, 2021). Schaftoside, a flavonoid molecule identified in DME also have shown strong binding affinity towards β tubulin protein (5IJ0 based modell) of pine wood nematode *Bursaphelenchus xylophilus* (Shanmugam et al., 2018). In all the three studies mentioned above, molecular docking studies of the ligand molecules were done against β tubulin protein of various organisms. In the case of lupeol, docking was carried out with *Bos taurus* β-tubulin (PDB-ID 7ODN) which has only 85.6% sequence similarity with *H. contortus* β-tubulin. In the case of thymol and albendazole, they used a modelled 3D structure of *H. contortus* β-tubulin (PDB-ID 1OJ0) but the binding affinity was much less compared to other active molecules and reference compound albendazole. This could also be attributed to the 'ligand binding site' selected in this study rather than using CBS. In the *in silico* analysis involving schaftoside, the target β tubulin was modelled using 5IJ0 (a microtubule assemble from human tubulin TUBB 3) as template owing to its sequence similarity of 86.38%.

Colchicine binding site stands out among other binding sites in tubulin owing to its unique ability to bind to small molecules and overcome drug resistance (Federico et al., 2021). Colchicine is known to block mitotic cells in metaphase by forming a tubulin-colchicine complex, thereby inhibiting the elongation of microtubule polymers (Leung et al., 2015). Consistent with these, the *Allium cepa* assay we conducted showed an accumulation of cells in the metaphase when treated with colchicine or extracts as compared to the vehicle controls (Table 7.10). For our

further *in silico* studies, CBS located at α and β chain interphase was chosen as the suitable target site. It was also noteworthy that the alpha and beta tubulin of *C. elegans* and *H. contortus* has a similarity of 87.11% and 93.6% respectively. Further, a homology modelling was performed to develop a 3D structure of *H. contortus* αβ tubulin using the closely related nematode *C. elegans* tubulin (PDB-ID 6E88) as the template.

Initially we repeated the interaction studies with colchicine and CBS of *C. elegans* tubulin (PDB-ID 6E88) and a modelled *H. contortus* tubulin *viz.* HcF and gave consistent results. Additionally, lupeol**,** detected in DME had a strong binding affinity with both *C. elegans* (-8.9 Kcal/mol) and *H. contortus* (-8.5 Kcal/mol) CBS. Similarly, Schaftoside also showed strong binding affinity towards *C. elegans* tubulin (-9.8 Kcal/mol) and HcF (-10.9 Kcal/mol). Quercetin detected in TME showed commendable binding affinity with the CBS of tubulin of *C. elegans* (-8.7 Kcal/mol) and *H. contortus* (-8.8 Kcal/mol). This was consistent with an earlier study that reports the tubulin binding efficacy of this compound identified from ethanolic extract of *Kaempferia rotunda* (Praveen, 2022). However, thymol showed a low binding affinity towards the tubulin protein of *C. elegans* (-6.0 Kcal/mol) and HcF (-6.1 Kcal/mol).

Apart from these compounds with previously known tubulin interactions, several other compounds present in TME viz; Isoquercetin, Cynaroside, Apigetrin, Leufolin A, Loquatoside, Agathisflavone and Echinacin also showed very high binding affinity towards the CBS of *C. elegans* and HcF tubulin. It was further interesting to note that several of these compounds had a higher binding affinity when compared to reference drugs used in the *in silico* study (Table 7.11). Since a diverse range of phytochemicals in DME and TME were found to have a great binding affinity towards the tubulin protein of nematodes, it may be noted that the synergestic effect of these phytocomponents may be the reason for tubulin depolymerisation followed by mitotic arrest, and nematicidal acticity. Fractionation and isolation of these specific components may be required to make more conclusive findings.

In the case of trematodes, tegument is a vital part of their body, as it is in direct contact with the host tissue as well as the body fluids. Several functions are attributed to the tegumental surface, such as maintaining homeostasis,

osmoregulation, absorption of nutrients and protection from the host's digestive enzymes. For the development of anthelmintic drugs, proper understanding of the tegumental structure is required (Panyarachun et al., 2010). In the present study, the ethyl acetate extracts, closely followed by ethanol extracts of both the plants have shown tegumental degradation, degradation of surface syncytium and blebbing in histological and morphological analysis. Sequential changes in the tegument observed, such as swelling, which may be the result of disrupted ionic pump, blebbing, sloughing off and detachment from the basement membrane, that are in accordance with the changes seen in the tegument of *F. gigantica* treated with crude extracts of *Terminalia catappa* L (Anuracpreeda et al., 2016). In the extract treated flukes, the teguments appear smooth due to loss of spines, when compared to the control. The loss of spines in tegument is in agreement with previously studied changes in the tegument surface of a liver fluke, *Fasciola gigantica* treated with the alcoholic leaf extracts of *Cassia fistula* (Sen et al., 2021). Further, the treated trematodes also showed loss of collagen. Interactions between collagen is inevitable for the production of structurally ordered cuticles. The final polymerised macromolecular structure is formed by interaction between collagen monomers, by forming a triple helix (Johnstone, 1994). The condensed tannins have high affinity for proteins and bind with them to alter its physical and chemical properties (Hoste et al., 2006). Presence of tannins were observed in phytochemical screening (Chapter 4; Table 4.2) and it may thus be one of the reasons for altered cuticle structure seen in the sections of amphistomes treated with ethyl acetate and ethanol extracts of the plants. The loss of this important protein interaction can lead to the cuticular damage and hence, mortality of amphistomes. The mechanistic aspect by which the phytochemical constituents exert this action is not fully clear. Also, the multicomponent extracts may have multiple targets especially since these small molecules can easily be absorbed in the damaged tegument system of these invertebrates (Hrckova & Velebny, 2013).

Table 7.1: Percentage inhibition of *H. contortus* egg hatch following its treatment with DEE, DME and DWE at various concentrations

Concentration (mg/ml)	DEE	DME	DWE
10	87.16±1.32$^{\$}$	90.83±0.98$^{\$}$	48±0.63$^{\$}$
5	83.83±1.16$^{\$}$	88.16±1.16$^{\$}$	45.6±2.25$^{\$}$
2.5	77.28±2.28$^{\$}$	87.33±0.81$^{\$}$	38.83±0.75$^{\$}$
1.25	53.83±2.31$^{\$}$	55±3.34$^{\$}$	36.66±1.50$^{\$}$
0.625	46.83±3.48$^{\$}$	50±1.26$^{\$}$	27.5±0.83$^{\$}$
DMSO (1%)	2±0.89	5.33±1.96	2.33±1.03

Comparisons were made between each treated group with the vehicle control (1% DMSO). Data are expressed as Mean ± SD. ($^{\$}$) shows significance $p \leq 0.001$

Table 7.2: Percentage inhibition of *H. contortus* egg hatch following its treatment with TEE, TME and TWE at various concentrations

Concentration (mg/ml)	TEE	TME	TWE
10	86.5±2.34$^{\$}$	90.5±1.64$^{\$}$	47.33±0.51$^{\$}$
5	73.83±1.94$^{\$}$	74.33±2.84$^{\$}$	46.66±2.33$^{\$}$
2.5	50.66±1.03$^{\$}$	61.83±1.83$^{\$}$	42.16±2.31$^{\$}$
1.25	45.3±1.03$^{\$}$	42.16±1.6$^{\$}$	37.16±1.72$^{\$}$
0.625	25.66±0.21$^{\$}$	25.66±0.21$^{\$}$	23±3.68$^{\$}$
DMSO (1%)	1.83±0.4	5.66±1.21	4±0.81

Comparisons were made between each treated group with the vehicle control (1% DMSO). Data are expressed as Mean ± SD. ($^{\$}$) shows significance $p \leq 0.001$

Table 7.3: Percentage inhibition of *H. contortus* egg hatch following its treatment with various concentrations Albendazole

Concentration (µg/ml)	Egg hatch inhibition
50	98±1.54
25	93±1.16
12.5	86±0.75
6.25	85±1.86

Data are expressed as Mean ± SD.

Table 7.4: EC_{50} and EC_{90} values (mg/ml) of egg hatch inhibition in *H. contortus*

Extracts Used	Egg Hatch Inhibition	
	EC_{50}	EC_{90}
DEE	0.771	>10
DME	0.661	6.407
DWE	>10	>10
TEE	1.827	>10
TME	1.664	>10
TWE	8.760	>10

Table 7.5: Larval Paralysis of *H. contortus* larva (L3) upon treatment with DEE, DME and DWE at various concentrations

Concentration (mg/ml)	DEE (%)	DME (%)	DWE (%)
10	57.5±2.5$^{\$}$	65.5±2.25$^{\$}$	39.33±0.81$^{\$}$
5	55.5±5.89$^{\$}$	59.66±1.36$^{\$}$	34.5±1.04$^{\$}$
2.5	42.5±2.07$^{\$}$	56±1.26$^{\$}$	34.33±1.03$^{\$}$
1.25	35.66±2.33$^{\$}$	32.66±2.06$^{\$}$	31.16±1.6$^{\$}$
0.625	25.33±0.51$^{\$}$	24.33±1.96$^{\$}$	21.66±1.86$^{\$}$
DMSO (1%)	2±0.57	2.16±0.75	2±0.57

Comparisons were made between each treated group with the vehicle control (1% DMSO). Data are expressed as Mean ± SD. ($^{\$}$) shows significance $p \leq 0.001$

Table 7.6: Larval Paralysis of *H. contortus* larva (L3) upon treatment with TEE, TME and TWE at various concentrations

Concentration (mg/ml)	TEE (%)	TME (%)	TWE (%)
10	60±4.42$^{\$}$	64.5±0.54$^{\$}$	48.83±0.4$^{\$}$
5	46.5±4.18$^{\$}$	51.16±0.98$^{\$}$	48.66±0.51$^{\$}$
2.5	42.3±3.71$^{\$}$	42±1.09$^{\$}$	47.16±1.47$^{\$}$
1.25	30.66±4.03$^{\$}$	41±1.26$^{\$}$	36.83±3.48$^{\$}$
0.625	19.66±0.51$^{\$}$	17.66±1.5$^{\$}$	18±1.67$^{\$}$
DMSO (1%)	2.5±0.54	2±0.63	1.66±0.83

Comparisons were made between each treated group with the vehicle control (1% DMSO). Data are expressed as Mean ± SD. (*) shows significance $p \leq 0.001$

Table 7.7: Paralysis of *H. contortus* larvae following its treatment with various concentrations of Albendazole

Concentration (µg/ml)	Larval Paralysis
50	97.33±1.86
25	96.33±2.16
12.5	90.83±2.13
6.25	88.33±1.86

Data are expressed as Mean ± SD.

Table 7.8: EC_{50} and EC_{90} values (mg/ml) of larval paralysis in *H. contortus*

Extracts Used	Larval Paralysis	
	EC_{50}	EC_{90}
DEE	4.370	>10
DME	2.931	>10
DWE	>10	>10
TEE	5.166	>10
TME	3.876	>10
TWE	6.412	>10

Table 7.9: EC_{50} and EC_{90} values (mg/ml) of *in vitro* adulticidal activity on *Fischoederius cobboldi.* at treatment duration of 2 hours

Extracts Used	*In vitro* adulticidal activity	
	EC $_{50}$	**EC $_{90}$**
DEE	3.477	9.189
DME	4.470	>10
DWE	>10	>10
TEE	3.845	9.427
TME	4.280`	9.458
TWE	>10	>10

Table 7.10: Effect of DME and TME on different stages of cell cycle

Concentration	Total number of cells counted	Total number of cells in division				Metaphase index (%)
		Prophase	Metaphase	Anaphase	Telophase	
Control (1% DMSO)	200	5.33±0.57	6.66±1.15	7.33±1.52	9.66±0.57	22.7
Colchicine (0.4 mg/ml)	200	8.66±1.15	10.66±0.57	7.33±0.57	6.33±0.57	32.3
DME (4 mg/ml)	200	9.33±0.57	11.33±0.57	6.33±0.57	4.33±1.15	36.1
TME (4 mg/ml)	200	10.33±0.57	12.33±0.57	6.33±0.57	5.66±1.15	35.5

Data represented as Mean ± SD.

Table 7.11: Binding affinity (Kcal/mol) of selected ligands and reference drugs on colchicine binding tubulin targets of nematodes.

Ligands	Binding affinity (Kcal/mol)	
	6e88	**Modelled-HcF**
Colchicine	-8.6	-8.2
Albendazole	-7.3	-7.1
Mebendazole	-9.1	-8.8
Thymol	-6.0	-6.1
Lupeol	-8.9	-8.5
Quercetin	-8.7	-8.8
Isoquercetin	-9.9	-9.6
Corilagin	-9.5	-9.7
Loquatoside	-9.5	-9.8
Echinacin	-10.9	-10.0
Agathisflavone	-9.7	-10.2
Apigetrin	-10.7	-10.6
Cynaroside	-10.8	-10.7
Schaftoside	-9.8	-10.9
Leufolin A	-10	-11.2

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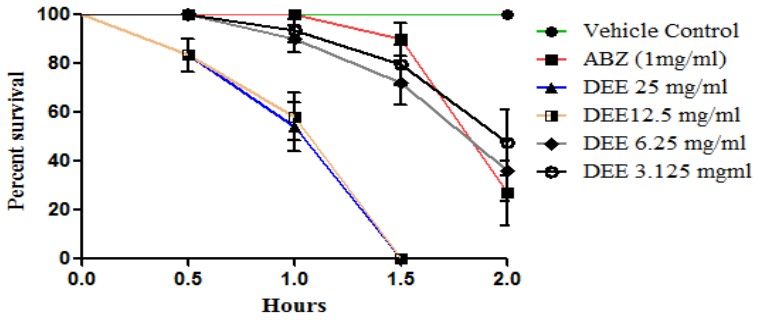

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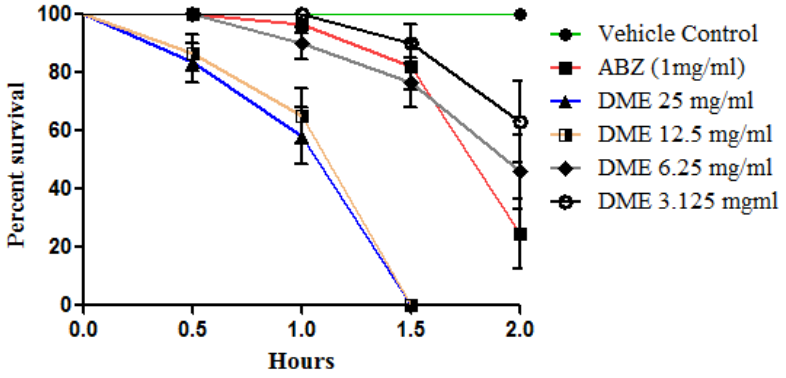

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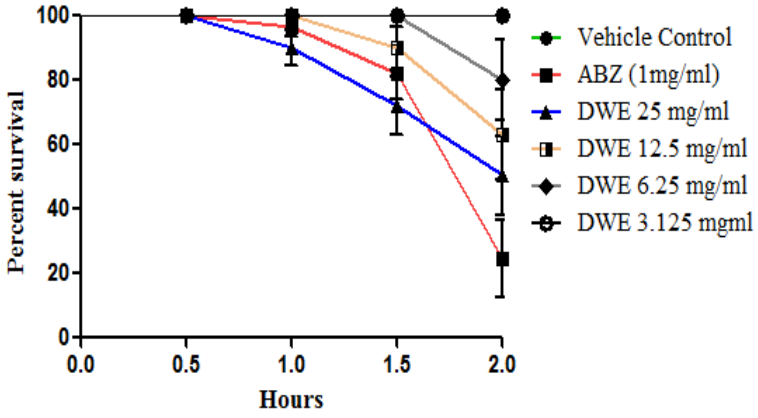
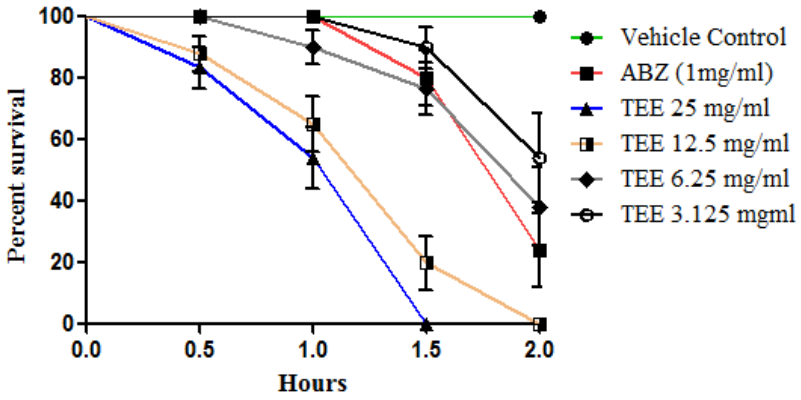
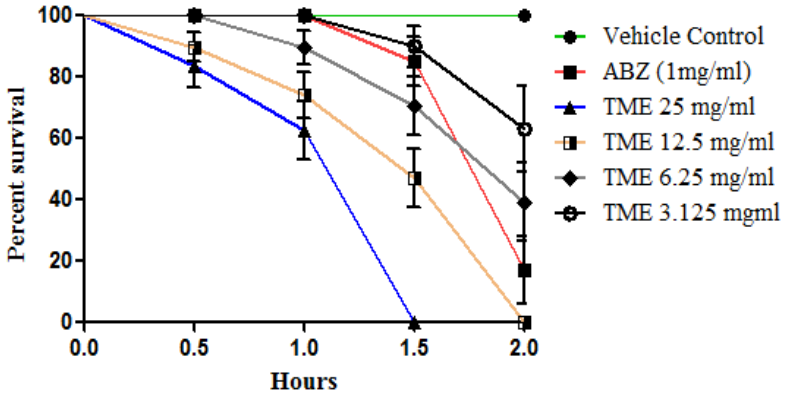


Figure 7.1: Kaplan-Meier survival curves of adult *F. cobboldi* treated with (A) DEE, (B) DME or (C) DWE at various concentrations for different time periods

A)



B)



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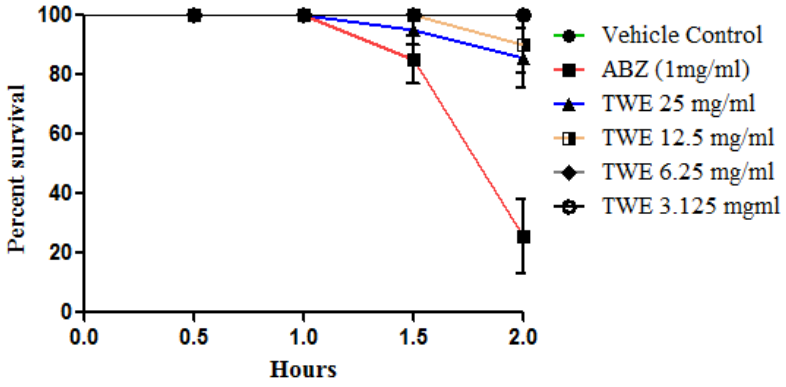

Figure 7.2: Kaplan-Meier survival curves of adult *F. cobboldi* treated with (A) TEE, (B) TME or (C) TWE at various concentrations for different time periods

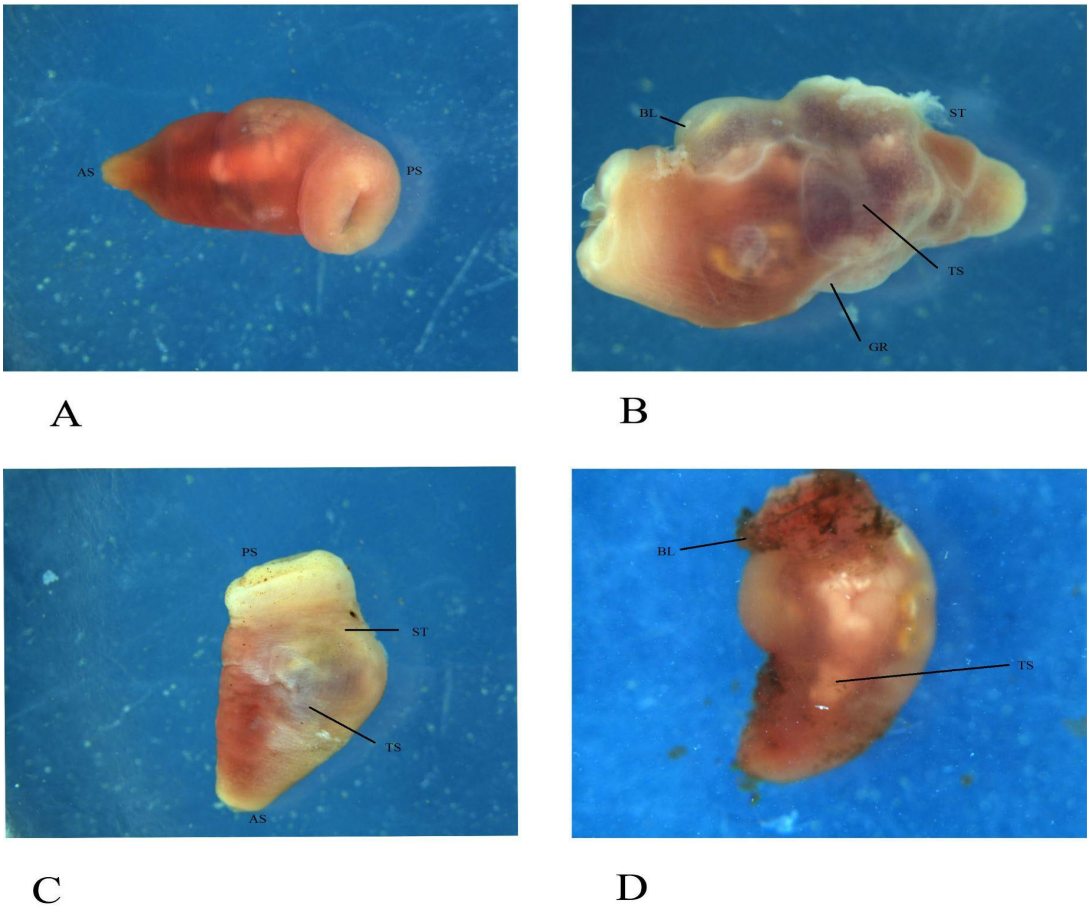

Figure 7.3: Stereo zoom microscopic image (40x magnification) of *F. cobboldi*. A; fluke treated in vehicle control (1% DMSO) with intact anterior suckers (AS) and posterior suckers (PS). C; shows fluke treated with albendazole (1 mg/ml) with swollen tegument (ST) and tegumental sloughing (TS). B and D; flukes treated in DEE and TEE respectively with grooves (GR), blebs (BL), tegumental sloughing (TS) and swollen tegument (SW).

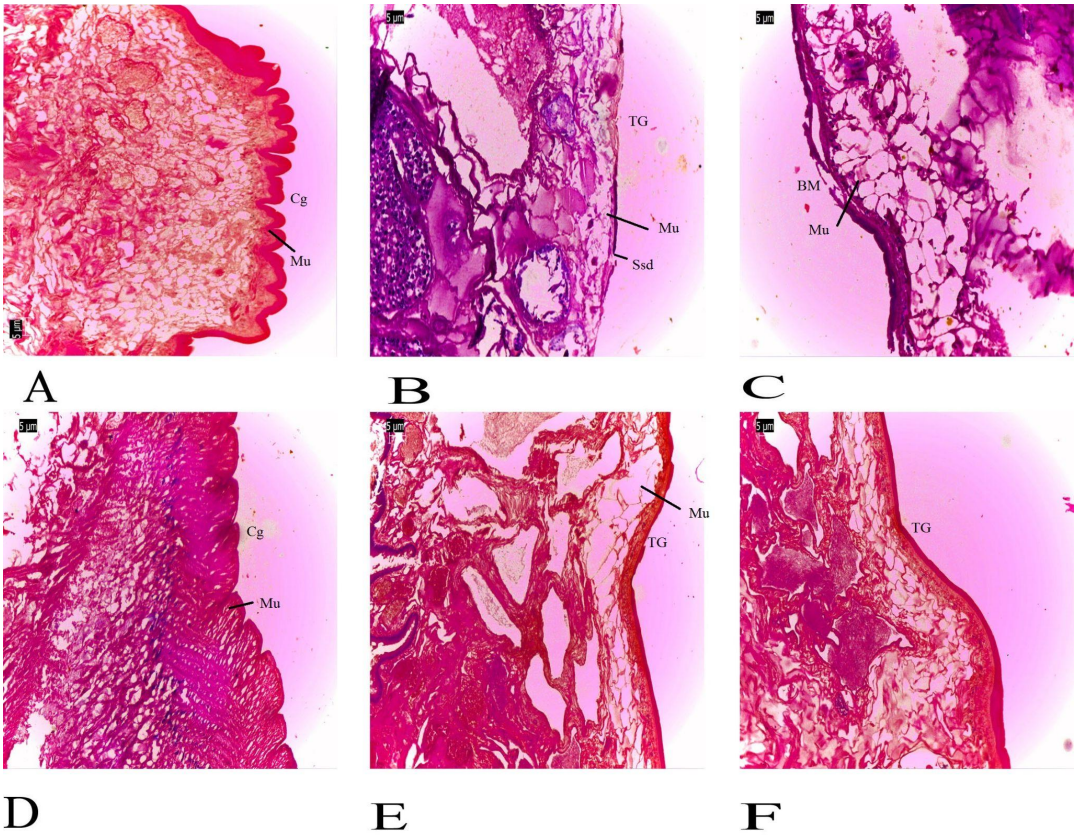

Figure 7.4: The histopathological analysis of *F. cobboldi* treated and control group of animals using H and E staining method (40x magnification). A and D: Vehicle control (1% DMSO) with intact tegument, having proper corrugations (Cg) and proper muscle layer (Mu) without any distortions beneath the tegument. B and E: Albendazole (50 µg/ml) treated with disrupted muscle layers (Mu) and smooth tegument (TG), without any corrugations. C and F: DEE and TEE respectively. In DEE detachment of the basement membrane is clearly visible (BM) along with disrupted mussel layer beneath (Mu). In TEE show smooth tegument (TG) which has lost all of its corrugations is clearly visible.

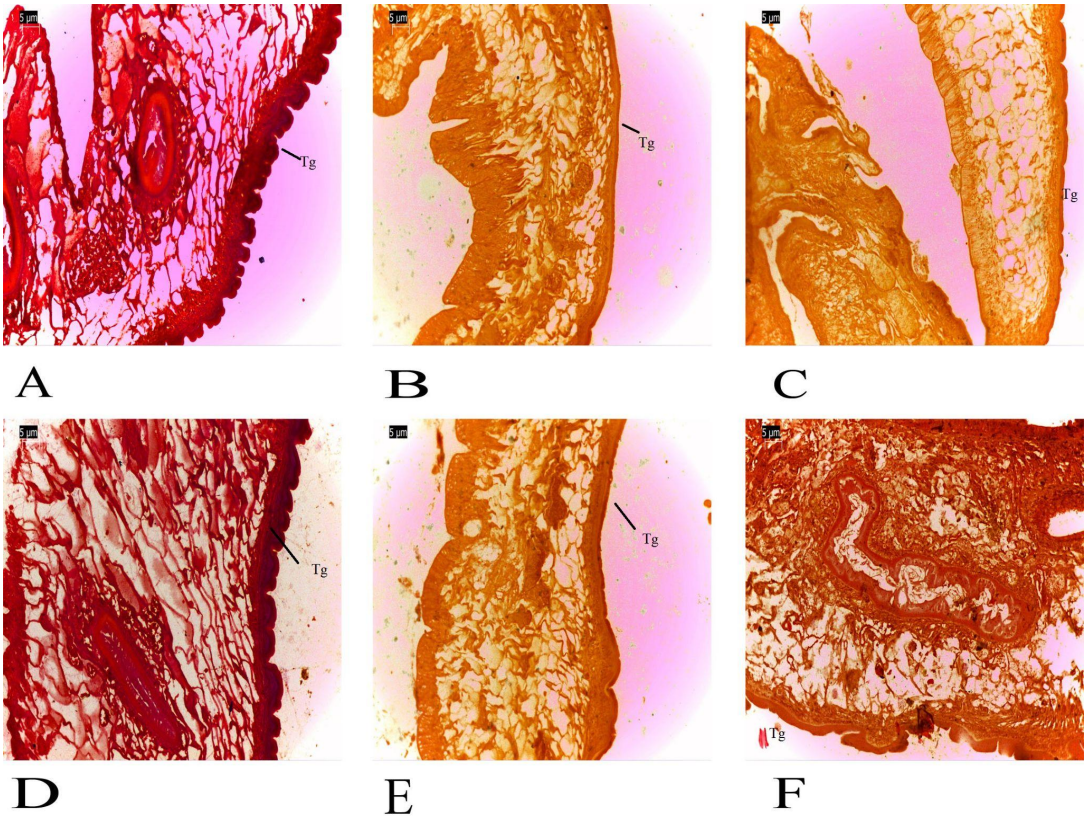

Figure 7.5: Masson's trichrome staining to analyse the changes in connective tissue (40x magnification). A and D: shows a bluish purple colour throughout the tissue which indicates intact collagen and muscle fibers along with an undisturbed tegument (Tg). B and C: albendazole (50 µg/ml) complete loss of intact collagen and muscle fibers as evident by the loss of the bluish purple colour. E and F: DEE and TEE loss of bluish colour indicating absence of collagen and also a smooth tegument (Tg) without corrugations.

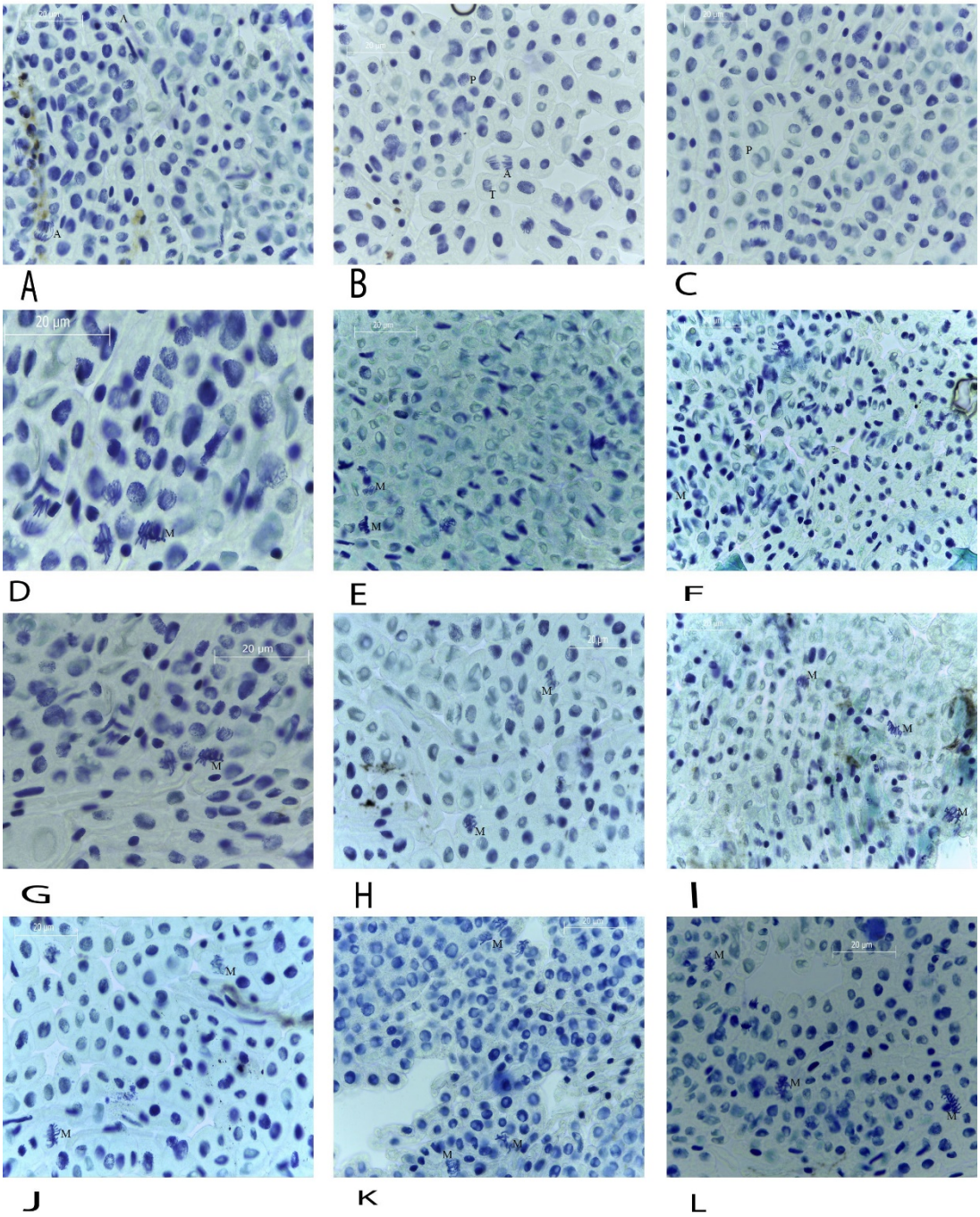

Figure 7.6: Photomicrographs of representative meristematic cells of *Allium cepa* root tips after exposure to control, colchicine, DME and TME for 4 h showing various stages of mitosis namely; prophase (P), metaphase (M), anaphase (A) and telophase (T). A, B and C; shows cells of root tips treated in control. D, E and F; shows cells of root tips treated in colchicine. G, H and I; shows cells of root tips treated in DME. J, K and L; shows cells of root tips treated in TME.

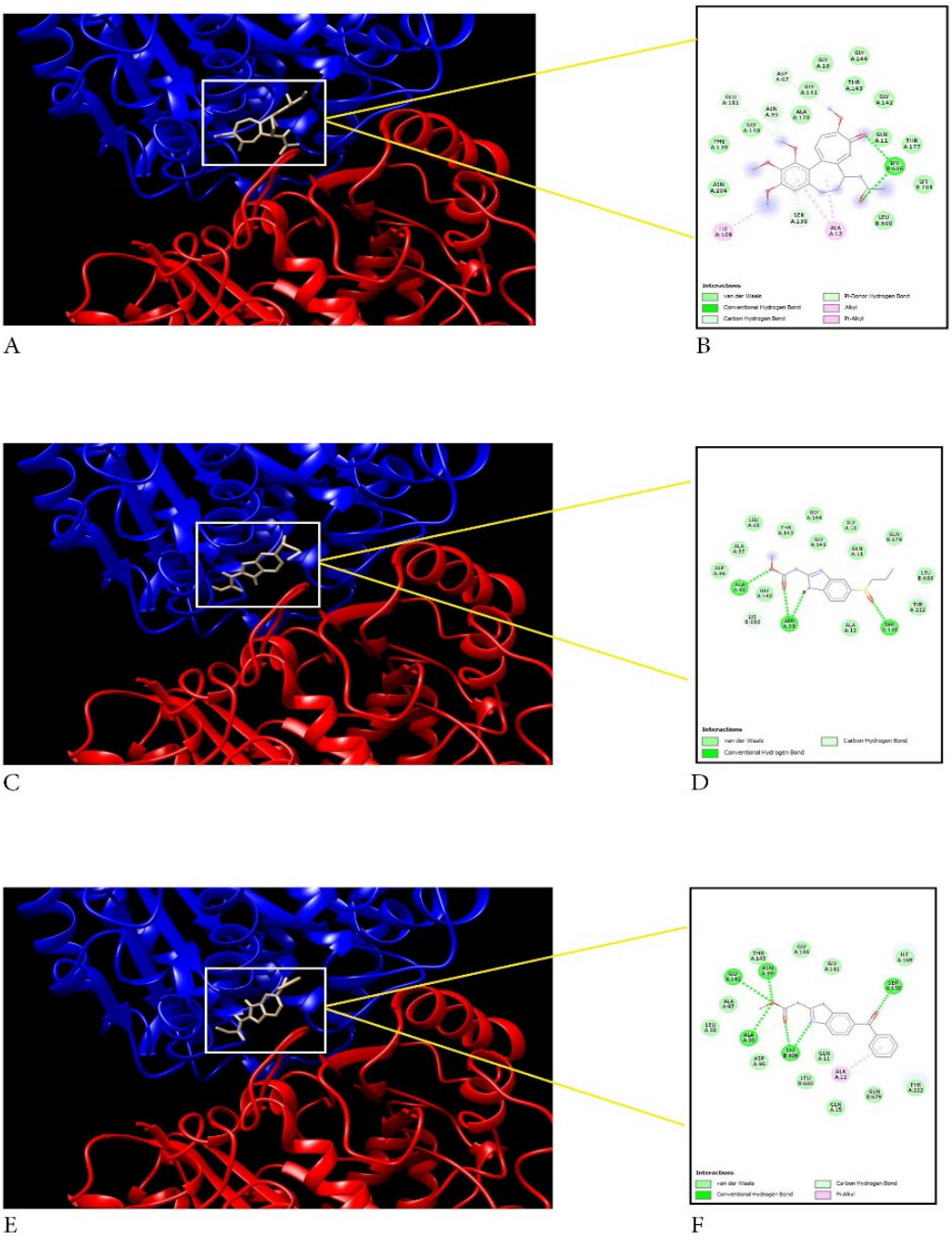

Figure 7.7: 3D and 2D images of validation studies of molecular docking using reference molecules and αβ tubulin of *C. elegans*. A and B: shows 3D and 2D images of interactions of colchicine with 6E88; C and D: shows 3D and 2D images of interactions of albendazole with 6E88; E and F: shows 3D and 2D images of interactions of mebendazole with 6E88.

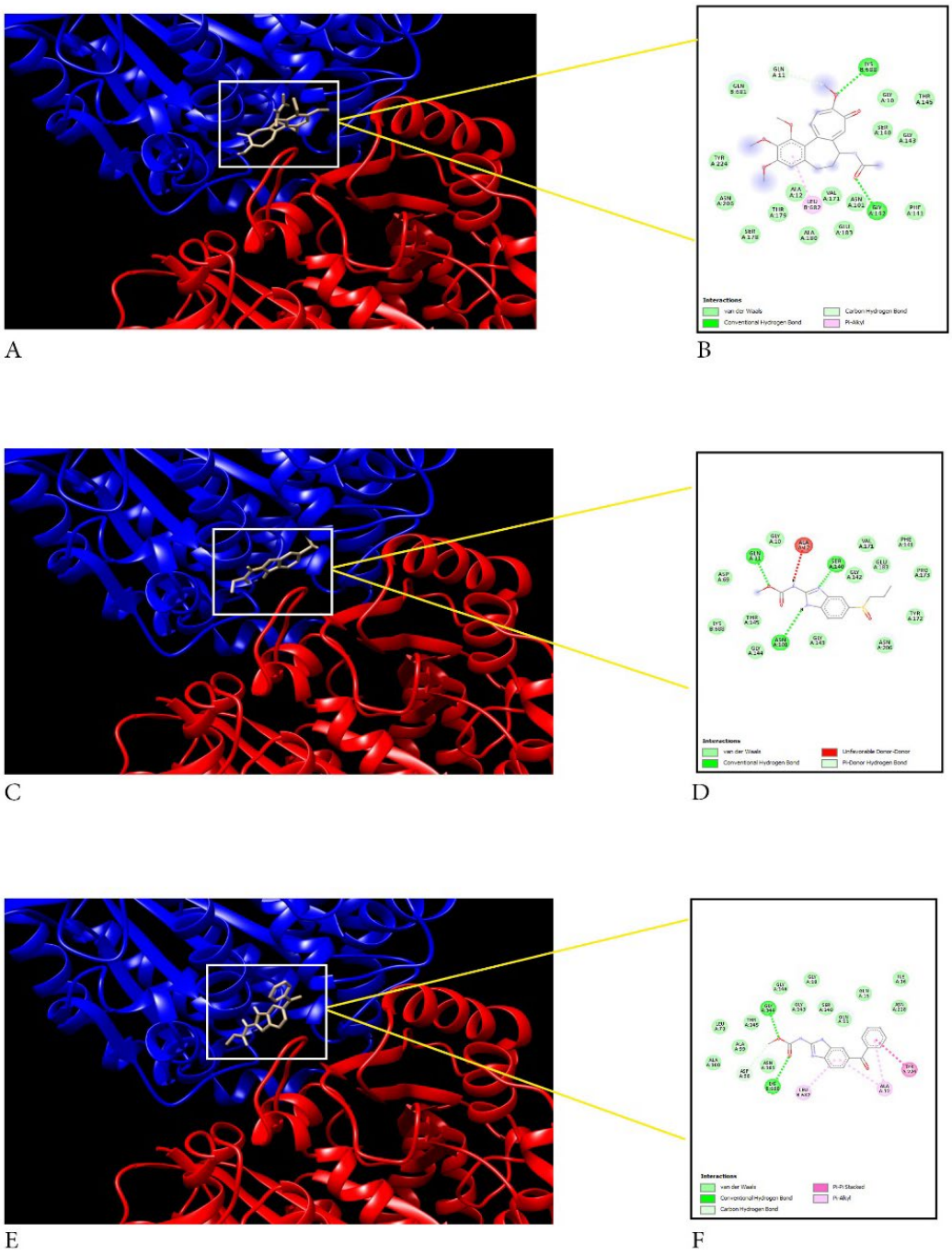

Figure 7.8: 3D and 2D images of validation studies of molecular docking using reference drugs and αβ tubulin of *H. contortus*. A and B: shows 3D and 2D images of interactions of colchicine with HcF; C and D: shows 3D and 2D images of interactions of albendazole with HcF; E and F: shows 3D and 2D images of interactions of mebendazole with HcF.

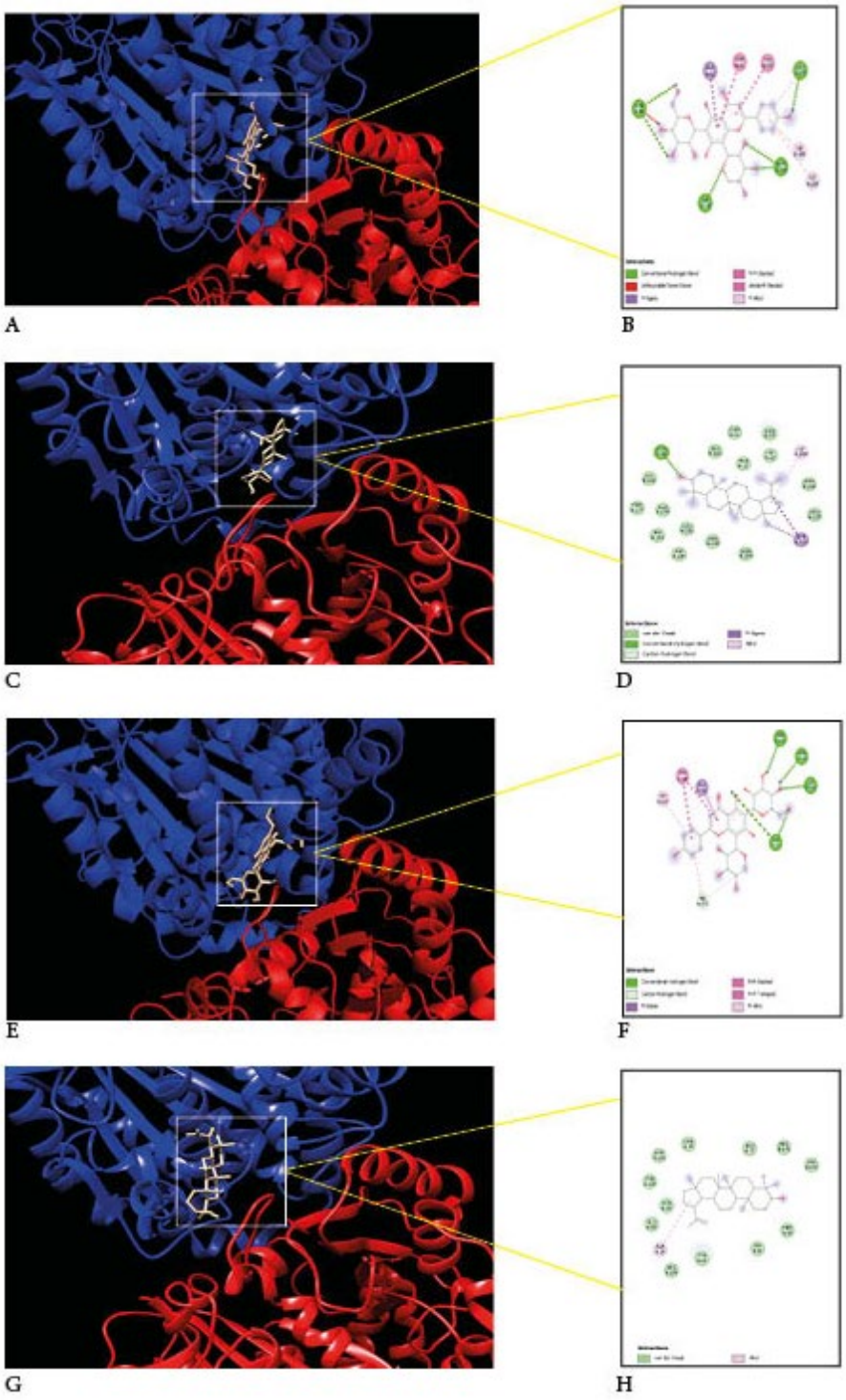

Figure 7.9: 3D and 2D images of validation studies of molecular docking using schaftoside, lupeol and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of schaftoside with 6E88; C and D: shows 3D and 2D images of interactions of lupeol with 6E88; E and F: shows 3D and 2D images of interactions of schaftoside with HcF; G and H: shows 3D and 2D images of interactions of lupeol with HcF.

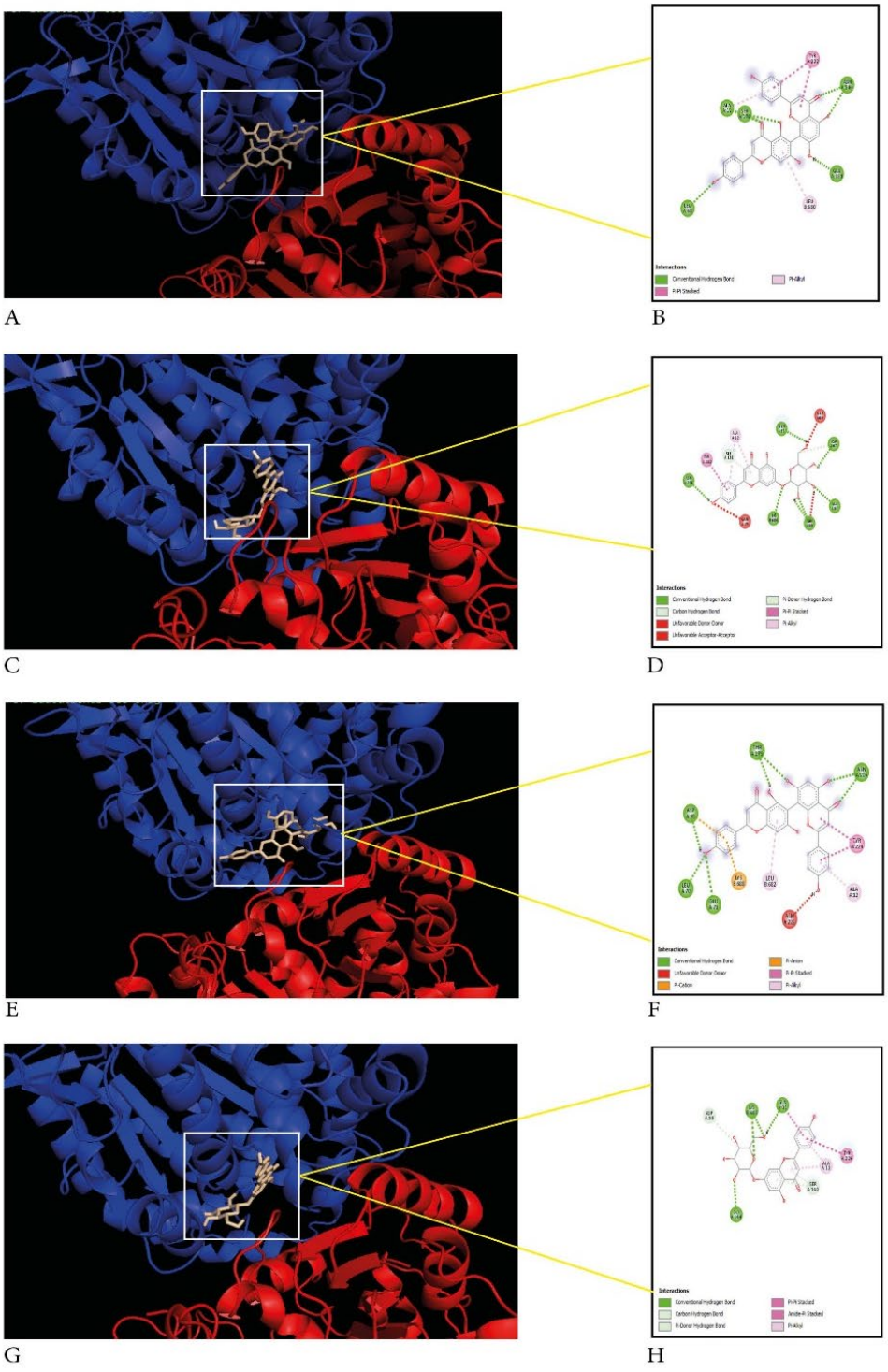

Figure 7.10: 3D and 2D images of validation studies of molecular docking using agathisflavone, apigetrin and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of agathisflavone with 6E88; C and D: shows 3D and 2D images of interactions of apigetrin with 6E88; E and F: shows 3D and 2D images of interactions of agathisflavone with HcF; G and H: shows 3D and 2D images of interactions of apigetrin with HcF.

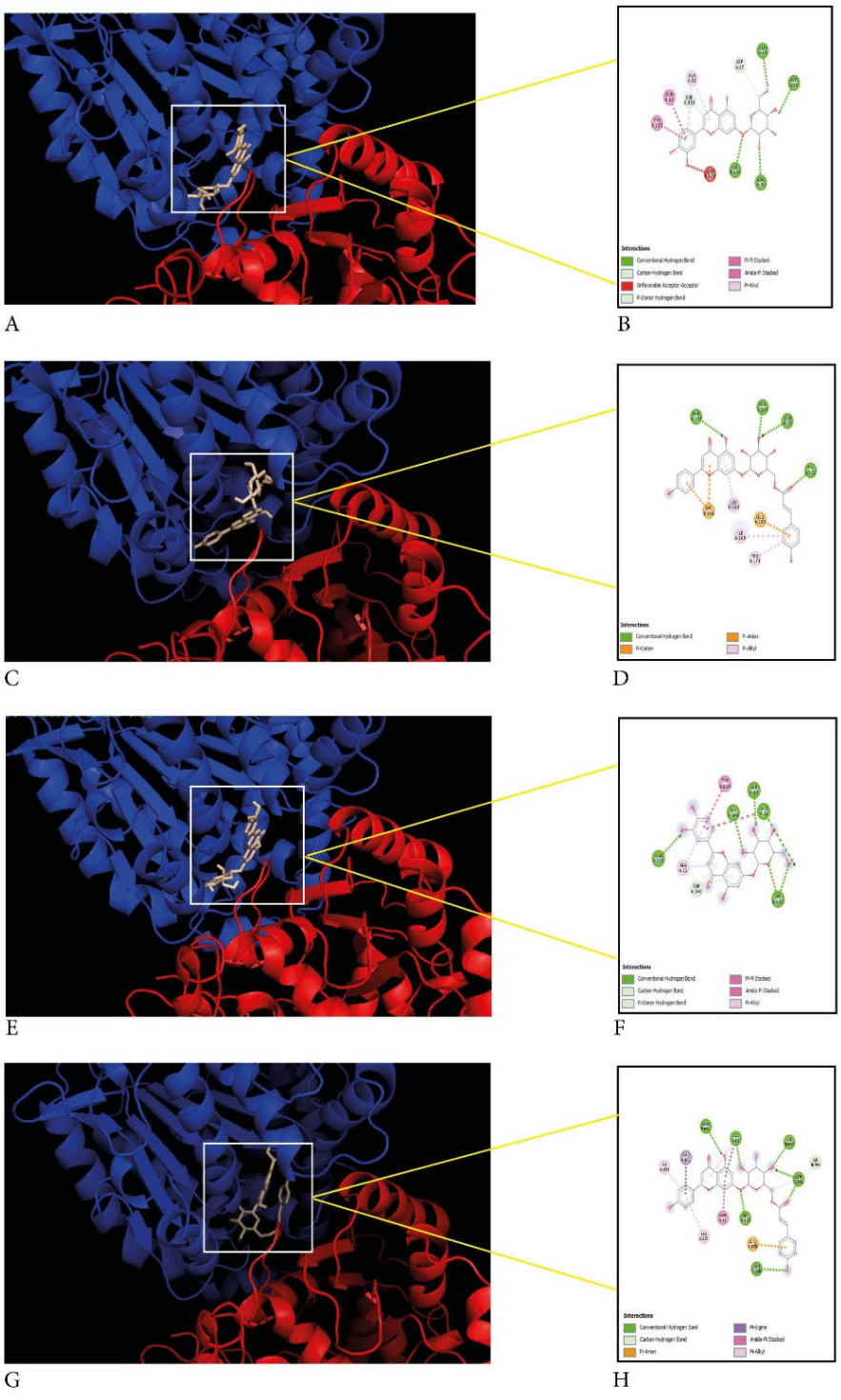

Figure 7.11: 3D and 2D images of validation studies of molecular docking using cynaroside, echinacin and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of cynaroside with 6E88; C and D: shows 3D and 2D images of interactions of echinacin with 6E88; E and F: shows 3D and 2D images of interactions of cynaroside with HcF; G and H: shows 3D and 2D images of interactions of echinacin with HcF.

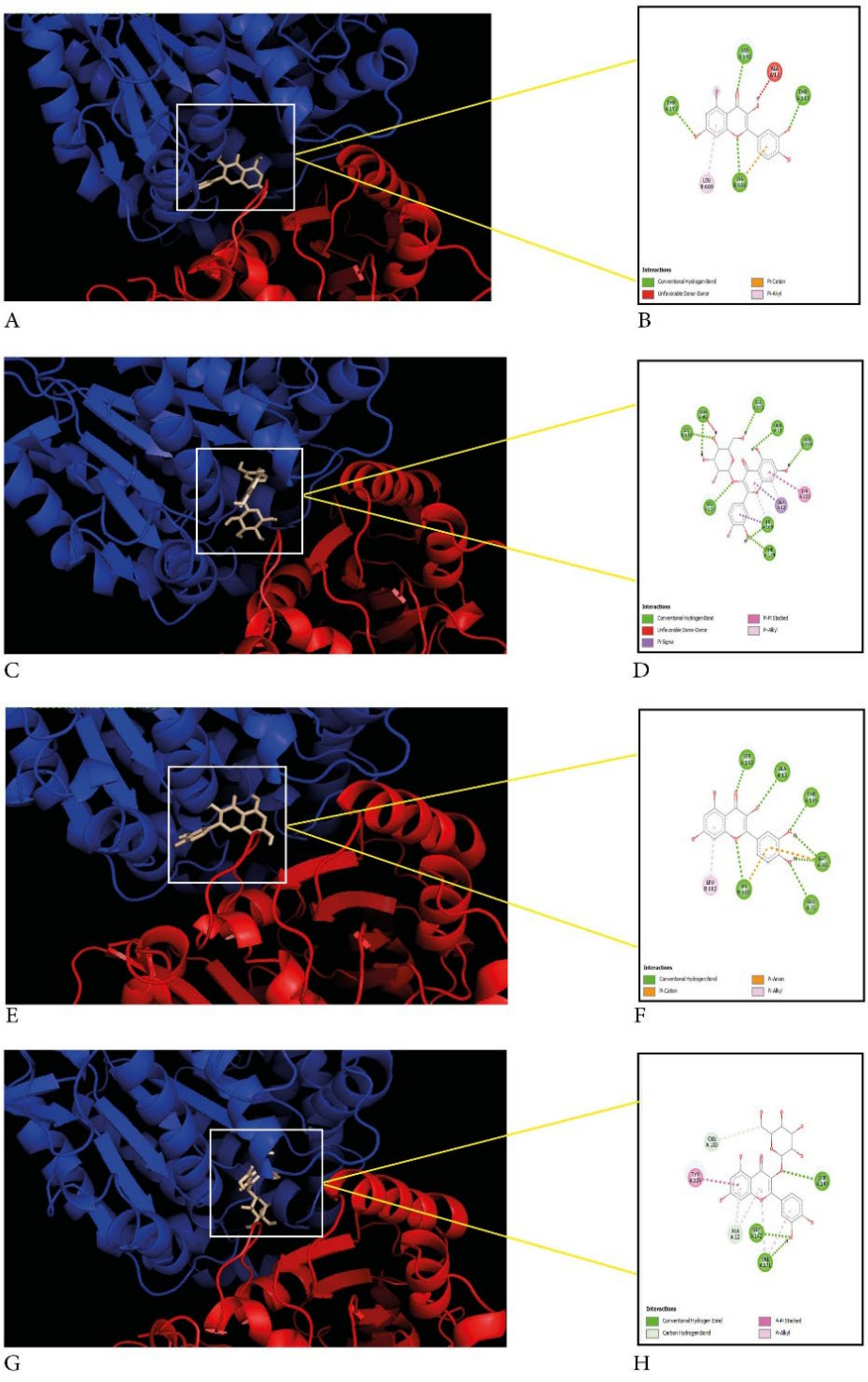

Figure 7.12: 3D and 2D images of validation studies of molecular docking using quercetin, isoquercetin and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of quercetin with 6E88; C and D: shows 3D and 2D images of interactions of isoquercetin with 6E88; E and F: shows 3D and 2D images of interactions of quercetin with HcF; G and H: shows 3D and 2D images of interactions of isoquercetin with HcF.

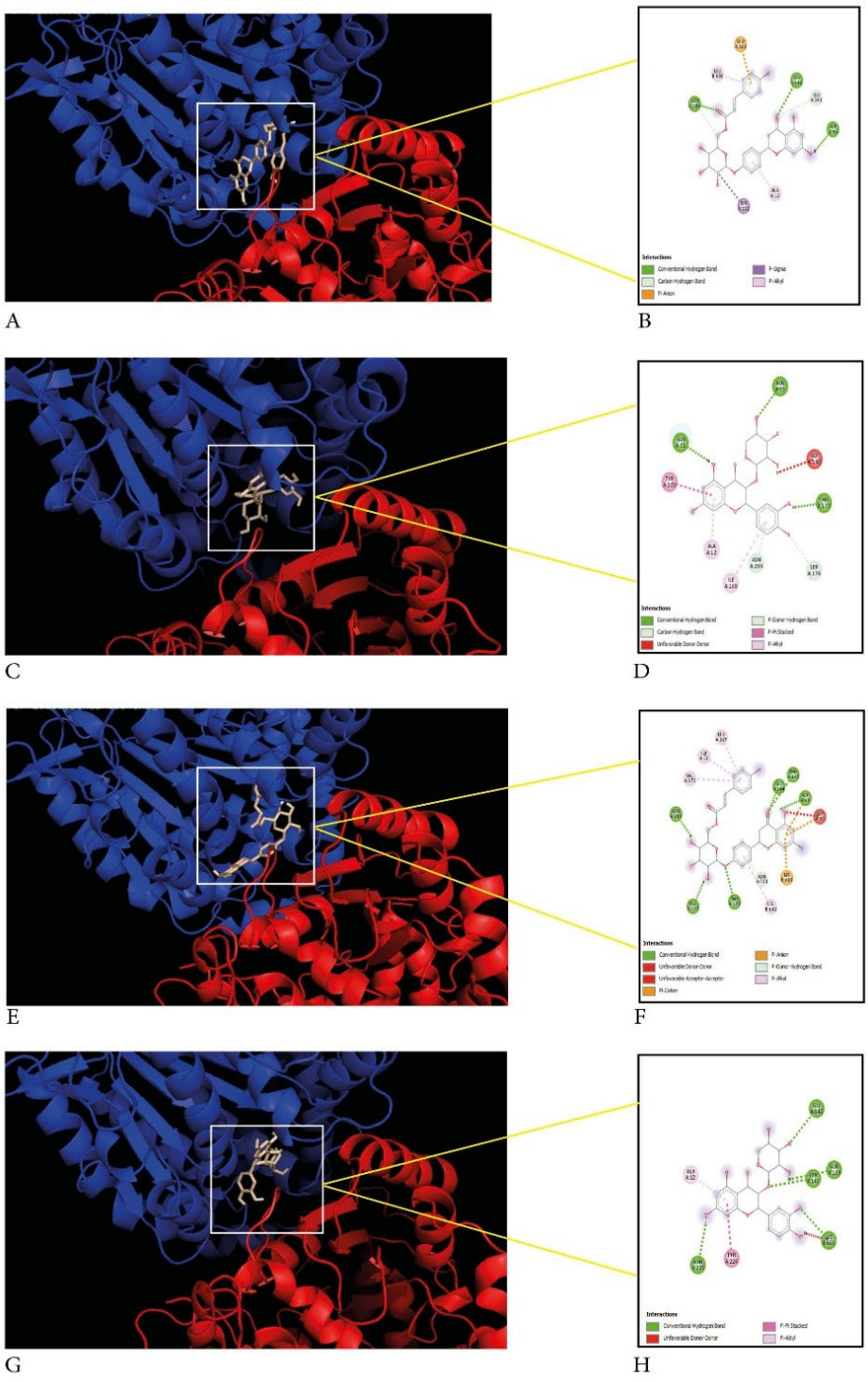

Figure 7.13: 3D and 2D images of validation studies of molecular docking using leufolin A, loquatoside and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of leufolin A with 6E88; C and D: shows 3D and 2D images of interactions of loquatoside with 6E88; E and F: shows 3D and 2D images of interactions of leufolin A with HcF; G and H: shows 3D and 2D images of interactions of loquatoside with HcF.

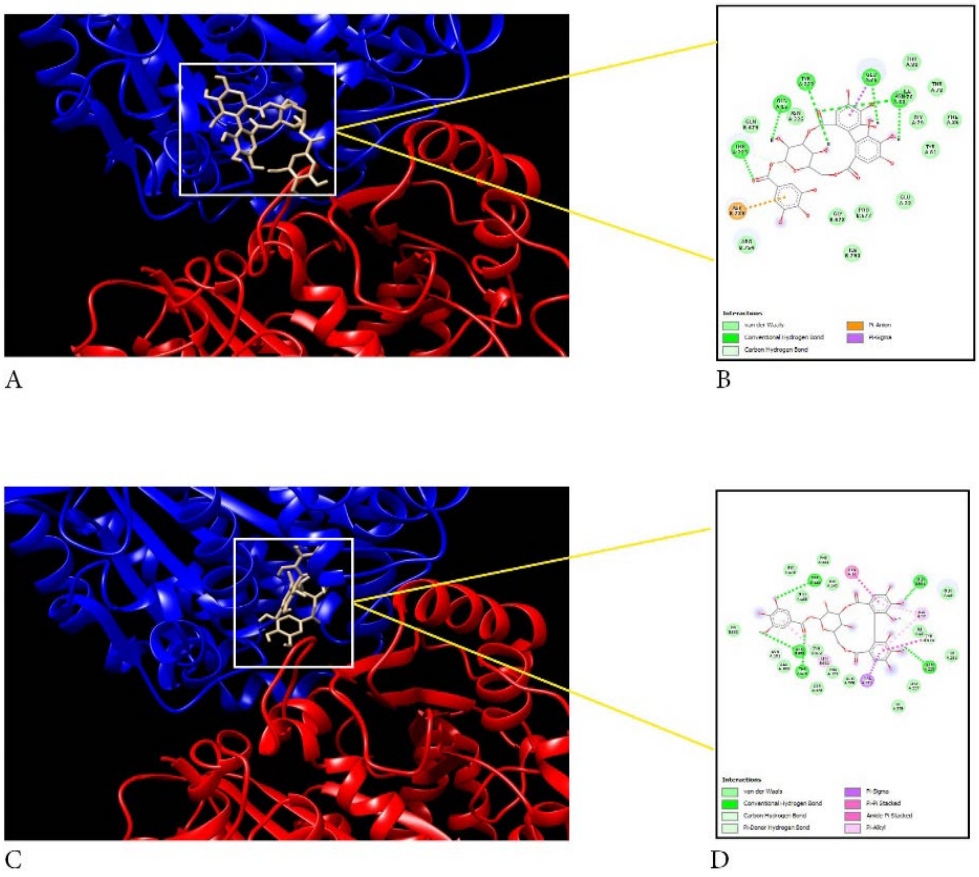

Figure 7.14: 3D and 2D images of validation studies of molecular docking using corilagin and αβ tubulin of *C. elegans* and *H. contortus*. A and B: shows 3D and 2D images of interactions of corilagin with 6E88; C and D: shows 3D and 2D images of interactions of corilagin with HcF.

SUMMARY & CONCLUSION

Search for therapeutically potent natural products goes on and plants are the precious storehouse of bioactive molecules that have the potential to be developed into new drugs. Population in developing countries heavily depend on herbal formulations for their health care needs, however, most of these herbal formulations in the traditional system are not properly validated. The broad objective of the current study was to test and scientifically validate the bioactive potentials of two herbs. *Pleurolobus gangeticus*, used in our study (Family Fabaceae) is a perennial shrub, found all over India, has been traditionally used in the treatment of scabies, ringworm, diarrhoea, cataract, fever, oedema, asthma, bronchitis and urogenital diseases (Singh et al., 2015). It is an important ingredient of several Ayurvedic preparations including 'Chyawanaprasha', 'Dasamula kwatha' and 'Dashmoolarishta' (Rastogi et al., 2011; Sharma et al., 2019). Another plant, *Tragia involucrata* is a perennial herb, that belongs to the Family Euphorbaceae, is also a medicinal plant used since time immemorial to treat various ailments mostly in the South Asian countries such as Sri Lanka, India and Bangladesh to treat cough, asthma, bronchitis, fever and several other disorders (Pallie et al., 2020). According to API (2004) this plant is used in preparing important ayurvedic formulations such as 'Vidaryadi Kvatha Churna' and 'Vidaryadi Ghrta'. The stem and roots of *P. gangeticus* and the whole plant parts of *T. involucrata* were selected for our studies. We evaluated the antitumor and anthelmintic potentials of solvent extracts from both plants and the ethanol extract showed strongest antitumor and anthelmintic potential followed by ethyl acetate extract. Aqueous extracts of both plants showed poor bioactive potentials.

The anticancer potential was evaluated using lymphoma cell lines, such as DLA and YAC-1. Ethanol extracts were capable of reducing cell proliferation in YAC – 1 lymphoma cell lines. It was also found to bring about a metaphase arrest in division induced lymphocytes. This probably reveals the ability of ethanol extracts to interfere with the microtubule network causing metaphase arrest and finally leading to apoptosis. The tumor reduction ability of these extracts were further tested using DLA solid tumor model in *Swiss albino* mice. The tumor mass extracted from the mice under study was subjected to histopathological analysis using H and E staining

method and TUNEL analysis. These studies also revealed the induction of apoptosis in the tumor tissues of ethanol extract treated animals.

The anthelmintic potential of the extracts was assessed against parasitic nematode *Haemonchus contortus* and trematode *Fischeoderius cobboldi*. The nematocidal potential of the organic extracts especially ethanol extract in both plants were evident from the egg hatch inhibition and larval paralysis assays conducted. An *Allium cepa* assay was conducted to confirm the ability of the ethanol extracts to bring about mitotic arrest. The trematocidal effects of the extracts were also highly pronounced as they induced mortality in the flukes. The histopathological analysis of its tissues revealed blebbing, degradation and detachment of the tegument. The Masson's trichrome staining also revealed collagen erosion in them. The mechanistic aspect by which the phytochemical constituents exert this action is not fully clear. It is inferred that the multicomponent extracts may have multiple targets, especially since these small molecules can easily be absorbed in the damaged tegument system of these invertebrates.

Phytochemical analysis of ethanol extracts was done using chromatographic techniques (GC-MS and LC- MS). Several components including several secondary metabolites were detected and some of them were reported in these plants for the first time. Since the ability of the molecules detected in both plants were known to interact with tubulin, further *in silico* molecular docking studies were performed using the identified molecules. The colchicine binding site in the interface of αβ tubulin were selected as the target site of action and the selected ligands were capable of binding to this target site thereby interfering with the tubulin protein leading to mitotic arrest and mortality. The biological activity elicited by the extract may be attributed this individual or synergistic efficacy of the phytocomponents present in the extracts.

RECOMMENDATIONS

Current study mainly focuses on exploring the antitumor and anthelmintic potentials of *Pleurolobus gangeticus* and *Tragia involucrata* extracts. Out of the three different solvent extracts used, ethanol extract was found to show the most effective antitumor and anthelmintic activities. We could find out the presence of many pharmacologically important molecules in the ethanolic extracts of both plants, a quantitative analysis is required for proper pre-clinical studies. Though several of the molecules identified in these extracts were reported previously, many molecules such as chavicine, okanin, rubone, corilagin, apigetrin, leufolin A, loquatoside, agathisflavone and echinacin are reported in these plants for the first time in our study. The presence of these secondary metabolites needs to be further confirmed and quantified using appropriate techniques. A bioassay guided fractionation studies are therefore highly desirable in this regard.

Coloumn chromatography can be used with appropriate solvent system in order to do the fractionation of the crude extract. This technique could ideally able to isolate different molecules in an acceptably purified form in-order to conduct bioassays. For the anthelmintic activity, we have evaluated the nematicidal and trematocidal potentails of the extracts using egg hatch, larvicidal and adulticidal assays. Apart from this, fecal egg count assay and larval migration inhibition assay could be considered further to substantiate our findings. Though we have used *Haemonchus contortus* as a model organism for evaluating nematicidal potential of the extracts, owing to its remarkable propensity to develop resistance to synthetic anthelmintic drugs, experimental amenability and economical significance, several other nematodes belonging to the genus Ostertagia, Trichostrongylus, and Cooperia could also be considered further for exploring the broad spectrum activity of the isolated molecules. An *in vivo* study is also recommended using Swiss albino mice infected with *Syphacia obvelata* (a pinworm nematode) as it is a nematode parasite infecting man and lab animals.

The tumor reduction potentials of the plants *P. gangetcus* and *T. involucrata* can also be evaluated after fractionating the ethanol extracts. Molecules that are identified to have tubulin binding activity can be of first priority in the anticancer studies. Molecules isolated from the fractions can initially be analysed for its effect

on cell cycle using FACS method. Tubulin interacting molecule is known to disturb the microtubule assembly, thereby accumulating cells in G2/M phase. This can be analysed in a vast variety of lymphoma cell lines such as DLA, L428, L540, L591, L1236, KM-H2, HDLM-2, UHO-1, SUP-HD1, and DEV after treating the cells with appropriate concentration and subjecting the treated cells to FACS analysis. To further delineate the mechanism of cell cycle arrest, different cell cycle proteins (cyclins, Cdks and other protein machineries) can be analysed using western blotting or similar techniques in the treated cells. *In vivo* studies to be followed in order to promote this novel anticancer molecule for pre-clinical studies. Essential toxicological studies such as acute, sub-acute and chronic toxicity estimation needs to be done in mice models before evaluation its tumor reduction potential. Once the anti tumour properties are proven, the molecule could be subjected for its bioavailability and ADME studies in order to promote it as a drug candidate.

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PUBLICATIONS

Research Publications

1. **Menon, M.,** Raj, S., Raghavamenon, A. C., & Varghese, L. (2023). Evaluation of the Tumor Reduction Potentials of *Pleurolobus gangeticus* Using *In Vitro* and *In Vivo* Models. *Revista Brasileira de Farmacognosia*, *33*(6), 1287-1297. https://doi.org/10.1007/s43450-023-00462-y
2. **Menon, M.,** & Varghese, L. (2023). Evaluation of the phytochemical constituents and tumor reduction potentials of *Tragia involucrata* Linn. *Journal of Applied Biology and Biotechnology*, *11*(4), 84-91. DOI:10.7324/JABB.2023.87672
3. Krishnakumar, P., **Menon, M.,** Rajagopal, A., & Varghese, L. Trematocidal activity of certain plant species against rumen fluke *Fischoederius cobboldi*. *Journal of Medicinal Plants Research*. (In press).
4. **Menon, M.,** Mariya, D., & Varghese, L. Studies on the nematicidal potentials of *Pleurolobus gangeticus* (L.) J. St. –Hil. ex H. Ohashi & K. Ohashi (Fabacea) and *Tragia involucrata* Linn (Euphorbaceae). *Indian Journal of Experimental Biology*. (In press).

Research paper presentations in national and international conference/seminars

1. "Evaluation of anthelmintic activity of a medicinal plant" in the national conference on "Bio radiance 2019 Toxicity-Current perspectives" Pushpagiri Research Centre at Pushpagiri Medical College Campus. Thiruvalla. 6 July 2019.
2. "Investigations on the anticancer potentials of a medicinal plant using MCF 7 cell lines" in the national seminar on "Recent Trends and Challenges in Developing Green Chemical Methods for Industrial and Medicinal Applications", P. G and Research department of Chemistry, Christ College, Irinjalakuda. 19-20 February 2020.
3. "Investigations on the anthelmintic potential of a medicinal plant against a ruminant gut parasite" in the International webinar and symposium on

"Trends in Modern Biology" Department of Zoology**,** University of Calicut, Kerala, India 2021. 25-27 August 2021.

4. "Effect of *Tragia involucrata* Linn. on Cell proliferation and Tumor reduction" in the "41st annual conference of Indian Association of Cancer Research (IACR)", Amity Institute of Molecular medicine and Stem Cell Research Uttar Pradesh, Noida. 2-5 March 2022.
5. "Investigations on the *in vitro* anticancer potentials of a medicinal plant" in the national seminar "RECENT TRENDS IN DISEASE PREVENTION AND HEALTH MANAGEMENT" CSIR-NIIST, Trivandrum. 14-15 December 2022.