Potentials of *Scoparia dulcis* and *Vernonia cinerea* to interfere with cell cycle and alleviate tumour burden

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

DOCTOR OF PHILOSOPHY IN ZOOLOGY

Under the Faculty of Science

University of Calicut

By

JIMSY JOHNSON MSc

Under the supervision of

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

2024

DECLARATION

I hereby declare that the work presented in the thesis entitled "**Potentials of *Scoparia dulcis* and *Vernonia cinerea* to interfere with cell cycle and alleviate tumour burden**" is based on the original work done by me under the guidance of Dr. Leyon Varghese, Assistant professor, Department of Zoology, Christ College, Irinjalakuda, Thrissur, Kerala and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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

Place: Irinjalakuda
Date: 11-04-2024

CHRIST COLLEGE (AUOTONOMOUS)

IRINJALAKUDA, THRISSUR, KERALA, INDIA 680125

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This is to certify that **Ms. JIMSY JOHNSON** has completed her research work for the full period prescribed under the Ph.D. ordinance of the University of Calicut. This thesis "**Potentials of *Scoparia dulcis* and *Vernonia cinerea* to interfere with cell cycle and alleviate tumour burden**" embodies the results of her investigations conducted during the period at which she worked as a research scholar in this institute. I recommend the thesis to be submitted for evaluation and further for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

PRINCIPAL
Fr. Dr. Jolly Andrews
Associate Professor -
In-Charge of Principal
Christ College (Autonomous)
Irinjalakuda

IMMUNOLOGY & TOXICOLOGY RESEARCH LABORATORY
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…27-07-2024

CERTIFICATE

This is to certify that the thesis entitled '**Potentials of *Scoparia dulcis* and *Vernonia cinerea* to interfere with cell cycle and alleviate tumour burden**' 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. Jimsy Johnson**, 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
ASSOCIATE PROFESSOR & PhD GUIDE
IMMTOX RESEARCH LAB, DEPT. ZOOLOGY
CHRIST COLLEGE (AUTONOMOUS) IRINJALAKUDA - 680 125

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

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JIMSY JOHNSON

ABSTRACT

ABSTRACT

Scoparia dulcis and *Vernonia cinerea* were explored for their chemopreventive and chemotherapeutic potentials, focusing on their effects on cell cycle and tumor reduction. Extracts were obtained using ethyl acetate, ethanol and water, to test their bioactivities. Phytochemical screening using advanced methods like GC-MS and LC-MS identified 17 compounds in *S. dulcis* ethyl acetate extract (SDEA) and 6 compounds in *V. cinerea* ethyl acetate extract (VCEA). Genoprotective potentials were evaluated using bacterial reverse mutation and cytokinesis-blocked micronucleus assays, showing strong antimutagenic properties in SDEA and VCEA. Both extracts exhibited low antioxidant activity but significant anti-cancer potential against lymphoma cells, with IC_{50} values of 15.41 µg/ml (SDEA) and 61.24 µg/ml (VCEA). Cell cycle analysis revealed S and G2/M phase arrests, and pro-apoptotic properties were confirmed via flow cytometry by the treatment of these extracts. *In silico* studies showed strong binding affinity of SDEA constituents to topoisomerase I, indicating S phase arrest, while VCEA constituents indicated ferroptosis induction, later confirmed by *in vitro* assays. Toxicological evaluations in mice showed no mortality or toxic effects, deeming lower dosages safe for further pharmacological testing. DLA-induced solid tumor model studies in mice showed significant tumor volume reduction with SDEA and VCEA treatments. Histopathological and TUNEL analysis of tumor tissues confirmed the apoptotic cell death in treated mice, highlighting the potential of *S. dulcis* and *V. cinerea* ethyl acetate extracts in reducing tumor burden. These findings underscore the potential of these plants in cancer prevention and treatment.

സംഗ്രഹം

ഔഷധസസ്യങ്ങൾ നൂതന മരുന്നുകളുടെ കണ്ടുപിടുത്തത്തിൽ പ്രധാനപ്പെട്ട പങ്കുവഹിക്കുന്ന പ്രകൃതിദത്ത സ്രോതസ്സുകളിൽ ഒന്നാണ്. മനുഷ്യജീവനെ ഭീഷണിയായി നിൽക്കുന്ന അർബുദകോശങ്ങളെ പ്രതിരോധിക്കുന്നതിനും നശിപ്പിക്കുന്നതിനും സസ്യങ്ങൾ ഉത്പാദിപ്പിക്കുന്ന ദ്വിതീയ മെറ്റാബൊളൈറ്റുകൾ ഉപകാരപ്രദമാണെന്ന് സമീപകാല പഠനങ്ങൾ വ്യക്തമാക്കുന്നു. ആസ്ട്രേസ്യ സസ്യകുടുംബത്തിൽപ്പെട്ട വെർണോണിയ സിനേറിയ, പ്ലന്റാജിനേസ്യ സസ്യകുടുംബത്തിൽപ്പെട്ട സ്കോപേറിയ ഡൾസിസ് എന്നീ ഔഷധസസ്യങ്ങളിൽ അടങ്ങിയിരിക്കുന്ന ദ്വിതീയ മെറ്റാബൊളൈറ്റുകളുടെ സത്തുക്കൾ വേർതിരിച്ച് അവയ്ക്ക് അർബുദകോശങ്ങളുടെ വളർച്ചയെ തടയുന്നതിനും നശിപ്പിക്കുന്നതിലുമുള്ള സാധ്യതകളെ ഈ ഗവേഷണത്തിലൂടെ പഠന വിധേയമാക്കിയിരിക്കുന്നു. ക്രോമറ്റോഗ്രാഫി, മാസ്സ് സ്പെക്ടോമെട്രി എന്നീ സാങ്കേതികവിദ്യകൾ ഉപയോഗിച്ച് ഈ സസ്യസത്തുകളിലെ ജൈവസജീവമായ സംയുക്തങ്ങളെ തിരിച്ചറിഞ്ഞിട്ടുണ്ട്. ഡിഎൻഎ നാശത്തിന് പ്രേരിപ്പിക്കുന്ന രാസവസ്തുക്കളുടെ പ്രവർത്തനത്തെ നിർവീര്യമാക്കുന്നതിനും മന്ദഗതിയിലാക്കാനും മേൽപ്പറഞ്ഞ സസ്യങ്ങൾക്ക് സാധിക്കും എന്ന് ബാക്ടീരിയയിലും മനുഷ്യകോശങ്ങളിലും നടത്തിയ പഠനങ്ങൾ വ്യക്തമാക്കുന്നു. ജനിതകഘടനയിൽ വരുന്ന വ്യതിയാനങ്ങൾ പ്രത്യേകിച്ച് മ്യൂട്ടേഷനുകൾ അർബുദത്തിലേക്ക് നയിക്കുന്ന പ്രാഥമിക ഘടകങ്ങളാണ്. ജനിതകഘടനയെ സംരക്ഷിക്കാനുള്ള കഴിവ് അർബുദപ്രതിരോധത്തിൽ ഇവയുടെ പ്രാധാന്യം വെളിപ്പെടുത്തുന്നു.

ലിംഫോമ അർബുദവിഭാഗത്തിലെ കോശങ്ങളെ നശിപ്പിക്കുന്നതിനും അവയുടെ വ്യാപനം തടയുന്നതിനും ഈ സസ്യങ്ങൾക്ക് സാധിക്കുമെന്ന് ഈ പഠനത്തിലൂടെ വെളിപ്പെട്ടിട്ടുണ്ട്. കോശവിഭജനചക്രത്തിന്റെ പ്രത്യേക ഘട്ടങ്ങളെ നിശ്ചലമാക്കി കോശങ്ങളെ അപ്പോപ്റ്റോസിസ് എന്ന പ്രതിഭാസത്തിലൂടെ നശിപ്പിക്കാൻ ഈ സസ്യങ്ങളിലെ ദ്വിതീയ മെറ്റാബോളൈറ്റുകൾക്ക് സാധിക്കും. കൂടാതെ വെർണോണിയ സിനേറിയ എന്ന സസ്യത്തിന് ഫെറപ്റ്റോസിസ് എന്ന പ്രക്രിയയയിലൂടെ അർബുദകോശങ്ങളെ അതിവേഗം നശിപ്പിക്കാൻ സാധിക്കും എന്നും ഈ പഠനത്തിലൂടെ കണ്ടെത്തിയിരിക്കുന്നു. അർബുദകോശങ്ങളുടെ വളർച്ച മുരടിപ്പിക്കുന്നതിൽ ഈ സസ്യങ്ങളുടെ പ്രാധാന്യം സ്ഥിരീകരിക്കുന്നതിനുവേണ്ടി സ്വിസ്സ് ആൽബിനോ വിഭാഗത്തിൽ പെട്ട എലികളിലും ഈ സസ്യങ്ങളുടെ ഈഥൈൻ അസറ്റേറ്റ് സത്തുക്കൾ പരീക്ഷിച്ച് ഇവയുടെ പ്രാധാന്യം സ്ഥിരീകരിച്ചു.

ആരോഗ്യമുള്ള മനുഷ്യകോശങ്ങളിൽ പാർശ്വഫലങ്ങളുണ്ടാക്കാതെ അർബുദ കോശങ്ങളെ മാത്രമായി നശിപ്പിക്കുന്നതിനും ഔഷധമൂല്യമുള്ള സസ്യ സംയുക്തങ്ങൾ സഹായിക്കുന്നു. കൂടാതെ ഇവ താരതമ്യേന എളുപ്പത്തിൽ ലഭ്യമാകുന്നതും പരിസ്ഥിതി സൗഹൃദവുമായ ഔഷധസ്രോതസാണ്. അതിനാൽ ഈ പഠനത്തിന്റെ കണ്ടെത്തലുകൾ മേൽപറഞ്ഞ സസ്യങ്ങൾ ഉൽപ്പാദിപ്പിക്കുന്ന ജൈവസജീവമായ സംയുക്തങ്ങളെ തുടർപഠനത്തിന് വിധേയമാക്കി നൂതന അർബുദ ഔഷധ നിർമ്മാണരംഗങ്ങളിൽ മുഖ്യധാരയിൽ കൊണ്ടുവരുവാൻ സഹായിക്കുന്നതാണ്.

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ABBREVIATIONS

ACSL4	–	Acyl-CoA Synthetase long chain family member-4
ASE	-	Accelerated Solvent Extractor
ATP	-	Adenosine triphosphate
B.WT.	-	Body Weight
BSA	-	Bovine Serum Albumin
CBPI	-	Cytokinesis-block proliferation Index
Cdk	-	Cyclin-dependent kinase
CHO	-	Cholesterol
CP	-	Cyclophosphamide
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
COX2	–	Cyclo Oxygenase 2
CSC	-	Cancer Stem Cells
DC	-	Differential Count
DDR	-	DNA Damage Response
DFO	-	Deferoxamine
DLA	-	Dalton's Lymphoma Ascites
DMSO	-	Dimethyl Sulfoxide
DMT1	–	Divalent Metal Transporter 1
dNTP	-	Deoxynucleotide triphosphate
DPPH	-	2, 2-Diphenyl-1-picrylhydrazyl
dUTP	-	Deoxyuridine triphosphate
ESR	-	Erythrocyte Sedimentation Rate
FACS	-	Fluorescence Activated Cell Sorting
FITC	-	Fluorescein isothiocyanate
FPN	-	Ferroportin
GCMS	-	Gas Chromatography Mass Spectrometry
GPX	-	Glutathione Peroxidase
GSH	-	Glutathione
GSR	–	Glutathione disulfide reductase
Hb	-	Haemoglobin
HDL	-	High-density lipoprotein

IAEC	-	Institutional Animal Ethics Committee
IC_{50}	-	Inhibition Concentration 50
JNK	–	Jun N-Terminal Kinase
LC-MS	-	Liquid Chromatography-Mass Spectrometry
LDL	-	Low-density lipoprotein
LPCAT3	–	Lyso Phosphatidyl Choline Acyl transferase-3
MCH	-	Mean Corpuscular Haemoglobin
MCHC	-	Mean Corpuscular Haemoglobin Concentration
MCV	-	Mean Corpuscular Volume
MIF	–	Macrophage migration Inhibitory Factor
MN	-	Micronuclei
NC	-	Necrosis
NCCS	-	National Centre for Cell Sciences
NER	–	Nucleotide Excision Repair
NF-κB	-	Nuclear factor kappa-light-chain-enhancer of activated B cells
OECD	-	Organization for Economic Co-operation and Development
PBS	-	Phosphate Buffered Saline
PCV	-	Packed Cell Volume
PDB	-	Protein Data Bank
PI	-	Propidium Iodide
PLT	-	Platelet
PMC	-	Pleomorphic cells
PUFA	-	Polyunsatuarated Fatty Acid
RBC	-	Red Blood Cells
RCSB	-	Research Collaboratory for Structural Bioinformatics
RLS	–	Reactive Lipid Species
RMSD	-	Root Mean Square Deviation
RNS	–	Reactive Nitrogen Species
ROS	-	Reactive Oxygen Species
RPMI	-	Roswell Park Memorial Institute
RT	-	Retention time

SDEA	-	*Scoparia dulcis* Ethyl Acetate extract
SDET	-	*Scoparia dulcis* Ethanol extract
SDWT	-	*Scoparia dulcis* Water extract
SEM	-	Standard Error of Mean
SGOT	-	Serum glutamic-oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic acid transaminase
SOD	-	Superoxide Dismutase
STEAP 3	–	Six Transmembrane Epithelial Antigen of Prostrate 3
TB	-	Total Bilirubin
TC	-	Total Count
TCF	-	Ternary Complex Factor
TFC	-	Total flavonoid content
TGL	-	Triglycerides
TLC	-	Thin Layered Chromatography
TNF	-	Tumour Necrosis Factor
TP	-	Total Protein
TPC	-	Total phenol content
TUNEL	-	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
VCEA	-	*Vernonia cinerea* Ethyl Acetate extract
VCET	-	*Vernonia cinerea* Ethanol extract
VCWT	-	*Vernonia cinerea* Water extract
VEGF	-	Vascular Endothelial Growth Factor
VLDL	-	Very Low Density Lipid

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CHAPTER 1

GENERAL INTRODUCTION

Cells undergo division through a highly co-ordinated and strictly regulated process in order to pass down their genetic material intactly into the daughter cells. Cell division is typically a cyclic process involving a series of events that occur in the cells and is also known as cell cycle. The cell cycle enables the precise duplication of the vast amount of DNA in the chromosomes and segregation of the copies to yield two genetically identical daughter cells (Philpott & Yew, 2008). Cell cycle progression is taking place under stringent genetic control to ensure tissue homeostasis. There are various cell cycle checkpoints to check the proper completion of each phase prior to the entry into the next phase in normal cells. It also integrates DNA repair with cell cycle progression (Wang, 2021). Deregulation of cell cycle thus leads to uncontrolled proliferation of abnormal cells resulting eventually into neoplasm (Park & Lee, 2003). Regardless of tumour type, multiple genes controlling the cell cycle are damaged or mutated in most tumour cells. The accumulation of such damages shifts the equilibrium between cell death (programmed cell death or apoptosis) and proliferation (cell division) which eventually lead to tumour development (Sandal, 2002).

The mostly accepted theory on the development of cancer is multistage carcinogenesis theory. It divides the entire process into three stages such as initiation, promotion and progression. Initiation stage commenced by the action of a genotoxic agent, producing a lesion on chromosomal DNA which will be either repaired by the cells itself or reproduced. There are both exogenous and endogenous factors that can cause DNA damage. Time factor is very essential in the repair

process. If the cell cycle is delayed, the cells will get enough time for appropriate repairing of the lesion. If not, the lesions will be transmitted to new cells. It is not easy to distinguish the initiated cells from normal cells. Once initiated, the cells bearing the irreversible alteration of the genetic material attains the potential to develop into neoplastic cell clones as a consequence of additional stimulus to tumour promoters which give growth advantages to cells. This stage is called promotion and is characterised by the insensitivity to growth signals. In this state, cells exhibit the phenotypic features of cancer cells. Recent perspectives about tumour initiation is that the genetic alterations priming the tumorigenesis can be occurs as a sudden one time catastrophe in contrary to the conventional somatic mutation theory which explains that the various mutations gradually accumulating over time in somatic cells leads to the onset of tumour (Shah, 2024). It is followed by progression where rapid proliferation of neoplastic cells with biochemical and morphological changes acquires invasive and metastasizing properties (Baba & Câtoi, 2007).

Cancer chemotherapeutic drugs are intended for delaying or prohibiting the aberrant proliferation of cells. Tumour cells are efficiently sensitized by chemotherapeutic drugs than the normal cells since rapid, continuous and uncontrolled cell division is a key hallmark of cancer cells. Chemotherapy to treat cancer initially comes from the successful evidence of nitrogen mustard in the treatment of lymphoma including oral derivatives like chlorambucil and ultimately cyclophosphamide (DeVita & Chu, 2008). Now, there are several classes of chemotherapeutic drugs in clinical practice based on their way of actions. Some of them are ‘alkylating agents, antimetabolites, topoisomerase inhibitors and microtubule poisons’. In addition to these primary mechanisms they exert additional

or secondary effects such as the production of reactive oxygen species (ROS) which further increases their cytotoxicity (Tilsed et al., 2022).

Sensitivity and resistance to a particular chemotherapeutic agent is different for each cancer type and are determined by the biochemical features of cancer cells. Sometimes, drug efflux pumps hinder the delivery of drugs to cell. Some cancer cells possess intrinsic mutations (mutations to tumour suppressors like TP53) which render them less sensitive to chemotherapy. Increased expression of repair related genes is found in association with the resistance to some platinum based drugs (Amable, 2016). Understanding about the mechanisms of chemotherapeutic drug resistance will be helpful to improve the efficacy of chemotherapetic drugs. For example the use of PARP (poly ADP-ribose polymerases - which recruits repair proteins at the lesions) inhibitors along with the drugs act by inducing DNA damage was found to overcome the drug resistance and improved the clinical efficacy (Zhu et al., 2020).

Combination therapy is a powerful approach to eradicate the cancer cells effectively by potentially reducing drug resistance. The use of multiple drugs having different anticancer mechanisms will act additively or synergistically which improve the total efficacy of therapy. Compared to the conventional mono-therapeutic approach, the toxicity and side effects exerted by the combination therapy is relatively less because of different pathways targeted. Additionally, in combination mode, the quantity of individual drug essential for causing therapeutic effects is often small. Besides, in most cases one drug in the combination regimen will be of antagonistic action in terms of cytotoxicity to normal cells, essentially protecting normal cells from cytotoxic effects of other drugs (Blagosklonny, 2005).

Combination therapy is also advantageous to reduce the susceptibility to drug resistance over mono-therapy. Combination therapy including the agents that target cancer stem cells (CSCs) can eliminate CSCs successfully, thus attenuating the likelihood of relapse (Bayat Mokhtari et al., 2017).

Researchers around the world are constantly looking for newer and better therapeutic options for cancer treatment in addition to the conventional treatment modes. The modern approaches recently emerged in this field include stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, chemodynamic therapy, sonodynamic therapy and ferroptosis-based therapy (Debela et al., 2021). Activating ferroptosis is a potential strategy to overcome the drug resistance which is often found in association with the traditional cancer chemotherapeutic treatments. Generation of lipid peroxides mediated by oxidative stress generally leads to ferroptosis. Iron in an oxygen-rich environment generates ROS through the Fenton reaction and is considered as the bioelement responsible to elicit ferroptotic mode of cell death. Ferroptosis is directly linked to iron metabolism, lipid metabolism and antioxidant metabolism. Since cancer cells avoid the occurrence of ferroptosis to some extent through metabolic reprogramming, ferroptosis inducing small molecules can easily sensitise the cancer cells specifically and efficiently. Interestingly recent studies have shown the immunogenic nature of ferroptosis which may elicit a vaccination-like effect to trigger antitumor immunity. Hence it will be also helpful to overcome immunotherapy resistance. Efimova et al. (2020) reported that induction of ferroptosis by a small molecule called RAS-selective lethal 3 in cancer cells in turn stimulated the adaptive immune system in preclinical models. Thus, ferroptosis

inducers can enhance the chemotherapeutic efficacy and long term survival of cancer patients.

Diverse types of pharmacologically active molecules are existing in nature. But only a few are explored yet. In search for novel active drugs with high selectivity, fewer side effects, cost- effectiveness and minimum level of drug resistance, natural sources especially plant-derived compounds are promising sources to be explored. It is noteworthy that more than 60% of synthetic drugs used in clinical practice are derived from natural sources and out of which 75% are contributed by plants (Newman & Cragg, 2016). Antitumour drugs with plant origin used in clinical practice include paclitaxel (Taxols) and its analogs docetaxel and cabazitaxel; camptothecin and its analogs belotecan, topotecan, and irinotecan; vinblastine, vincristine, and their analogs vindesine and vinorelbine; podophyllotoxin and analogs etoposide and teniposide (Sharifi-Rad et al., 2019). Taxol is a taxane diterpene obtained from the crude extract of *Taxus brevifolia* Nutt. (Western yew) bark. An alkaloid, Camptothecin isolated from *Camptotheca acuminata* is acting as DNA topoisomerase I inhibitor. Vinblastine and vincristine are the alkaloids having cell cycle specific activity in M phase by preventing the formation of mitotic spindles and these are isolated from the plant called *Catharanthus roseus*. Podophyllotoxin is an antimitotic lignan isolated from the plants of the Podophyllum genus.

The knowledge about the medicinal plants used in various traditional medicinal practices (ethnomedicine) is helpful in discovering the new drugs with high therapeutic potential successfully. Fabricant and Farnsworth (2001) estimated that out of 122 globally used drug compounds of plant origin, 80 % are of

ethnomedical importance. Thus integrating the ethnomedicinal information into drug discovery will obviously help to tackle the serious challenges including the time and expense in new drug discovery. The incomparable chemical diversity, structural complexities and steric properties, novel mechanism of action and selectivity of molecular targets, etc are some of the remarkable and valuable features of phytochemicals in drug discovery (Yuan et al., 2016).

Plantaginaceae and Asteraceae are large, diverse families of flowering plants with worldwide distribution. Many plants from these families are used for medicinal purposes. The well-known cardiac glycoside, digoxin, was isolated from the *Digitalis* species of plants belonging to the Plantaginaceae family. The sesquiterpene lactones namely artemisinin and arglabin are approved drugs for human malaria and cancer respectively and both are isolated from *Artemisia* species of Asteraceae family (Sülsen et al., 2017). *Scoparia dulcis* L. (family-Plantaginaceae) and *Vernonia cinerea* (L.) Less. (family-Asteraceae) are the plants explored in this study for their chemopreventive and/or therapeutic effect in curing tumours. These plants are often seen in similar habitats.

Scoparia dulcis L. is commonly known as Kallurukki in Malayalam and Sweet broom weed in English. Various Indian ethnic groups use this plant for curing diseases like diabetes, jaundice, stomach problems, piles, skin diseases, fever and headaches (Bhuyan & Baishya, 2013; Jeeva & Femila, 2012; Jeyaprakash et al., 2011). Previous reports on its anti-diabetic, anti-inflammatory, antioxidant properties were available (Mishra et al., 2013; Tsai et al., 2011). However, studies regarding the chemotherapeutic potentials of chemical constituents from this plant are very limited. *Vernonia cinerea* (L.) Less. is commonly known as

Poovamkurunnila in Malayalam and Ash coloured fleabane in English. This Indian medicinal plant is used for curing several illness (The Ayurvedic pharmacopoeia of India, 2001). Combination of this plant with other herbal ingredients is used to cure breast tumours by the tribal people in the southern region of Western Ghats of India (Ayyanar and Ignacimuthu, 2005). Various pharmacological potentials such as anti-inflammatory, anti-diabetic, nephroprotective and antimicrobial properties were studied in different extracts of this plant (Amuthan et al., 2021; Gupta et al, 2003; Dhanalakshmi et al., 2013; Haque et al., 2013; Pratheeshkumar and Kuttan, 2009; Sreedevi et al., 2011). Anticancer potential of this plant was reported in the dichloromethane fraction of ethanolic extract and was found to be exerting cytotoxicity against various human epithelial cancer cells (Beeran et al., 2014). Methanol extract of *V. cinerea* was reported to be effective in interfering with the B16F-10 melanoma cells invasion (Pratheeshkumar & Kuttan, 2011). Its ethanol and chloroform extracts decreased the cancer cell count and increased the lifespan of mice inoculated with lymphoma cells into their peritoneal cavity to develop ascites tumour (Sangeetha & Venkatarathinakumar, 2011).

Since different tumours exhibit widely varied reactions in response to each chemotherapeutic agent, there is a necessity to assess the antitumour activities of drugs in a particular tumour model. This study investigate the potentials of *S. dulcis* and *V. cinerea* in protecting DNA from genotoxic agents as well as their chemotherapeutic prospects with special emphasis on their effects on cell cycle and tumour reduction.

Objectives of the study are:-

- Extraction of *Scoparia dulcis* L. and *Vernonia cinerea* (L.) Less. using ethyl acetate, ethanol and water and their phytochemical characterization.
- Study the genoprotective potential of *S. dulcis* and *V. cinerea* extracts in prokaryotic and eukaryotic systems.
- Study the cytotoxic/cytostatic potential of *S. dulcis* and *V. cinerea* extracts using cell proliferation, cell cycle and apoptosis assays in mice lymphoma cell line.
- *In vivo* toxicity profiling and antitumour studies of the active extracts using mice model.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Cancer

Cancer is a highly complicated and devastating disease that is impacted by several factors. It is often described as unrestrained multiplication of aberrant cells. Oncogenic transformation of normal cells results in neoplastic cells acquiring some adaptive capabilities. It involves both genetic as well as metabolic transformation. According to Hanahan & Weinberg (2000) the important hallmarks of cancer include "self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evading programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis". Two additional hallmarks of cancer cells referred to as 'emerging hallmarks' were identified later. It includes reprogramming of cellular metabolism and avoiding immune destruction. Tumour microenvironment plays an integral role in the tumorigenesis and malignant progression and it is composed of heterogeneous and interactive populations of cancer cells and cancer stem cells together with the recruited stromal cell types (Hanahan, 2022). Complex molecular signalling pathways are involved in tissue homeostasis and their dysregulation due to genetic mutations and epigenetic changes drive cancer progression and enable the cancer cells to attain the above mentioned characteristic features (Sever & Brugge, 2015).

Sustained action of mitogenic signals render growth advantages to cancer cells. Some cancer cells synthesise growth factors by themselves and act in an autocrine manner. In other cases, cancer cells induce the nearby normal cells to produce growth factors and work by paracrine signalling. Upregulation of receptor

proteins in the cell surface is another adaptive feature of cancer cells. Structural modifications to receptors enabling ligand independent receptor activation often results in excessive multiplication of cancer cells (Hanahan & Weinberg, 2011). Epidermal growth factor receptor (EGFR) mediated signalling pathway is the prime most pathway in oncogenesis. EGFR activation stimulates several downstream signalling pathways such as "RAS-RAF-MEK-ERK MAPK and AKT-PI3K-mTOR" which ultimately trigger the transcription factors essential for cellular multiplication. Ligand-receptor interactions, dimer formation and subsequent trans-autophosphorylation of receptors and the recruitment of signalling proteins or adaptors are key events occuring during the signal transduction. Mutations to EGFR accompanied by the ligand independent signal transduction can initiate an oncogenic process (Wee & Wang, 2017). RAS activating mutations are seen as the causative alteration in some cancers since RAS can activate three important effector proteins such as RAF-1, PI3K and RalGDS (Ras-like guanine nucleotide-dissociation stimulator) (Fernández-Medarde & Santos, 2011). RAF activates MEK and ERK. Passage of these into nucleus triggers TCF transcription factors that in turn trigger immediate early genes including c-FOS and c-MYC which further turn on late-response genes. The c-FOS and c-JUN function in the transcriptional activation of cyclin D1 thus mediating G1 progression in cell cycle. The PI3K-AKT-mTOR pathway also comes downstream to the EGFR pathway. It regulates various cellular functions like metabolism, proliferation, cell survival, protein synthesis and migration. The PI3K converts PIP2 (phosphatidylinositol (3, 4)-bisphosphate) to PIP3. Akt, also known as PKB on binding to PIP3, attains partial activation. The mTOR or DNA PK phosphorylates Akt, thus fully activated. Fully active Akt phosphorylates several proteins in the nucleus as well as cytoplasm and

intervene in cellular metabolism, apoptosis and cell division. PP2A and PTEN are the negative effectors of Akt signalling (Hemmings & Restuccia, 2012). The mediators of negative feedback regulatory mechanisms to check the excess cell proliferation are often downregulated during oncogenesis. Loss of function mutations in PTEN gene leading to amplification of PIP3 signalling is found in some human cancers (Jiang & Liu, 2009).

Apoptosis is the intrinsic death strategy of the cell that controls a number of pathological and physiological events and has been remarkably preserved throughout evolution. The BCL-2 family of proteins are the chief regulators of this process. Cancer cells acquire the capability to evade this phenomenon during tumorigenesis. During oncogenic transformation, cells experience a variety of stressful conditions that elicit stress response in cells that favour pro-survival or anti-apoptotic signals (Fulda, 2011). Cancers arising from haematopoietic systems are more vigorously inhibiting apoptosis since these cell types are more sensitised for such death and often tend to change the expression of BCL-2 family members. The mechanisms by which cancer cells resist apoptosis consists of overexpression of proteins linked to survival (BCL-2, and BCL-XL) and diminished expression of pro-apoptotic proteins (BIM, BID, BAX, Noxa and PUMA) and suppression or inhibition of the pore-forming proteins (BAK and BAX) (Singh et al., 2019).

In order to maintain the biosynthetic, bioenergetic requirements tumour cells have altered nutrition uptake and metabolic patterns. As the consequences of mutations to multiple oncogenes and tumour suppressor genes, the various signalling pathways regulating the metabolic processes inside the cells are fluctuated. The most important alteration is the constitutive activation of aerobic glycolysis (Warburg effect) in cancer cells regardless of the oxygen availability

inside the cells. Glucose is the main energy source in cells which is used in the generation of ATP by glycolysis and oxidative phosphorylation. Due to the increased energy demands for the rapid proliferation, cancer cells undergo metabolic reprogramming into the fast but less efficient glycolysis and convert glucose to lactate rather than moving on to mitochondrial respiration even in the presence of functional mitochondria. Thus the oxygen consumption in cancer cells is extraordinarily high compared to normal cells. The alterations in the genes such as PI3K, PTEN, Myc and P53 are chiefly involved in the metabolic shifts occurring in cancer cells (Jang et al., 2013). Stimulation of PI3K signalling in turn activates the downstream effector AKT and stabilises the HIF-1. AKT stimulates several glycolytic enzymes and the mTOR pathway. It also causes the overexpression of transmembrane glucose transporters to meet the increased glucose uptake of cancer cells. HIF-1 can activate pyruvate dehydrogenase kinases denying the entry of pyruvate to Krebs cycle. MYC activation has a positive impact in the stimulation of lactate dehydrogenase and some glycolytic enzymes. Due to inhibitory relationship of p53 with glycolysis (deactivation of one glycolytic intermediate), loss of function mutation of this gene contributes to enhancing glycolysis in cancer cells (Vousden & Ryan, 2009). Inherent connections between the cancer cell proliferation and glycolysis (fermentation) are also consistent with the recent studies (Martins Pinto et al., 2023).

Angiogenesis, the generation of new vascular channels from the already existing ones, plays a pivotal role in tumorigenesis since it helps in the supply of nutrients for the rapidly dividing cells. Endothelial cells in the normally quiescent stage undergo proliferation only in the presence of proangiogenic signals generated on nutrient and oxygen deprivation (Kazerounian & Lawler, 2018). The shifts in

equilibrium between the pro-angiogenic and anti-angiogenic signals is found in association with tumour progression. In opposition with physiological angiogenesis, tumour angiogenesis results in the partially dysfunctional and irregular tumour endothelial cells showing altered metabolism and gene expression and are resistant to senescence (De Palma et al., 2017). VEGF is regarded as the key angiogenic factor which mediates other angiogenic signalling. In addition to this there are several growth factors which can act as pro-angiogenic factors. It includes fibroblast growth factors, tumour necrosis factor-α, transforming growth factor -β), and angiopoietin. Matrix metalloproteinases are an important class of proteins involved in angiogenesis since they mediate the breakdown of the basement membrane of already existing blood vessels and the extracellular matrix and enable the release of proangiogenic factors (Ansari et al., 2022). Tumour cells can generate proangiogenic signals as their own and trigger immune cells especially neutrophils which further facilitates angiogenesis. Tumour related inflammation can also contribute to angiogenesis since oxygen deprived conditions found in tumour cells activates the expression of pro-inflammatory factors (Zhao et al., 2021; Kammerer et al., 2020).

Inflammation is an inevitable part of cancer progression. It is the body's reaction to various kinds of tissue trauma aimed for tissue repair and it involves the functioning of specific immune cells and the associated cellular alterations. The persistent inflammatory response resulting from the extrinsic factors such as viral attacks, autoimmune disorders, obesity, the use of tobacco and alcohol sometimes leads to chronic states which generate the environment suitable for tumorigenesis. Conversely, the genetic alterations in the cancer cells can activate the expression of inflammatory factors leading to the creation of tumour microenvironment (Singh et

al., 2019). The immune cells like macrophages and leukocytes produce ROS to combat against infectious agents as part of chronic inflammatory response. This may eventually cause alterations in the genetic materials of adjacent cells. Also, the TNF-α and the MIF liberated by these immune cells augments the DNA damage. The macrophage inhibitory factor often hinders the action of P53 and favours tumorigenesis (Pollard, 2004). Tumour associated Macrophages originated from monocytes attracted by the chemokines are important among the immune cells infiltrated into the inflammatory site and they produce the factors activating growth, angiogenesis and metastasis. The TNF, IL-1 and 6, and chemokines released by tumour cells or tumour associated immune cells facilitate malignancy. TNF stimulates NF-κB and JNK signalling. TNF is actually a "double edged sword" which has both tumour promoting as well as inhibiting roles (Wang & Lin, 2008).

Each normal cell maintain its own phenotypic identity and functional features intactly as obtained during organogenesis and is referred to as phenotypic plasticity. Tumour specific microenvironment pushes the cells to escape from their terminally differentiated state and acquire further phenotypic changes facilitating tumour progression. This enables the tumour cell to better adapt in the unfavourable environment (Yuan et al., 2019). Senescence is a phenomenon taking place in cells with aging and are influenced by DNA damage, free radicals, telomere shortening, oncogene overexpression and a variety of stress factors which pushes the cells to arrest their further growth. This process is found to be a part of immune surveillance and thus, a barrier for tumorigenesis. Loss of function mutation to $p16^{INKA4}$ or p53 help cells to evade this process leading to malignancy. SASP (Senescence Associated Secretory Phenotype) factors released by the senescent cells sometimes trigger inflammation in adjacent cells and evoke tumour growth

according to the host cell types (Wyld et al., 2020). Though the senescent cells are irreversibly withdrawn from cell cycle, their capability for retaining their status for an extended time periods (usually years), there are chances for the cells to resume their growth and promote tumorigenesis (Zeng et al., 2018a).

2.2. Genomic Instability and Initiation of Tumour

Genetic stability is essential for cells to get away from the errors occurring during DNA replication, endogenous genotoxic stress factors such as reactive oxygen species (ROS) generated as part of cellular metabolism and exogenous carcinogenic agents (Yao & Dai, 2014). Cells preserve the genomic stability by DNA damage and mitotic checkpoints as well as by DNA repair. Alteration to these mechanisms or damage to the genes or chromosomes result in carcinogenesis. Thus loss of genomic stability is a most prominent hallmark of cancer cells. According to Loeb (2001) loss of function mutation of genes involved in DNA repair and maintenance of genomic integrity might be a reason for genomic instability and initiation of tumour phenotype. Microsatellite (simple tandem nucleotide repeats, repetitive motifs of 1 to 6 nucleotides, scattering widespread the human genome) instability due to the defects in the DNA mismatch repair system and chromosome instability as a result of chromosome missegregation (due to incorrect chromosome number and abnormal chromosome structure) are other leading factors (Kunkel, 1995; Rao et al., 2009).

The DNA damage checkpoint exists in the cells where a tumour suppressor gene called p53 stops cell division to check the transfer of damaged genetic materials and direct them to repair. If the damage remains to be unsolved, the same gene induces apoptosis (programmed cell death) or senescence thereby suppresses the tumour initiation (Efeyan et al., 2006; Zhang et al., 2011). Thus p53 gene is

considered as the guardian of genome which initiate several DNA damage responses (DDR) when the genes get damaged (Williams & Schumacher, 2016). DNA repair pathways which take part in the maintenance of genomic stability include 'nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and DNA double strand break repair (DSBR)'. DNA lesions formed by single strand breaks or by base modifications are generally repaired by NER, BER and MMR. Whereas the DNA double strand breaks are repaired specifically by 'homologous recombination' or 'non-homologous end joining'. Variations, more specifically the mutations to the genes involved in DDR often enables the cells to survive with the unrepaired DNA damages which ultimately results in cancer. At present, induction of "synthetic lethality" to repair genes in cancer cells which were formed due to previous genomic insults are suggested as a strategy for the removal of cancer cells (Abuetabh et al., 2022). For instance, conventional chemotherapies in cancer cells negative for BRCA proteins (involved in homologous recombination) results in SSBs which can be repaired by the cancer cells itself thus exhibit resistance. In such cells, treatment with PARP inhibitors create more SSBs leading to the formation of DSBs which cannot be repaired by these cell since they possess mutated BRCA proteins (Livraghi & Garber, 2015; Helleday, 2011).

Chromosome stability is maintained by mitotic checkpoint where a number of conserved proteins monitor whether the spindle microtubules oriented correctly with all chromosomes before proceeding to nuclear division. Telomere maintenance is another mechanism by which cells maintain genomic integrity. Telomeres are the conserved tandem repeats which project the ends of chromosomes from fusion. Excessive telomere shortening results in chromosome instability and tumour initiation (Maser & DePinho, 2002). Unstable telomeres results in the fusion of

chromosome ends leading to the loss of genomic integrity. Telomere shortening is counteracted by the functioning of telomerase and by means of alternative lengthening of telomeres (ALT). Recent studies indicate that telomere shortenting is linked to activation of retrotransposones such as LINE1 and SINE that leads to chromatin openness and inductions several types of genetic mutations thus compromising genomic integrity. Cells with short telomeres are more prone to genomic instability and it is associated to early tumorigenesis (Lu & Liu, 2023).

In addition to the alteration of genes controlling DNA repair, deregulation of proteins involved in antioxidant defences and liver xenobiotic metabolism are also the causative factors of genomic instability. Glutathione, glutathione peroxidases, superoxide dismutase and catalases could scavenge the free radicals formed inside the body. However in the presence of excess free radicals, the endogenous antioxidant enzymes might not be sufficient leading to oxidative stress and results in radical induced DNA damage and onset of cancer. Liver metabolising enzymes (phase I and II) are essential for the removal of toxic compounds from the body. Phase I reactions mediated by p450 cytochromes convert the xenobiotic compounds into reactive forms which further undergoes conjugation reactions by phase II enzymes into non-reactive water soluble forms and hence easily eliminated from the body. However, phase I reactions sometimes convert certain non-cancerous compounds into DNA reactive compounds which causes mutations in DNA and initiates carcinogenesis (Langie et al., 2015).

2.3. Chemotherapeutic Agents

Cancer chemotherapy means the administration of the cytotoxic drug aimed to eliminate or lessen the tumour burden which helps to improve and prolong the patient's life. Cancer chemotherapeutic drugs can be classified into two groups

such as plant-derived (natural) and synthetic based on their origin. Classification based on mechanism of action include the following categories such as 'alkylating agents, antimetabolites, topoisomerase inhibitors, mitotic spindle inhibitors, and others'. In any case all of them are targeting cells undergoing division and are therefore non-selective to tumour/normal cells and is the case for all side effects.

Alkylating agents generate cross links between DNA strands. It blocks replication of DNA and transcription of RNA. "Aziridine, chlorambucil, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, melphalan, nitrogen mustards, thiotepa" are some examples of classical alkylating agents. Platinum complexes like Carboplatin, Cisplatin and Oxaliplatin are also included in this category. Alkylating drugs are usually used in the treatment of lymphoma, breast cancer and multiple myeloma. Clinical toxicities often seen associated with the use of these drugs include nausea, vomiting, bone marrow depression with anaemia, leukopenia and thrombopenia (Finch & Burns-Naas, 2014).

Antimetabolites are cytotoxic chemotherapeutic agents which interfere with the DNA replication or make DNA strand breaks. This group consists of 'pyrimidine antagonists like cytarabine, 5- fluorouracil (5-FU), gemcitabine, and capecitabine, purine antagonists like fludarabine, purine analogs such as 6-mercaptopurine, azathioprine, and cladribine, antifolates such as methotrexate, pemetrexed, and pralatrexate, and ribonucleotide reductase inhibitors like hydroxyurea'. These drugs inhibit essential biosynthetic pathways involved in nucleotide synthesis, DNA replication or cause the formation of DNA strand breaks when they get inserted into nucleic acids leading to cell death (Lansiaux, 2011).

A third group of molecules that prevent the activity of topoisomerases I and II in DNA replication leads to the accumulation of single strand breaks

resulting in cell death. Unwinding of strands are necessary for the execution of both replication and transcription in prokaryotes and eukaryotes. But this process often causes topological changes making overstrain to DNA strand. Cell tackle this tortional strain by the help of enzymes called DNA topoisomerases. It create temporary breaks at the necessary region by making a bond between tyrosine residue at the enzymes' active site and a phosphodiester of DNA by a transesterification processes. So that the DNA strand can rotate freely to release the strain and after this re-annealing is executed by a second esterification reaction and release the enzyme from the site (Woodfield et al., 2000). Topoisomerase are broadly categorised as type I and type II, each of them are further classified into sub types (A and B). Type I enzymes often induce breaks at any one strand, whereas, type II induce lesions at both strands. Topoisomearase inhibitors are important target cancer therapy since several cancer cell types especially breast cancer cells over expresses these enzymes (Millis et al., 2015). Irinotecan and topotecan are examples of topoisomerase I inhibitors. These drugs are water soluble derivatives of camptothecin which was initially isolated from the plant *Camptotheca acuminata*. These drugs directly bind with topoisomerase and creates DNA breaks and preventing the sealing of DNA strand breaks by stabilising the enzyme-DNA complex. Belotecan is a novel, less toxic drug in this category having identical action (Park et al., 2016). Topoisomerase II inhibitors include etoposide, teniposide and anthracyclines like doxorubicin and mitoxantrone. Etoposide and teniposide are the derivatives of podophyllotoxin isolated from the extracts of *Podophyllum peltatum* (American May apple) and are applied for the treatment of many solid and haematological malignancies (Buzun et al., 2020).

Mitotic spindle inhibitors are another class of chemotherapeutic agents.

They interfere with the assembling and working of mitotic spindle tubules leading to mitotic arrest in metaphase and ultimately results in cell death. Vinca alkaloids are binding with the protein tubulin, thus preventing microtubule polymerisation, whereas, docetaxel and paclitaxel make stable and nonfunctional microtubules by enhancing the microtubule formation (Fischer, 2005).

In addition to the above mentioned major categories, there are some other classes of chemotherapeutic agents. The enzyme l-asparaginase could cleave the amino acid l-asparagine which is a prerequisite for normal cell metabolism. Bortezomib exerts apoptosis by suppressing the apoptotic protein degradation. Imatinib and erlotinib block the activities of tyrosine kinases involved in cell signalling tahen place during cell proliferation. Bleomycin, generates the accumulation of reactive radical species that make DNA lesions leading to G_2 phase arrest. Actinomycin D, a DNA transcription inhibiting agent now known to inhibit the transcription of some cyclins leading to cell cycle arrest and apoptosis in osteosarcoma cells (Lu et al., 2015). Doxorubicin is a chemotherapeutic agent of Anthracycline group well known for its efficiency intreating haematological cancers though they accompanies many side effects and toxicities. They work by several mechanisms which include intercalation in DNA duplex, inhibiting DNA topoisomerase activities and inhibit the action of DNA and RNA polymerase (Tacar et al., 2013). Immunotherapy, endocrine therapy and gene therapy are the other treatment modes to cure cancer in addition to chemotherapy.

2.4. Cell Cycle Arrest and induction of apoptosis - Anticancer Mechanisms

Cell cycle progression is a strictly regulated, ordered series of events. Multiple checkpoints are operating in this process. The four distinct phases of the cell cycle are 'G_1, S, G_2 and M phases'. 'Cyclins and cyclin dependent kinases'

function as the accelerators or positive regulators of the cell cycle. 'Cyclin-dependent kinase inhibitors' are the negative regulators which halts cell division upon appropriate times. Cancer arises when these regulators express abnormally resulting defective cell cycle progression (Park & Lee, 2003). Defects in cell cycle machinery and loss of checkpoints regulation leads to tumorigenesis. Studies have shown that cells with defective checkpoint control are more vulnerable to anticancer agents than the normal cells. Based on the effects on cell cycle, chemotherapeutic agents are classified into cell cycle nonspecific agents (CCNSA) which act on any stage of cell cycle including G_0 phase and cell cycle specific agents (CCSA) which act on a certain phase of cell cycle (Ocio et al., 2014). Alkylating agents and anthracyclines come under the CCNSA category. 6-mercaptopurine (6-MP) as an antimetabolite drug act in the S phase and the plant alkaloids functioning on M-phase are examples of CCSA group (Shpigun & Andryukhina, 2019; Wang et al., 2016a). In clinical practice, surgery and radiation is usually used primarily to eradicate tumour mass, but in most cases these will stimulate the dormant cell (G_0) to re-enter into replication. In such situations, cell cycle specific chemotherapeutic drugs will be advantageous in curing the disease (Sun et al., 2021a).

Cell cycle arrest is a mechanism by which the cell can survive from the propagation of damaged DNA or chromosomes by getting the opportunity to repair the damage. If the damage is repaired properly, the cell cycle will resume. If it fails, the cell will be eliminated through apoptosis. Cell cycle is found to be the target of many chemotherapeutic agents. Drugs targeting the cell cycle progression are relatively non-toxic to normal cells since most of the normal cells remain in the terminally differentiated and quiescent stage. Generally cancer cells overexpress cyclins and are unable to express critical CDKIs (cyclin dependent kinase

inhibitors). Flavopiridol, a antineoplastic drug which act as a CDKI due to its considerable affinity to bind and directly inhibit CDK1 (cyclin B1-cdc2 kinase), CDK2, CDK4, and CDK6 thereby inducing cell cycle arrest and induction of apoptosis (Carlson et al., 1996; König et al., 1997). Bryostatin-1 stimulate the induction of p21 and subsequent dephosphorylation and inactivation of CDK2. It is a lactone produced by a marine invertebrate called *Bugula neritina.* Bryostatin could also decrease cyclin B expression in tumour cells resulting in the arrest of cells at G2 (Asiedu et al., 1995; Koutcher et al., 2000).

Apoptosis is a regulated killing mechanism by which the body can get rid of the unnecessary or impaired cells. Apoptosis involves a cascade of molecular events and is a complex and energy dependent process. Two important pathways by which cells elicit apoptosis are 'extrinsic or death receptor pathway and intrinsic or mitochondrial pathway'. In the mitochondrial pathway, proapoptotic factors are released from mitochondria into cytoplasm which activates the initiator caspase-9 which further activates the effector caspases 3/7. The extrinsic pathway is initiated by the activation of death receptors from the tumour necrosis factor (TNF) receptor family that promote the recruitment and activation of initiator caspase-8 through adaptor proteins called Fas-associated death domain (FADD) or TNFRSF1A-associated via death domain (TRADD) (Martin & Pognonec, 2010). Bcl-2 family proteins (both pro and anti-apoptotic factors) and p53 (tumour suppressor protein) are the key regulators or modulators of apoptotic pathways (Fridman & Lowe, 2003; Tsujimoto, 2003). Both pathways ultimately enter into the execution pathway. It induces 'DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells'. A

separate caspase independent cell death pathway is also occurring called perforin/granzyme pathway through the induction of single stranded DNA damage (Martinvalet et al., 2005).

Induction of apoptosis in cancerous cells is an effective chemotherapeutic strategy. Cell cycle is directly linked with apoptosis since it is regulated by some common genes that are involved in cell cycle progression. Evading apoptosis is a key characteristic of cancer cells which acquire this resistance by disrupting the balance between pro- and anti-apoptotic proteins, impairing the signalling through death receptors and reducing the function of caspases (Wong, 2011). Development of strategies focusing on these pathways will be helpful in counteracting the chemoresistance associated with cancer therapy. The tumour suppressor protein p53 (known as guardian of genome) is required for the induction of apoptosis, cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence (Lane, 1992; Oren & Rotter, 1999). More than 50% of cancers are associated with defects in p53 gene expression (Bai & Zhu, 2006). An earlier study conducted by Seitz et al, (2010) indicated that the administration of agents which upregulate the functioning of p53 protein along with the DNA damage causing chemotherapeutic drugs helps in eliciting apoptosis via extrinsic or intrinsic pathway and is a useful strategy to deal with chemoresistance. Resveratrol, a polyphenolic compound with anticancer properties promotes apoptosis in cancer cells by the activation of p53 (Ferraz da Costa et al., 2017). Gallic acid, another polyphenol compound induces G_2/M arrest and subsequently triggering apoptotic mode of death by elevating p53 and caspase 3. These effects of Gallic acid are found to improve the effectiveness of the paclitaxel/Carboplatin combination used to treat cervical cancer when administered

together (Aborehab & Osama, 2019). A recent study revealed that chemotherapeutic effects of ethanolic extract of leaves from a south Asian traditional medicinal plant, *Lagerstroemia speciosa* against hepatocellular carcinoma is due to its ability to trigger G_1 arrest and apoptotic mode of death in HepG2 cells. The underlying mechanism behind this effect was found to be the upregulation of p53, p21, p27, FOXO1 and downregulation of p-Akt, MDM2, CDK4, cyclin D1, and E1 expressions (Rohit Singh & Ezhilarasan, 2020). Another study indicated that sodium propionate, a short chain fatty acid, a metabolite produced by gut microbiota could induce G_2/M cell cycle arrest and apoptosis in H1299 and H1703 lung cancer cell line through the down-regulation of survivin (an antiapoptotic protein overexpressed in most cancer cells), and up-regulation of p21(a cyclin-dependent kinase inhibitor) (Kim et al., 2019).

2.5. Ferroptosis – a novel weapon in cancer therapy

Ferroptosis is a recently discovered rapid cell death process associated with intracellular iron accumulation and lipid peroxidation and plays a remarkable role in cancer chemotherapy. Its biochemical, morphological and genetic features are different from 'apoptosis, necrosis and autophagy'. Cells undergoing ferroptosis are characterized by mitochondrial shrinkage along with dense membrane and disappearing cristae. Dependance on intracellular iron is a key feature of ferroptosis. Dixon et al. (2012) first termed this unique iron-dependent form of non-apoptotic cell death as ferroptosis in their work on RSL (RAS selective lethal compounds) that were selectively lethal to oncogenic RAS-mutant cell lines. In this work they found that the cell death induced by RSLs especially erastin was linked to ROS accumulation and this is reversed when treated with iron chelators or hindering the entry of iron into cells. Flow cytometric analysis revealed that erastin

(10 μM) could enhance the lipid peroxides level inside the fibrosarcoma cells rapidly depending on the incubation time. They also observed that the co-treatment with the iron chelator deferoxamine (DFO, 100 μM) resulted in the suppression of ROS accumulation and cell death. Besides, the incubation with three different exogenous sources of iron also induced death in these cells. But the same did not happen with other metal ions (Cu^{2+}, Co^{2+}). Microscopic examination of erastin treated cells expressed smaller mitochondria with increased membrane density and more importantly none of the morphological features of apoptosis, necropsy and autophagy were visible. Since then several consistent data have accumulated emphasising and detailing the process of ferroptosis. A brief review on the pathways and underlying mechanisms are as below (Figure 2.1).

2.5.1. GPX4-regulated canonical pathway

GPX4 is a selenoprotein catalyzing the conversion of phospholipids to alcohols by the aid of their selenocysteines and electrons from GSH. The GSSG to GSH conversion is activated by GSR utilizing $NADPH/H^+$. Synthesis of GSH requires the availability of cysteine and the activity of glutamate-cysteine ligase. Cystine is the precursor of cysteine and is obtained through the exchange of extracellular cystine for intracellular glutamate through the cysteine-glutamate antiporter System xc^- (xCT-4F2) which is composed of two subunits namely SLC7A11 and SLC3A2 (Tang et al., 2021). Inhibition to System xc^- in turn inhibits the absorption of cysteine and the synthesis of GSH will be affected, leading to the reduction in antioxidant capacity of cells resulting in the accumulation of lipid ROS causing cell death. Yang et al. (2014) found that RSL3, a member of RSL compounds induces ferroptosis in cells by downregulating the expression of GPX4

and its upregulation reverses the effect. P53 protein is also now known to induce such cell death by inhibiting the expression of SLC7A11 (Jiang et al., 2015).

2.5.2. Lipid oxidation

Lipid oxidation is an important element in ferroptosis. It has three phases such as initiation, propagation and termination. At the initial phase, ROS, RNS or RLS withdraw a hydrogen atom from PUFA and making them lipid radicals (L•). In the next phase oxygen interacts with (L•) and form peroxyl radical (LOO•). This LOO• then withdraw one hydrogen atom from PUFA to form a new L• and LOO•. Interaction of lipid or peroxide radical with endogenous antioxidant compounds helps in the elimination of such radicals (Yin et al., 2011). In the presence of iron, continuous Fenton reaction mediated by Fe^{2+} in the cytosol produces excessive number of HO• to promote the peroxidation of PUFA through the sustained lipid radical chain reaction (Li & Li, 2020). Since the membrane PUFAs are affected in the ferroptotic process rather than the free PUFAs, the enzymes catalyzing the incorporation of PUFAs on the phospholipid bilayer are also playing an important role in ferroptosis. It includes ACSL4 and LPCAT3. ACSL4 catalyses arachidonoyl (AA) and adrenoyl (AdA) into acyl Co-A derivatives. Then, LPCAT3 esterifies them into phosphatidylethanolamines (AA-PE and AdA-PE) (Lee et al., 2021). There are some iron containing enzymes which cause lipid peoxidation. Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases catalyzing the stereospecific addition of oxygen onto PUFAs to produce lipid peroxides (Kuhn et al., 2005). These are also the interesting targets of ferroptosis.

2.5.3. Iron metabolism

Iron metabolism has a crucial role in ferroptosis. Iron is required for vital biological functions and its amount is strictly controlled at the cellular and systemic level to avoid iron overload and iron related toxicity in humans. Inorganic iron (Fe^{3+}) in food is turned to Fe^{2+} by duodenal cytochrome B reductase (DCYTB). It is then imported into the intestinal cells by apical DMT1. At the basolateral membrane of enterocytes, an iron exporting protein called ferroportin (FPN), transports Fe^{2+} into the bloodstream and is subsequently oxidized to $Fe3^{+}$ by membrane ferroperoxidase and then again bind to plasma transferrin. Transferrin receptor protein-1 (TRF1) is a ubiquitous protein present in the plasma membrane. Interaction of iron bound transferrin to TRF1 initiates its internalization. Inside the endosomes, the acidic environment cause the release of Fe^{3+} from Tf and is then converted to Fe^{2+} by STEAP3. It is then sent to the cytosol by endosomal DMT1. Iron thus released to the cytosol is either kept as ferritin or effluxed by ferroportin to keep the free iron level at minimum. Excess cytosolic iron promotes ROS formation by the Fenton reaction which leads to lipid peroxidation and ultimately results in ferroptosis (Capelletti et al., 2020). Heme oxygenase is another source of intracellular iron. This is the enzyme which converts heme to carbon monoxide (CO), biliverdin, and iron (Fe^{2+}). Silencing of TFRC (the gene encoding TFR-1) suppressed the erastin-induced ferroptosis while Upregulation of Heme oxygenase-1 (HO-1) accelerated erastin-induced ferroptosis by supplementing iron via catabolizing the heme (Gao et al., 2015; Kwon et al., 2015).

2.5.4. Role of Mitochondria in ferroptosis

Mitochondria is a major cellular organelle playing a key role in cellular metabolism including cellular respiration, energy production, fatty acid oxidation,

and iron metabolism (Nunnari & Suomalainen, 2012). Mitochondria effectively participate in various forms of regulated cell death including apoptosis, pyroptosis, necroptosis and ferroptosis (Tang et al., 2019). Iron exists in cells in two forms. One is chelatable iron which is either free or loosely bound to anionic metabolites. The other one is non-chelatable forms which are bound to ferritin or prosthetic groups (heme, Fe-S cluster, etc) of proteins. Oxidative stress is elicited by the chelatable iron through Fenton reaction in the cytosol. Chelatable iron content in cytosol is kept as low by storing in lysosomes at normal physiological conditions. During abnormal conditions, lysosomes release these iron to cytosol. This excess iron gets into the mitochondria (center of iron utilization) by two mitochondrial channel proteins namely mitochondrial calcium uniporter (MCU) and mitoferrin (Mfrn). Under elevated iron levels in cytosol MCU transport iron from cytosol to mitochondria in addition to their calcium transport potential. Mfrn also mediates iron transport across the mitochondrial membrane.

The core function of mitochondria is energy supply which is executed by the electron transport chain (ETC) and oxidative phosphorylation. It needs a sufficient amount of iron to facilitate the complex redox chemistry of ETC. Several enzymes taking part in ETC, contains haem. Mitochondria is the sole site of heme biosynthesis and Fe- S cluster synthesis. Electron transport chain reactions are often accompanied by the generation of ROS due to the unique redox properties of iron (Duan et al., 2022).

Approximately 90% of the cellular ROS is generated by mitochondria in the eukaryotic cells. The ROS generated in mitochondria (as part of the electron transport chain) reacts with the free iron (Fenton reaction) inside the mitochondria and results in the generation of excess ROS. Electrons escaped from ETC

(specifically from complex 1 and 3) generates (O2•−), which is further converted to H_2O_2 by superoxide dismutase (SOD)–mediated dismutation. H_2O_2 is further react with ferrous ion (Fe^{2+}) in the matrix to generate hydroxyl radicals (•OH) which reacts with the bis-allylic hydrogen in PUFAs to generate PUFA radicals (PUFA•) which then reacts with oxygen to form PUFA peroxyl radicals (PUFA-OO•) and converted to PUFA hydroperoxides (PUFA-OOH) resulting in the membrane damage (Murphy, 2009; Zheng & Conrad, 2020). This lipid peroxidation occurring in mitochondria has a key role in induction of ferroptosis (Gan, 2021). In order to counteract the accumulation of ROS mitochondria possess an antioxidant system including the mitochondrial version of GPX4. Mitochondrial lipid peroxidation is also facilitated by some iron dependent enzymes called lipoxygenases and cytochrome P450 oxidoreductase (Yang et al., 2016; Zou et al., 2020).

2.5.5. Drugs inducing ferroptosis

Various drugs having the potential to initiate ferroptosis were reported in some studies. Sulfasalazine, an anti-inflammatory drug was found to be the potent suppressor of breast cancer growth executes its action by a depletion of GPX4 and system xc- leading to ferroptosis (Yu et al., 2019). Cisplatin, a most commonly used chemotherapeutic agent, can induce both apoptosis and ferroptosis in A549 and HCT116 cells. Due to the high affinity of platinum compounds to thiol groups, it can conjugate with glutathione and cause inactivation of glutathione peroxidase leading to overload of lipid peroxides which ultimately lead to cell death. Antitumor potential of Cisplatin was reported to be increased while treatment with a common inducer of ferroptosis called erastin. Drug resistance associated with cisplatin induced apoptosis is not found with the ferroptotic process (Guo et al., 2018). A study has indicated that Trigonelline, an alkaloid present in *Trigonella foenum-*

graecum L. (fenugreek) and coffee seeds was the potent inhibitor of NRF2 which blocks the expression of MT-1G leading to reduced GSH content and causing ferroptosis (Boettler et al., 2011; Shin et al., 2018). Recently, β-elemene, a natural product in combination with cetuximab was reported to elicit ferroptosis in KRAS mutant colorectal cancer cells by iron mediated ROS accumulation, glutathione depletion, upregulation of heme oxygenase-1 and down regulation of GPX4, SLC7A11, SLC40A1 and FTH1(Chen et al., 2020a).

2.6. Herbal Medicine and Ethnopharmacology

Herbal drugs are gaining more acceptance around the world due to its safety and efficiency in curing various ailments. Emergence of the field phytopharmacology which addresses the concepts such as herb- drug interactions, mechanism of drug action, and it contributes to the discovery of novel drug candidates from plant resources. Even though the synthetic drugs are good for fast and strong medicinal effects, they often create unwanted side effects. Good therapeutic efficiency, low side effects and low prices of natural drugs compared to synthetic drugs forces the public to go back to the natural ones today (Nisar et al., 2018). One third of the top pharmaceutical compounds selling in the markets comes under the category of natural compounds (from plants and microorganisms) or their derivatives (Watkins et al., 2015). Selection of plants for the development of new drugs based on their traditional medicinal uses is known as ethnopharmacological approach (Atanasov et al., 2015). About 74% of bioactive molecules are discovered by employing this ethnopharmacological approach (Ncube et al., 2008). Knowledge about traditional medicinal plants was acquired by human beings as part of their prolonged and continuous trial and error learning processes which enabled him to discriminate useful plants from those which cause toxic or neutral effects and the

processing method or combinations of herbs for obtaining therapeutic effects. These types of knowledge about the medicinal applications of plants are specific to certain ethnic groups and have been transmitted by generations but it lacks well documented and scientific validation. Ayurveda is one of the prevalent traditional medical practices in India that uses a holistic approach in healing a disease incorporating polyherbal mixtures rather than single constituents isolated from plants. Though ayurvedic treatment of many chronic diseases including cancer are highly efficient, it has the drawback of poor scientific validation in terms of pharmacology and mode of action (Jaiswal & Williams, 2017). In the phytotherapeutic approach of drug development, fractions of extracts, mixtures of fractions or crude active extracts are found to be of therapeutically effective, less toxic and inexpensive compared to the pure isolated drug compounds. Herbal drugs (single plant extract or mixtures) should be standardised carefully for their efficacy and safety. Such standardised herbal drugs will be useful and affordable to many people. The integrated approach incorporating the knowledge from traditional medicinal practices and the modern drug discovery procedures may lead to standardised extracts or individual bioactive druggable compounds as the novel therapeutic drug (Katiyar et al., 2012).

2.7. Plant Secondary Metabolites

Plants contain a variety of chemical compounds of which only a few are responsible for the specific therapeutic activity. Bioactive compounds from plants are referred as the secondary metabolites which can elicit a particular pharmacological or toxicological effects in humans or animals and are considered as the products of biochemical 'side tracks' in the plants as they are not required for the daily functioning of the plant (Bernhoft, 2010). Plants synthesise secondary

metabolites with the intention to be competitive in the adverse environmental conditions where they inhabit. These compounds can exert their effects in plant cells and other organisms (Teoh, 2016). Plant secondary metabolites are broadly categorised as ‘terpenoids, phenolic compounds and alkaloids’.

Essential oils extracted from plant parts mainly composed of several kinds of terpenes and terpenoids. The basic structural unit of terpenes are isoprene units. Based on the repeats of isoprene part, terpenes are classified as ‘hemiterpenes, monoterpenes, sesquiterpenes, triterpenes and tetraterpenes’. Terpenes and terpenoids are formed by the mevalonic acid pathway in the cytosol and the precursors are synthesised in plastid by 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (De Oliveira et al., 2020). Terpenoids are the terpenes containing oxygen molecules which are formed through some biochemical modifications. Carvacrol, geraniol, piperitone, menthol and thymol are examples of some terpenoid compounds with pharmacological properties (Hyldgaard et al., 2012). These compounds are mainly produced by aromatic plants and many terpenoids exerts antimicrobial properties by promoting cell rupture and inhibition of protein and DNA synthesis (Álvarez-Martínez et al., 2021). Some antibacterial monoterpenes act by disrupting the microbe multiplication and development and also by interfering with their physiological and metabolic activities (Burt, 2004). Azadirachtin, carvone, menthol, ascaridole, methyl eugenol, toosendanin, and volkensin are some examples of terpenes showing antimicrobial properties as well as insect pest repellent properties (Isman & Machial, 2006; Pandey et al., 2016a; Pandey et al., 2016b). Many terpenoid compounds studied for their therapeutic effectiveness in preventing several malignancies. Citral, a monoterpene compound, showed antiproliferative effects in human colorectal cancer (HCT116 and HT29)

cells through mitochondrial mediated apoptosis (Sheikh et al., 2017).

Phenolic compounds are the most obvious secondary metabolites of plants. Benzene rings with hydroxyl substituents is the structural peculiarity of these compounds. They are produced through the involvement of shikimic acid, pentose phosphate and phenylpropanoid pathways. Phenolic compounds exerts health benefits like anti-aging, anti-inflammatory, antioxidant and antiproliferative effects. Most phenolic compounds are potential free radical scavengers which can reduce, counteract and repair the damage resulting from oxidative stress and inflammation associated with some chronic diseases (Lin et al., 2016). Phenolic compounds are generally classified as simple phenolic compounds and polyphenols. Simple phenolic compounds contain only one phenol ring. These are further classified into Simple phenolics (catechol, resorcinol, and hydroquinone) and Phenolic acids (Hydroxybenzoic acids, Hydroxycinnamic acids and coumarins). Polyphenols contain more than one phenol ring. It includes flavonoids (flavone, flavonol, chalcone, anthocyanin, flavanone and isoflavone), tannins (hydrolyzable, condensed and complex) and other phenolic compounds like stilbenes, lignans and lignins (Vermerris & Nicholson, 2007; Vuolo et al., 2019). Phenolic compounds are acidic in nature and can undergo oxidation reactions to form phenolic radicals which are stabilised by resonance through delocalization of the resultant single electron over the ring. Phenolic compounds have many industrial applications like therapeutic agents, cosmetic agents, food preservatives and additives (Al Mamari, 2021).

Alkaloids are produced in about 20% of plant species and are often synthesised in small quantities. They are often found in a variety of cyclic structural forms and contain at least one nitrogen atom. They play roles in protecting plants from predators and regulating growth. Naturally they are synthesised from amino

acids (Srivastava & Srivastava, 2013). Pharmacological properties of alkaloids include anaesthetic, cardioprotective and anti-inflammatory agents. Morphine, strychnine, quinine, ephedrine, and nicotine are examples of some therapeutic alkaloids in clinical practice (Kurek, 2019). Vinca alkaloids such as vinblastine, vincristine, and vindesine, isolated from the *Catharanthus roseus* and camptothecin isolated from *Camptotheca acuminate* are some extensively studied anticancer agents (Mondal et al., 2019).

2.8. Role of Phytochemicals in Cancer Chemoprevention

Prevention of development of tumours at the early stages is the most effective strategy in cancer management than the attempts to eradicate fully developed tumours with chemotherapeutic agents. Chemoprevention means the treatment used to suppress, prevent or reverse the initial phases of carcinogenesis or blocking the invasion of premalignant cells (Sporn, 1976). The term chemoprevention was coined by Michael Sporn in the middle of 1970s to describe strategy of preventing tumour onset using relatively non-toxic substances (Surh, 2003). Clinically, chemoprevention is categorized as 'primary, secondary and tertiary'. The first one is focused on cancer unaffected people as well as those with high risk for cancer. The secondary prevention is for people at premalignant stage. The tertiary prevention is referred to cure or prevent the recurrence of cancer (Pitot, 1993). According to Wattenberg (1985) the chemopreventive agents are classified into two categories. They are blocking agents and suppressing agents. Blocking agents act by preventing carcinogens from reaching the target sites, from undergoing metabolic activation process or from subsequent interaction with crucial cellular macromolecules (DNA, RNA and proteins). Suppressing agents block the promotion or progression stage.

Plants are excellent sources of chemopreventive agents since several phytochemicals, though non-nutritive, possess antimutagenic and anticarcinogenic activities. Chemopreventive phytochemicals can interfere in various cellular and molecular events which include 'carcinogen activation or detoxification by xenobiotic metabolising enzymes, DNA repair, cell-cycle progression, expression and functional activation of genes involved in cell proliferation and apoptosis, angiogenesis and metastasis'. The activation of the transcription factor called NF-κB has been associated with stimulation of proliferation in malignant cells and the inhibition of apoptosis. Some chemopreventive agents act by suppressing the overexpression of NF-κB. Curcumin, a phytochemical isolated from the rhizome of *Curcuma longa* L. exerted chemopreventive potential in a mouse model of skin carcinogenesis where it suppressed tumour promotion by inhibiting TNF-α mediated COX2 expression and stimulating NF-κB. In addition to this, Curcumin inhibited IκB degradation by downregulation of NF-κB-inducing kinase (NIK) and IκB kinase (IKK) α/β (Plummer et al., 1999). Resveratrol, a phytoalexin compound occurring in some plants like red grapes, berries and peanuts, has proven to facilitate chemoprevention by various mechanisms. This compound can modulate the enzymes involved in the detoxification and carcinogen activation reactions in the liver (Chow et al., 2010)**.** Resveratrol also promotes immunosurveillance by enhancing the expression of NKG2D, an antigen receptor expressed by cytotoxic lymphocytes including natural killer cells which identify specific surface ligands expressed in transformed cells for exerting cytotoxicity and enhancing elimination of spontaneous tumour cells prior to proliferation (Guerra et al., 2008).

Reactive oxygen species are formed inside the cells as part of aerobic cellular metabolism. An equilibrium is usually present in cells between the

generation of ROS and the antioxidant defence. Oxidative stress results when this balance is disrupted due to the accumulation of ROS. Excess ROS are often produced in cancer cells due to their increased and unchecked proliferation and accelerated metabolism (Sosa et al., 2013). Many phytochemicals such as lycopene, beta carotene are exogenous sources of antioxidants which can prevent inflammation, cancer and other stress related diseases. However, positive results are still lacking in the clinical studies on antioxidant chemopreventive therapy. More encouraging results might be obtained by the appropriate selection of balanced compositions of vitamins, antioxidants and minerals (Kotecha et al., 2016). Lycopene, a carotenoid compound found in tomatoes, is capable of exerting the chemopreventive potential by lowering the intracellular generation of ROS by activating the proteins of cellular antioxidant defense. It also reduces the oxidative stress by down-regulating expression of ROS generating proteins (Palozza et al., 2010). Folate, a phytochemical found in green leafy vegetables, asparagus and broccoli could lower the risk of several cancers including pancreatic,stomach and colorectal cancer by its role in DNA repair and in modulating S-adenosylmethionine, a universal methyl donor group for DNA methylation reactions (Lamprecht & Lipkin, 2003; Tio et al., 2014).

2.9. Advantages of Plant Based Chemotherapeutic Drugs over Synthetic Drugs

Multiple drug resistance and systemic toxicity are the main obstacles in the use of most currently administered chemotherapeutic agents. Overexpression of a plasma membrane glycoprotein (P-gp) seen on the cell surface (encoded by the MDR1 gene) is the main mechanism responsible for multiple drug resistance (Bellamy, 1996). In addition to decreased accumulation of drugs in cells, insufficient activation of the drug, utilisation of alternate metabolic pathways,

mutations in the p53 gene and overexpression of the Bcl-2 gene family are the causes of multiple drug resistance which leads to the failure of chemotherapy (Gottesman, 2002). The structural diversity, more chiral centres, varied ring systems and complex scaffold capable of binding with the proteins involved in the metabolic processes enabled the phytochemicals to act as good chemotherapeutic agents over synthetic drugs. This complex structure of the phytochemicals helps them in interacting with the molecular targets thus reducing non-specific binding and adverse side effects (Wang et al., 2012). Side effects like 'myelosuppression, mucositis, hair loss, cardiotoxicity, neurotoxicity and immunosuppression' are other limitations of conventional chemotherapeutic agents since they target dividing cells. In some instances, the amount of chemotherapeutic agents to be administered increased, due to the rapid elimination of drugs and widespread distribution of introduced drugs to non-targeted organs. This in turn, enhances the side effects and also is not economic (Singh et al., 2016).

The use of combination drugs is an effective strategy to overcome drug resistance. Traditional herbal medicinal formulations are based on the concept that synergistic action of various phytoconstituents, each with extremely low doses of each active component and different mechanisms of action are beneficial in enhancing the effectiveness and lowering the resistance in treatment of diseases. Single compound capable of eliciting a number of molecular events (pleiotropic effects) is another remarkable feature of phytochemicals in search of new chemotherapeutics. Plant derived anticancer agents currently available in the market comes under the following four classes such as vinca alkaloids (vinblastine, vincristine and vindesine), epipodophyllotoxins (etoposide and teniposide), taxanes (docetaxel and paclitaxel) and camptothecin derivatives (camptothecin and

irinotecan) (Desai et al., 2008). Selection of plants with ethnomedicinal importance is found to be worthy in terms of greater success rate and reduced cost, time, and toxicity parameters (Singh et al., 2016).

2.10. Family: Plantaginaceae

The family Plantaginaceae consists of plants distributed all over the world consisting of more than 3000 species belonging to 292 genera (Luu & Van, 2022). This family is commonly known as the 'Snapdragon'. In India, Snapdragons is represented by 273 species and many plants from this have been used in folk medicines. They possessed diverse classes of phytochemicals with proven pharmacological activities. A popular plant from this family is *Digitalis purpurea* (known as the foxglove plant), a poisonous plant which contains the cardioactive glycosides namely digitoxin and digoxin used in heart diseases (Banasik & Stedeford, 2014). *Verbascum thapsus* L. is another plant in this family having uses in pulmonary problems and asthma (Turker & Gurel, 2005). *Bacopa monnieri* (commonly known as Bramhi) also belongs to this family. It is useful for improving memory and to cure neurological diseases. Anti-epileptic, anticancer, anti-ulcer and anti-inflammatory potential of this plant was also reported (Mukherjee et al., 2022).

Earlier studies have also shown the anticancer potentials of certain plants in this family. *Scrophularia amplexicaulis*, an Iranian endemic plant exhibited *in vitro* cytotoxicity effects to MCF-7 and WEHI-164 cancer cells in a dose-and time-dependent manner with little cytotoxicity to normal HUVECs (Valiyari et al., 2020). *Gratiola officinalis* L. of this family could induce caspase-dependent apoptosis in Jurkat T-cell lymphoblastic leukaemia tumour cells (Polukonova et al., 2022). An earlier study showed that *Scrophularia umbrosa,* a medicinal plant from this family, induced apoptosis in MCF-7 breast cancer cells (Mansouri et al., 2019).

Veronica is the largest genus under this family comprising more than 500 species. Iridoid glycosides including aucubin, catalpol, and 6-O-catalpol derivatives - are characteristic of this genus, most of which have many pharmacological properties, such as anti-inflammatory, anti-oxidative, anticancer, anti-bacterial, anti-angiogenic, anti-neurodegenerative, neuroprotective and hepatoprotective effects (Xue et al., 2019).

2.11. *Scoparia dulcis* (L.)

This plant is commonly known as Kallurukki (Malayalam), Sweet broom weed (English), Ghoda Tulsi (Hindi) and Sarakkottini (Tamil). This plant is abundantly found in grazed grasslands, wastelands and cultivation lands of tropical or subtropical areas around the world including India, South East Asia, Brazil, West Indies, Philippians, Myanmar and America. It is an herbaceous perennial plant in which flowering and fruiting occurs in the monsoon season. The roots are about 7-13 cm long with pale yellow to brown colour. Tap roots along with many hair-like extensions called lateral roots are found. Stem is wooden at the base and branched which attains 1 meter length. Leaves are serrated with acute apex and tapering base and are arranged in whorled pattern. The flowers have 5 - 7 mm diameters and are hermaphrodite and usually auxillary. Sepals are with oval-oblong and persistent calyx lobes ciliated at the margins. Corolla consist of thickly hairy tubes especially near the throat, long lobes and obtuse apex. Four greenish stamens are present with filaments inserted at the top of the corolla tube. Dorsi-fixed and erected style anthers and truncate to 2-partite stigma are seen in the flowers. Long dehiscent capsulated and ovoid shaped fruits which open into two halves upon ripening are seen in this plant. Obconical shaped seeds are seen which are usually dispersed by cattle and buffaloes (Sarkar et al., 2020).

2.11.1. Ethnomedicinal uses of *Scoparia dulcis* (L.) in India

Some tribal people from Assam state, India use the leaves of *Scoparia dulcis* for treating diabetes, jaundice, stomach problems, piles and skin diseases. It is one of the ingredients in their traditional rice beer preparations known as Sujen which has a great role in the socio-cultural life of the Deori tribe of Assam (Bhuyan & Baishya, 2013). The plant sap was used by the Nadar tribes from Kanyakumari district of Tamilnadu state, India for curing fever and headaches. The juice from leaves is orally consumed for curing kidney stones by the same people (Jeeva & Femila, 2012). The decoction prepared from the roots is orally taken for reducing stomach problems by the Paliyar and Muthuvan tribes of Theni district of Tamilnadu, India (Jeyaprakash et al., 2011).

2.11.2. Pharmacological prospects of *Scoparia dulcis* (L.)

The traditional use of *Scoparia dulcis* as the antidiabetic drug was evaluated in various studies. Misra et al. (2013) performed both *in vitro* and *in vivo* studies in order to assess the antidiabetic potential of this plant and found that the methanol extract could effectively check the postprandial glucose level. This extract showed significant reduction in blood sugar level similar to the standard antidiabetic drug glibenclamide. Scoparic acid D, a diterpenoid isolated from the ethanol extract, also shows antihyperglycemic activity *in vivo* (Latha et al., 2009). Ethanol extract and one of its major triterpene, glutinol showed analgesic and antipyretic activities in mice and rats. They could also exert inhibitory activities on early stages of the acute inflammatory process (De Farias Freire et al., 1993). Anti-Inflammatory activity of Ethanol extract was also confirmed by Tsai et al. (2011). Antimicrobial potential of this plant was demonstrated in a number of studies (Niveditha et al., 2015 ; Zulfiker et al., 2011). Antisickling properties of this plant

was investigated by Abere et al. (2015) and it was found that crude extract prepared from leaves inhibited the percentage sickling of erythrocytes taken from blood samples collected from sickle cell anaemia patients. Aqueous extract of *S. dulcis* could increase the levels of antioxidant enzymes and decrease the extent of lipid peroxidation in oxidative stress induced diabetic mice (Pari & Latha, 2004). Antioxidant activity of this plant was also confirmed in other *in vitro* studies (Coulibaly et al., 2011; Patra et al., 2014.; Ratnasooriya et al., 2005). Antitumor potential of *S. dulcis* was revealed in some studies. Ethanol extract could cause inhibitory effects on the proliferation of cholangiocarcinoma cell lines (Promraksa et al., 2019). Scopadulcic acid, a tetracyclic diterpenoid isolated from this plant could suppress the promoting effect of TPA (12-O-tetradecanoylphorbol-13-acetate) on the formation of skin tumours in mice (Nishino et al., 1993). Crude ethanol extract and its ethyl acetate fraction possess cytotoxic activity against HepG2 cells with IC_{50} value less than 50µg/ml (Thanh Tin et al., 2019). Ethanol extract could induce the activation of several apoptotic proteins in A549 cells.

2.11.3. Phytochemicals from *Scoparia dulcis* (L.)

The presence of alkaloids, flavonoids, tannins, saponins and terpenoids are reported from aqueous and methanol extract of *S. dulcis* (Wankhar et al., 2015). Triterpenoids identified from this plant include friedelin, glutinol, α-amyrin, betulinic acid, ifflaionic acid and dulcioic acid (Mahato et al., 1981). Flavonoids like scutellarein, hispidulin, apigenin and luteolin were identified from this plant (Liu et al., 2014). Three different types of diterpenoids were isolated from the chloroform fraction of ethanol extract such as labdane type (‘scoparic acid A, B and C’), scopadulan type (‘scopadulcic acid A, B and scopadulciol’) and aphidicolan type (scopadulin). Among them, Scopadulcic acid B possesses various biological

activities including antitumour activities (Hayashi, 2000). Two acyclic di-terpenes were isolated from the acetonitrile fraction of dichloromethane fraction of *S. dulcis* for the first time by Nur-e-Alam (2020) namely, Acetic acid 6-hydroxy-2-(6-hydroxy-4-methyl-hex-4-enylidene)-4,8-dimethyl-undeca-4,8-dienyl ester and Acetic acid 8-hydroxy-2-(6-hydroxy4-methyl-hex-4-enylidene)-6,10-dimethyl-undeca-5, 9-dienyl ester (Nur-e-Alam et al., 2020). Methanol fraction of ethanol extract has yielded four bioactive compounds such as 2-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one 2-O-βD- glucopyranoside, ferruginoside C, isorhoifolin and 3, 4'-O-dimethylcedrusin 9'-O glucopyranoside (Thanh Tin et al., 2019). Two cytotoxic terpenoids namely 1β-Hydroxydulcinol (scopadulane-type diterpenoid) and scoparinol (labdane-type diterpenoid) were present in the ethanol extract (Wang et al., 2022).

2.12. Family: Asteraceae

The Asteraceae family is considered as one of the most advanced families of dicotyledonous plants with diverse chemical composition. It is also called as the 'sunflower family' and contains more than '1600 genera and 25000 species' around the world. Most plants in this family have been traditionally used for therapeutic purposes. They are distributed worldwide though commonly grown in the arid and semiarid areas of subtropical regions. Members of this family showed several kinds of biological and pharmacological effects. Chemical composition of plants from this family was found to be similar. Bitter taste of most plants in this family is due to the presence of sesquiterpene lactones (Rolnik & Olas, 2021). Nutritional value of plants belonging to this family is remarkable as these contain high protein and fibre content, vitamins and minerals with low fat content (García-Herrera et al., 2014). A study conducted in various members of the Asteraceae family such as *Cirsium*

arvense, *Onoporidium acanthium*, *Centaurea solstitailis* and *Carduus acanthoides* revealed their total phenolic content ranging from 8.035 to 90.305 mg gallic acid equivalent/L and total flavonoid content from 18.031 to 185.437 mg quercetin/L (Koc et al., 2015). Due to the higher phenolic content, many Asteraceae plants possessed good free radical scavenging properties (Yuan et al., 2012). In addition to phenolic compounds, a number of triterpenes were identified from members of this family. Triterpenes, such as taraxacin, taraxacin acid, fardiol, arnidiol, taraxasterol, α-amiryn and β-amiryn, have been identified in *Taraxacum* species, an important member of the family (Hu, 2018). The genus *Vernonia* is an important genus in this family which contains more than one thousand species of plants, mainly used for medicine and food.

2.13. *Vernonia cinerea* (L.) LESS

Vernonia cinerea (L.) Less is a herbaceous medicinal plant of Asteraceae family. It is seen everywhere in India. It is commonly called as Ash coloured fleabane in English and Poovamkurunnila in Malayalam. This plant is considered as one among the top 5 most frequently used species in the genus Vernonia (Toyang & Verpoorte, 2013). *V. cinerea* grows favourably in sunny or slightly shaded habitats and it reproduces and spreads by seeds upon wind dispersal. It can be seen abundantly on roadsides and waste lands. It is seen especially in Asian countries. It is an erect, slender, annual herb that can grow up to 1.3 metres in height. The stems are hairy and glandular. The leaves are arranged alternatively with petiolated lower leaves while the upper ones are reduced and sessile. The leaves have length between 2- 6 cm and are hairy. The head has 7 mm length and 2.5 mm diameter. The terminal corymbose cymes are peduncled. The flowers are tubular, and have bright purple, pink, or white colour. About 20 flowers are seen in each head. The

achenes (a small, dry one-seeded fruit that does not open to release the seed) are rounded, ribless and length about 1.5 mm with outer pappus 1 mm long, setaceous, inner 3 mm long (Verma, 2018).

2.13.1. Ethnomedicinal uses of *Vernonia cinerea* (L.) LESS in India

Ayurvedic pharmacopoeia of India described traditional uses of *V. cinerea* in treating diseases like 'intermittent fever, blisters, boils, lymphatic filariasis, vaginal discharges and psychoneurosis' (The Ayurvedic pharmacopoeia of India, 2001). This plant, in combination with other herbal ingredients is used externally to cure breast tumours by the tribal people in the southern region of Western Ghats of India and the traditional healers from Chandauli District of Uttar Pradesh, India (Ayyanar & Ignacimuthu, 2005; Singh & Singh, 2009). A survey on ethnomedicinal plants used in Sirumalai Hills of Eastern Ghats, Dindigul district of Tamilnadu, India indicated that the leaves of *V. cinerea* has been used for treating malaria and rheumatism and their roots are used to cure helminthic infections, diarrhoea and stomach problems (Alagesaboopathi, 2012).

2.13.2. Pharmacological prospects of *Vernonia cinerea* (L.) LESS

Anti-inflammatory, anti-diabetic, nephroprotective and antimicrobial potentials of *V. cinerea* was reported in many studies. Crude aqueous extract of this plant could reverse the cisplatin induced nephrotoxicity and regenerated the proximal tubular epithelial cells in tumour bearing mice without compromising the effectiveness of the anticancer drug cisplatin (Amuthan et al., 2021). Another study also indicated the protective activity of this plant against cisplatin-induced kidney damage by analysing the serum markers levels of renal functional parameters and revealed the promising curative activity of alcoholic extract and prophylactic

activity of ethyl acetate extract (Sreedevi et al., 2011). Ethyl acetate extract of *V. cinerea* leaves has shown good anti-dandruff activities against *Pityrosporum ovale* and *P. folliculitis* (Dhanalakshmi et al., 2013). The benzene extract of *V. cinerea* (50-500µg/mL) was reported to have a broad spectrum of antibacterial activity. The activity at 500 µg/mL was similar to that of chloramphenicol (10 µg/mL), against *Bacillus subtilis* and *Psudomonas aeruginosa* (Gupta et al., 2003). The carbon tetrachloride fraction of methanolic extract of *V. cinerea* exhibited a time and dose dependent reduction of the blood glucose levels of the alloxan-induced diabetic rats when compared to the untreated group indicating its anti-diabetic potential (Haque et al., 2013). Pratheesh Kumar and Kuttan (2009) has studied the antioxidant and anti-inflammatory activities of *V. cinerea* and found that this extract possesses good scavenging properties against hydroxyl, superoxide and nitric oxide radicals. Administration of this extract induced the increase in the level of antioxidant enzymes in treated mice. Also, this extract inhibited carrageenan induced oedema.

The ethanolic extract of *V. cinerea* was found to be cytotoxic to various human epithelial cancer cells and triggers apoptosis. Additionally, this fraction inhibited functional activity of MDR transporters (ABC-B1 and ABC-G2) in cancer cells and sensitised the cancer cells to the action of chemotherapeutic drugs (Beeran et al., 2014). Treatment with ethanol and chloroform extract of this plant was found to be effective in decreasing the cancer cell count and increasing the lifespan of mice inoculated with lymphoma cells into their peritoneal cavity to develop ascites tumour (Sangeetha & Venkatarathinakumar, 2011).

2.13.3. Phytochemicals from *Vernonia cinerea* (L.) LESS

Sesquiterpene lactones and steroids are the most commonly found phytochechemical groups among the members of Vernonia. NMR data on the

hexane fraction of methanolic extract of *V. cinerea* indicated the presence of Lupeol, 12-oleanen-3-ol-3ß-acetate, Stigmasterol, ß-sitosterol (Haque et al., 2012). An earlier study has also reported the presence of phytosterols namely stigmast 5, 17(20)-dien-3β-ol, stigmasterol and sitosterol in *V. cinerea* (L.) Less (Misra et al., 1984). The sesquiterpene lactones such as 8*α*-(2′*Z*-tigloyloxy)-hirsutinolide, 8*α*-(2′*Z*-tigloyloxy)-hirsutinolide-13-*O*-acetate, 8*α*-(4-hydroxytigloyloxy- -hirsutinolide, and 8*α*-hydroxy-13-*O*-tigloyl-hirsutinolide were identified from the chloroform fraction of methanolic extract (Youn et al., 2014). Khay et al. (2012) has also identified and isolated the sesquiterpene lactone, 8alpha-tigloyloxy-hirsutinolide-13-O-acetate from this plant which was cytotoxic against the cancer cell lines (IC_{50} = 3.50 μM for HT29 and IC_{50} = 4.27 μM for HepG2). Two cytotoxic sesquiterpene lactones named as 'Vernolide-A and Vernolide-B' were reported from this plant's ethanolic extract. Vernolide-A is cytotoxic to 'KB, DLD-1, NCI-661, and Hela tumour cell lines'. Vernolide-B had only marginal cytotoxicity against these cell lines (Kuo et al., 2003). Another study showed that Vernolide A could induce of apoptosis and inhibit the metastatic progression of B16F-10 melanoma cells (Pratheeshkumar & Kuttan, 2011).

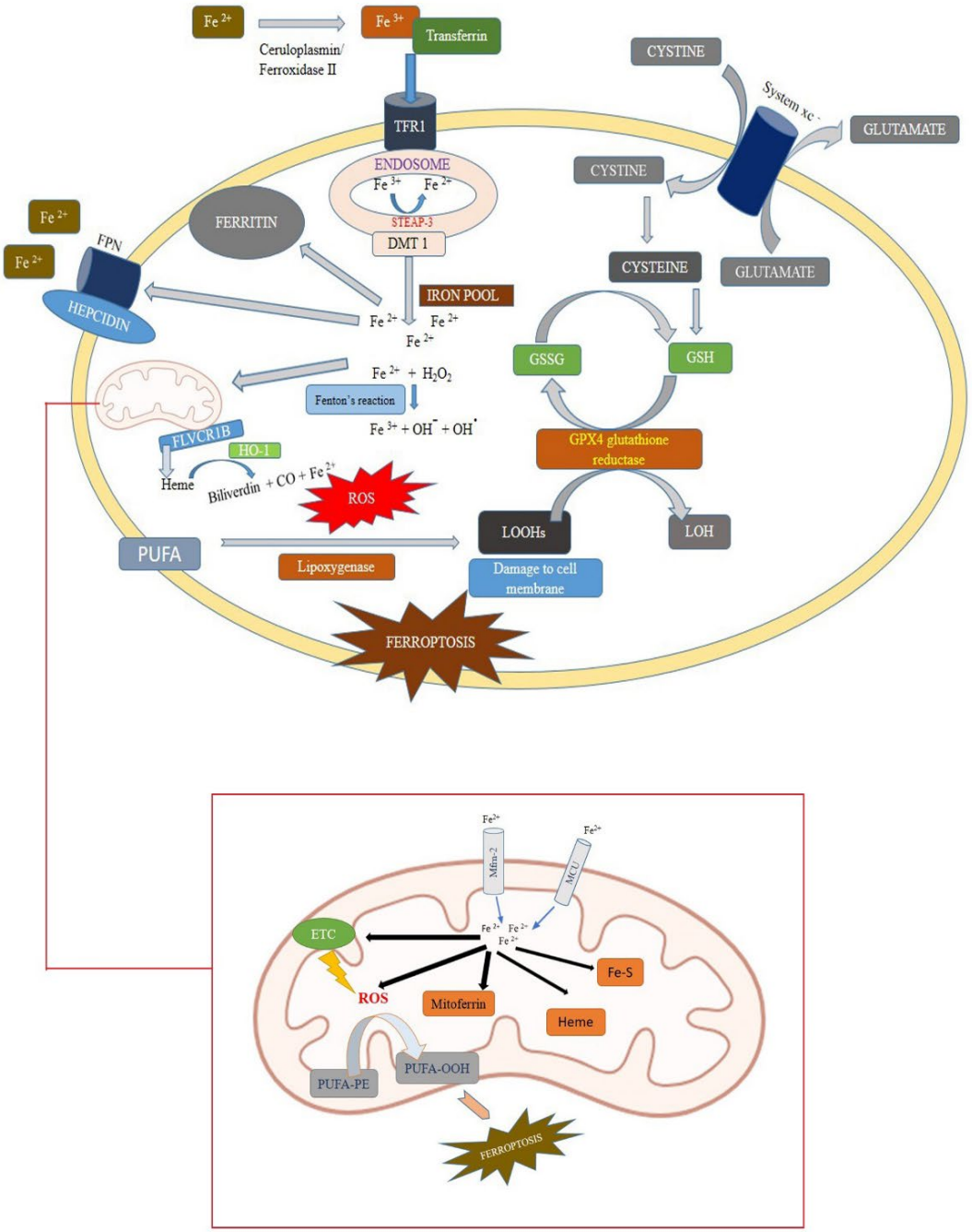


Figure 2.1: Schematic representation of the various pathways involved in ferroptosis. Abbreviations: TFR1- transferrin receptor-1; FPN – ferroportin; GSH – glutathione; GSSG – glutathione reduced; HO-1 – heme oxygenase 1; LOOHs – lipid peroxides; LOHs – lipid alcohols; ROS – reactive oxygen species; ETC – electron transport chain.

CHAPTER 3

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. CHEMICALS AND REAGENTS

Acetic anhydride	: MERCK, India
Agar agar	: Hi-Media, India
Alkaline phosphatase	: Agappe diagnostics Ltd., India
Aluminium chloride	: MERCK, India
Ammonia	: Sigma Aldrich, USA
Ammonium chloride	: NICE, India
Amoxicillin	: Sigma Aldrich, USA
Annexin V - FITC	: Sigma Aldrich, USA
Bilirubin	: Agappe diagnostics Ltd., India
Biotin	: Hi-Media, India
Bromine water	: NICE, India
Chloroform	: NICE, India
Cholesterol (Total)	: Agappe Diagnostics Ltd., India
Citric acid monohydrate	: MERCK, India
Creatinine	: Euro Diagnostic Systems Pvt. Ltd.,
Crystal violet	: Sigma Aldrich, USA
Cyclophosphamide	: Neon Laboratories Ltd
Deferoxamine (Desferal)	: Novartis, India
Dextrose	: Hi-Media, India
Dimethyl sulfoxide (DMSO)	: MERCK, India
DMEM medium	: Sigma-Aldrich, St.Louis, USA

Drabkin’s reagent	: Agappe Diagnostics Ltd., India
Dragendroff’s reagent	: NICE, India
Eosin	: Hi-Media, India
Ethyl acetate	: MERCK, India
Ferric chloride	: MERCK, India
Folin-Ciocalteau reagent	: Sigma Aldrich, USA
Gallic acid	: Sigma Aldrich, USA
Gallic acid	: Sigma Aldrich, USA
Glacial acetic acid	: Nice, India
Hematoxylin	: Hi-Media, India
Histidine	: Hi-Media, India
Hoechst dye	: Sigma Aldrich, USA
Hydrochloric acid	: MERCK, India
K_2HPO_4	: MERCK, India
Lead acetate solution	: MERCK, India
Leishman’s stain	: Hi-Media, India
Mayer’s reagent	: NICE, India
Methanol	: Sigma Aldrich, USA
$MgSO_4$	: MERCK, India
MTT	: Sigma-Aldrich, St.Louis, USA
Nutrient broth	: Hi-Media, India
Petroleum ether	: MERCK, India
Potassium acetate	: MERCK, India
Potassium dihydrogen phosphate	: MERCK, India
Propidium iodide	: Sigma Aldrich, USA
Quercetin	: Sigma Aldrich, USA

RPMI medium	: Sigma-Aldrich, St.Louis, USA
SGOT diagnostic kit	: Agappe diagnostics Ltd., India
SGPT diagnostic kit	: Agappe diagnostics Ltd., India
Sodium azide	: Sigma Aldrich, USA
Sodium carbonate	: Nice, India
Sodium chloride	: Hi-Media, India
Sodium phosphate dibasic	: MERCK, India
Sodium phosphate, monobasic	: MERCK, India
Sodium sulphate	: MERCK, India
Sulphuric acid	: MERCK, India
Total protein	: Agappe diagnostics Ltd., India
Triglycerides	: Agappe Diagnostics Ltd., India
Trypan blue	: MERCK, India
Trypsin	: Sigma-Aldrich, St.Louis, USA
TUNEL label mix	: Roche-Sigma Aldrich, USA
Urea diagnostic kit	: Euro Diagnostic Systems Pvt. Ltd.,
Wagner's reagent	: NICE, India
Xylene	: Hi-Media, India

3.1.2. REAGENTS AND MEDIA PREPARED

a. Phosphate buffered saline (PBS)

NaCl - 8.00 g

KCl - 0.20 g

KH_2PO_4 - 0.20 g

Na_2HPO_4. $2H_2O$ - 1.44 g

Dissolved the above contents in distilled water, made up to 1000 ml. Adjusted the pH to 7.2 with 1N NaOH/ HCl. Autoclaving at 15 lbs for15 minutes, cooled and stored at 4 ˚C until used.

b. Vogel Bonner Solution

Warm distilled water	: 65 ml
$MgSo_4$	: 1 g
Citric acid monohydrate	: 10 g
K_2HPO_4	: 50 g
Ammonium Sodium Phosphate	: 175 g

The above chemicals are added one after another with constant stirring using a magnetic stirrer. Adjusted the volume to 100 ml. Autoclaved at 121 ˚C, 15 lbs pressure for 30 minutes and allowed to cool to room temperature and were stored at 4 ˚C until used.

c. M9 solution

Ammonium chloride	: 25 g
Sodium phosphate dibasic	: 150 g
Potassium dihydrogen phosphate	: 75 g
Sodium chloride	: 125 g

The above chemicals are added one after another with constant stirring using a magnetic stirrer. Adjusted the volume to 500 ml. Autoclaved at 121 ˚C, 15 lbs pressure for 15 minutes and allowed to cool to room temperature and were stored at 4 ˚C until used. This serve as an alternative for sodium ammonium phosphate

d. 10% Glucose solution

Dextrose	: 10 mg
Distilled water	: 70 ml

Add dextrose into distilled water taken in a standard flask and stirred using a magnetic stirrer. Distilled water was added to bring the final volume to 100ml. Autoclaved at 121 ˚C, 15 lbs pressure for 20 minutes and allowed to cool to room temperature and were stored at 4 ˚C until used.

e. Glucose minimal agar plates

Distilled water : 900 ml

Agar : 15 g

VB salt solution : 10 ml

M9 solution : 10 ml

Glucose solution 10% : 50 ml

Add the agar to the water and autoclaved for 30 min at 121 ˚C. Allowed to cool for about 45 minutes to about 65 ˚C. Add sterile VB salt solution and M9 solution. Mixed thoroughly, then sterile glucose (10% v/v) solution was added and again swirled thoroughly. This medium was dispensed to petri dishes (approximately 20ml/plate). Allowed the plates to solidify and then stored at 4 ˚C. Before use, the plates warmed up to room temperature or incubated overnight at 37 ˚C in the cases of excess moisture content.

f. Top Agar

Agar : 0.6 g

NaCl : 0.6 g

Agar was dissolved in 90 ml of distilled Water and the solution was autoclaved at 121 ˚C, 15 lbs pressure for 15 minutes, allowed to cool to 50-45 ˚C and was added with 10ml of Histidine-Biotin solution.

g. Histidine-Biotin Solution (0.5mM)

Sterile Distilled water : 20 ml

Histidine : 1.92 mg

Biotin : 2.48 mg

The solution was filtered through 0.45 mm membrane filter and was stored at 4 ˚C until used.

h. Ampicillin solution

Distilled water : 100 ml

Ampicillin : 8 mg

Dissolved the ampicillin in warm (65 ˚C) water and sterilised using a 0.45 mm membrane filter. Stored at 4 ˚C.

i. Sodium phosphate buffer, 0.1 mM, pH 7.4

Sodium phosphate, monobasic (0.1 M)

$NaH_2PO_4.H_2O$: 13.8 g

Distilled Water : 1 l

Sodium phosphate, dibasic (0.1 M)

$Na_2HPO_4.H_2O$:14.2 g

Distilled Water : 1 l

120 ml of Sodium phosphate (0.1 M), monobasic and 880 ml of Sodium phosphate dibasic (0.1 M) solutions was mixed well and adjusted the pH to 7.4 using 0.1 M dibasic sodium phosphate solution. Autoclaved for 30 minutes at 121 ˚C and cooled. Stored the bottles at room temperature in the dark.

3.1.3. INSTRUMENTS

Accelerated solvent extractor	: Dionex ASE 150, Thermo Scientific, USA
Centrifuge	: REMI (column oven CTO-20A), India
Electronic weighing balance	: Shimadzu Corporation Ltd, India
GC-MS analyser	: Shimadzu; Model Number: QP2010S

Acquity UPLC system	: Waters
Q-TOF- mass spectrometer	: Waters Xevo G2 QTOF
Hot air oven	: Rotek Instruments Pvt. Ltd., India
Light microscope	: Leica (DM 500) German Radicle, Ambala
Magnetic stirrer	: Spinit, Tarsons Products Pvt. Ltd., Kolkata
Rotary evaporator	: Hahnshin Scientific, HS2005 V-N
Spectrophotometer	: Agilent (Carry 60 UV vis), USA
Stereo zoom microscope	: Leica (M205 C) German Radicle, Ambala
Sterile laminar air flow	: Labline, India
Vortex mixer	: Rotek Instruments Pvt. Ltd., India
Water bath	: Rotek Instruments Pvt. Ltd., India
Flow cytometer	: BD FACSAria III, BD Biosciences
Autoclave	: Rotek Instruments Pvt. Ltd., India
Fluorescent Cell Imager	: Bio-Rad, India

3.1.4. STAINS

a. Giemsa stain

Giemsa powder	: 800 mg
Glycerol	: 50 ml
Methanol	: 50 ml

Giemsa powder was dissolved in glycerol at 60 ˚C with shaking and the mixture was cooled to room temperature. To this methanol was added and mixed well for 5 minutes and allowed to stand overnight. It was filtered through Whatman No.1 filter paper and stored at 4 ˚C.

b. Trypan blue dye

Trypan blue powder	: 100 mg

Normal saline (0.9% NaCl) : 100 ml

Trypan blue powder was dissolved in saline and then filtered using Whatman No.1 filter paper

c. Haematoxylin solution

Haematoxylin : 5 g

Ethyl alcohol : 50 ml

Potassium alum : 50 mg

Potassium iodide : 50 mg

Distilled water : 950 ml

Haematoxylin was dissolved in ethyl alcohol with gentle heating. The alum was dissolved in distilled water by heating along with continuous stirring and maintained at 4 ˚C overnight. Alcoholic haematoxylin was mixed with the alum solution. This mixture was allowed to cool and potassium iodide was added and filtered.

d. Eosin solution

Eosin : 500 mg

Ethyl alcohol : 100 ml

Eosin was dissolved in 5 ml of ethyl alcohol and it was made up to 100 ml with ethyl alcohol

e. Leishmann's stain

Leishmann's stain : 150 mg

Methanol : 100 ml

Leishmann's stain was dissolved in methanol and filtered.

f. Turk's fluid

Acetic acid : 1.5 ml

10% crystal violet : 1.0 ml

Distilled water : 98 ml

Stirred overnight and filtered.

3.1.5. CELL LINES

- Dalton's Lymphoma Ascites (DLA) cell lines were procured from Amala Cancer Research Institute, Thrissur, Kerala, India.
- YAC-1 (mice lymphoma) and $HepG_2$ (human hepatic) cell lines were obtained from National Centre for Cell Sciences (NCSS), Pune, India.

3.1.6. BACTERIAL STRAINS

- The bacterial strains *Salmonella typhimurium* TA100 (MTCC 1252) were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.1.7. ANIMALS

Male and female Swiss albino mice (25–30 g) used in this study were obtained from Small Animal Breeding Station, Kerala Veterinary University, Small Animal breeding station, Mannuthy, Kerala, India. Animals were maintained under controlled conditions of temperature (22-28 ˚C) and humidity (60-70%) in ventilated polypropylene cages with paddy husk bedding and top grill of stainless steel with facilities for getting food and water. Studies using experimental animals were conducted with prior permission from the Institutional Animal Ethics Committee, Amala Cancer Research Centre (Approval No. ACRC/IAEC/20(1)–P15) according to the rules and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

3.1.8. PLANT MATERIALS (Figure 3.1)

a. *Scoparia dulcis*

Common names: Licorice weed, goat weed, scoparia-weed and Sweet broom (English), Mithi patti (Hindi), Kallurukki (Malayalam), Sarakkotthini (Tamil) and Pashanabheda, (Sanskrit).

Habitat: Waste and cultivated ground, moist thickets or along sandy stream beds.

Habit: Herb

Taxonomic position

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Lamiales
Family	:	Plantaginaceae
Genus	:	Scoparia
Species	:	*Scoparia dulcis* L.

b. *Vernonia cinerea* (L.) Less

Common names: Ash fleabane, Purple fleabane (English), Sahadevi (Hindi and Sanskrit), Poovamkurunthala, Puvamkozhinjal, Puvamkurunal (Malayalam), Sirusengalaneer (Tamil).

Habitat: Roadsides, open waste places, dry grassy sites and plantations

Habit : Herb

Taxonomic position

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Magnoliopsida
Order	:	Asterales
Family	:	Asteraceae
Genus	:	Vernonia
Species	:	*Vernonia cinerea* (L.) Less

3.2. METHODS

3.2.1. COLLECTION AND AUTHENTICATION OF PLANT MATERIALS

Scoparia dulcis L. and *Vernonia cinerea* (L.) Less were collected locally (10°36'39.7"N76°02′10.7"E) from Thrissur district, Kerala, India during the months of June, July and August in the years 2018, 2019 and 2020. Authentication of the plant materials was done by Dr. V. B. Sreekumar, Senior scientist and head, Forest Botany Department, Kerala Forest Research Institute (KFRI), Peechi, Thrissur, Kerala and the voucher specimens were deposited with accession numbers 17684 (*S. dulcis*) and 17685 (*V. cinerea*).

3.2.2. EXTRACTION

The whole parts of each plant material were dried using a hot air oven with temperature set at 50 °C. The dried materials were then powdered using mixer grinder. 15 grams of this dried powder was weighed and mixed with an inert compound called diatomaceous earth and packed in the extraction cell of an accelerated solvent extractor (Dionex ASE 150, Thermo Scientific). The cellulose filters of 1.91 cm in diameter were laid down on both ends of the cell. The cell was then closed and kept inside the ASE. The samples were extracted separately with solvents *viz* ethyl acetate, ethanol and water. The solvent was kept in a separate chamber having 200 ml of capacity. Extraction was done at 1500 psi pressure and temperature ranging from 75-125 °C depending on the boiling point of solvents used for extraction. At this condition 60% solvent flushed into the extraction cell and purged with nitrogen. The extracts were collected in separate collection vials. Rinsing of complete system was done between each extraction procedure to avoid any cross contamination. The extracts obtained were evaporated to dryness using a vacuum evaporator (Hahnshin Scientific, HS2005 V-N) and were stored in amber

coloured bottles under refrigeration (4 °C) until further use. The extraction yield of individual extracts was calculated. The extracts were dissolved in appropriate vehicle solvents for further *in vitro* and *in vivo* studies.

3.2.3. PRELIMINARY PHYTOCHEMICAL ANALYSIS

3.2.3.1 Qualitative testing of phytochemicals

The qualitative phytochemical screening of various plant extracts of *S. dulcis* and *V. cinerea* was performed to identify the occurrence of some important secondary metabolites. The concentrated crude extracts were used for the screening of alkaloids, polyphenols, flavonoids, tannins, saponins, terpenoids and phytosterols according to the standard procedures (Sofowora, 1996; Trease & Evans, 1989; Harborne, 1999).

i. Test for alkaloids

a. Mayer's test: To 0.2 g of plant extract, 5 ml of 1% hydrochloric acid was added and then boiled in a steam bath. This was then allowed to be cooled and filtered. To the filtrate, 2 drops of Mayer's reagent was added. Appearance of creamy white precipitate indicates the presence of alkaloids.
b. Dragendroff's test: To 0.2 g of plant extract, 5 ml of 1% hydrochloric acid was added and then boiled in a steam bath. This was then allowed to be cooled and filtered. To the filtrate, 2 drops of Dragendroff's reagent was added. Appearance of brown to reddish brown precipitate indicates the presence of alkaloids.
c. Wagner's test: To 0.2 g of plant extract, 5 ml of 1% hydrochloric acid was added and then boiled in a steam bath. This was then allowed to be cooled and filtered. To the filtrate, 2 drops of Wagner's reagent was added. Appearance of reddish brown precipitate indicates the presence of alkaloids.

ii. Test for polyphenols

a. Ferric chloride test: To 1 ml of plant extract (0.5 g), equal volume of ferric chloride (5%) was added. Appearance of deep blue colour indicates the presence of polyphenols.

b. Lead acetate Test: To the plant extracts (0.5 g), 10% lead acetate solution was mixed and the colour of precipitate was observed. Formation of white precipitate shows the presence of polyphenols.

iii. Test for flavonoids

a. Ferric chloride test: Plant extract (0.5 g) was boiled with water and filtered. To 1 ml of this filtrate two drops of ferric chloride solution (10%) was added and observed for the colour formation. Bluish green or violet colours show the presence of flavonoids.

b. Sulphuric acid test: To the plant extracts, concentrated sulphuric acid was added. Formation of yellow colour indicates the presence of flavonoids.

iv. Test for tannins

a. To 2 ml of the extract (0.5 g), 10 ml of distilled water and a drop of 10 % $FeCl_3$ was added. The development of blue colour shows the presence of tannins.

b. To about 0.5 g of the extract, 5 ml of 45% ethanol was added and boiled in a water bath for about 5 minutes. The mixture was allowed to be cooled and filtered. To 1 ml of the filtrate 3 drops of lead acetate (1 %) solution was added. Formation of a gelatinous precipitate shows the presence of tannins.

v. Test for saponins

a. 2 ml of the extract (0.5 g) was mixed with 4 ml of distilled water and it was shaken vigorously for 1 minute. Stable froth formation indicates the presence of saponins.

b. About 0.1 g of the extract was boiled with distilled water (5 ml) for 2-3 minutes. And then filtered. To 1 ml of the filtrate 2 drops of olive oil was added and shaken for a minute. Formation of emulsion indicates the presence of saponins.

vi. Test for terpenoids

a. Salkowski test: To 2 ml of plant extract (0.5 g), acetic anhydride and concentrated sulphuric acid was added gently. Bluish green ring formation indicates the presence of terpenes.

vii. Test for phytosterols

a. Liebermann Buchard test: To 1 ml of acetic anhydride, chloroform was added in equal amounts and then cooled to 0 °C. To this 1 ml of plant extract (0.5 g) and concentrated sulphuric acid was added. Formation of green, red, orange colour changes indicates the presence of phytosterols.

b. Salkowski test: 2 ml of plant extract (0.5 g) was mixed with acetic anhydride and concentrated sulphuric acid was added gently. The formation of a brownish red colour shows the presence of phytosterols.

3.2.3.2. Quantitative testing of phytochemicals

i. Estimation of Total Phenolic Content (TPC)

Total phenol content in the plant extracts was estimated by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). 100 µl of the extract (100 mg/ml) was mixed with 3.9µl of distilled water and 500 µl of Folin-Ciocalteu reagent. After 3 minutes, 2 ml of 20 % sodium carbonate was added and mixed well. This mixture was incubated for 1 minute in a boiling water bath and the absorbance was determined using Ultraviolet-Visible spectrophotometer (Agilent carry 60 UV- vis) at 650 nm. All samples were prepared in triplicate. The same

procedure was done with gallic acid (the standard solution). TPC was expressed in terms of mg of Gallic Acid Equivalents/ gram of the extract (mg GAE/g).

ii. Estimation of Total Flavonoid Content (TFC)

The TFC of plant extracts was estimated colourimetrically using the aluminium chloride method (Chang et al., 2002). The extracts were diluted using methanol to a concentration of 100 mg/ml. 100 µl of this sample was mixed with 0.1 ml of 0.1 mM potassium acetate solution and 0.1 ml of 10% (w/v) aluminium chloride solution. This mixture was incubated for 30 minutes at room temperature. Same procedure was repeated with the standard solution, quercetin. After incubation, the absorbance was measured at 415 nm using UltraViolet-visible spectrophotometer (Agilent Carry 60). A calibration curve was plotted to determine total flavonoid content and the TFC was expressed as milligram of quercetin equivalents of extract (mg QCE/g).

iii. Estimation of Total Terpenoid Content (TTC)

Total terpenoid content of plant extract was estimated according to Indumathi et al., 2014. To 100 mg (wi) of plant extract, ethanol (9 ml) was added and kept for 24 hours. Then it was filtered and the filtrate was extracted with petroleum ether (10 ml). Ether layer was collected using a separating funnel to a preweighed glass vial. Evaporation of ether was done and the dry weight of ether extract was calculated (wf). Total terpenoid content was estimated using the equation (wi-wf) / wi ×100.

3.2.4. IDENTIFICATION OF COMPOUNDS USING CHROMATOGRAPHY AND MASS SPECTROMETRY

i. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Identification of volatile compounds of ethyl acetate, ethanol and water extracts of *S. dulcis* and *V. cinerea* was performed using GC-MS (Shimadzu ; Model Number: QP2010S) equipped with a capillary column (ELITE-5MS) of 30 metre length, 0.25 mm internal diameter and 0.25 μm film thickness. Ultra-pure Helium (99.99%) was employed as carrier gas at a linear velocity of 36.5 cm/sec. The sample volume of 1 μl was injected by maintaining the injector temperature at 260 °C in splitless mode. The oven temperature was programmed from 60 °C (hold time of 5 min) to 280 °C. Other instrument conditions for the GC programme include Ion source temperature 200 °C, Interface temperature 280 °C, Flow control mode-Linear velocity, Pressure-57.5kPa, solvent cut time-5 minutes, Detector gain mode-Relative, Threshold-500. For the MS, start time 5.10 min, end time 49 min, ACQ mode- Scan, Event time 0.50 seconds and scan Speed 1250. The start m/z 50.00 and finish time m/z 650.00 were used. The mass spectra obtained were compared and matched with those reference spectra available in the database of NIST 11 and WILEY 8 for the identification of compounds.

ii. Liquid Chromatography-Mass Spectrometry analysis

Ultra performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-ToF-MS) was used for the chromatographic separation and detection of compounds in the extracts. Acquity UPLC system (Waters) comprises a TUV detector (J12TUV750A), a column chamber (J12 CHA730G), a quaternary solvent manager (H12QSM632A) and a sample manager FTN (K12 SD1069G). A reversed-phase BEH C18 column having the dimensions

50 mm × 2.1 mm × 1.7 μm with a flow rate of 0.3 ml min^{-1} was applied for chromatography. Water and acetonitrile with 0.1% formic acid in gradient mode was taken as the mobile phase. A quadrupole time-of-flight mass spectrometer (Waters Xevo G2 QTOF) with electrospray ionisation (ESI) interphase working in positive and negative ionisation mode was connected with the UPLC system. 10 μl of sample was injected into the column. All samples were analysed using ESI positive ionisation mode and scanned for the m/z range between 50 and 1000. The desolvation gas flow used was 900 l/h and 350 ^{o}C temperature was maintained. The mass spectra obtained in the range of 5 to 30eV of collision energy. MassLynx software (4.1) was used for managing the instrument and also for data acquisition.

3.2.5. GENOPROTECTIVE POTENTIALS OF PLANT EXTRACTS

3.2.5.1 Ames assay

Ames assay or bacterial reverse mutation test detects reversion of mutations present in the genes of tester bacterial strains involved in the synthesis of an essential amino acid, histidine. This test is used for the preliminary screening for the genotoxicity (especially, for point mutation-inducing activity) of chemicals. This test, with some modifications, can also be used for testing antigenotoxic potentials of chemicals. When the *Salmonella* tester strains are grown on glucose minimal agar plates containing a trace of histidine, only those bacteria that revert to histidine-independence (*his*$^{+}$) are able to form colonies. The number of revertant colonies per plate is increased usually when the bacteria are treated with mutagenic compounds. Standard plate incorporation method with pre-incubation procedure was used to test the mutagenic/ antimutagenic potential of plant extracts using *Salmonella typhimurium* TA100 (base pair substitution). In plate incorporation method, the suspension containing bacteria and the test chemical are mixed with an

overlay agar and plated immediately onto a minimal medium. After 48 hours of incubation, revertant colonies are counted and compared to the number of spontaneous revertant colonies on solvent control plates (Vijay et al., 2018).

Procedure

Preincubation mixture containing 100 µl of overnight bacterial culture, 50 µl of mutagen or 50 µl of plant extract at different concentrations and 500 µl of sodium phosphate buffer was incubated for 20 minutes at 37 ^{o}C. After incubation, 2 ml of molten top agar supplemented with 0.5mM L-histidine and 0.5mM D-biotin (maintained at 45 ^{o}C) was mixed and poured directly over the surface of glucose minimal agar plates and rotated quickly to spread the top agar evenly on the plate. The plates were then allowed to solidify and incubated at 37 °C in an inverted position for 48 hours using an incubator. Post incubation the background lawn of the plates were checked carefully to know the viability of bacterial cells and counted the number of revertant colonies. Negative control (without test chemicals) was used to determine spontaneous reversion activity. Sodium azide (5 µg/ml) was used as positive control. The standard mutagen (Sodium azide) was added simultaneously with the plant extracts during preincubation in antimutagenicity protocol.

Percentage inhibition rates of mutagenicity was calculated using the formula:

$$\text{Inhibition rate } (\%) = 1 - (\text{T} / \text{M}) \times 100$$

Where T = No. of revertants in the plate with the mutagen and the plant extract, M= No. of revertants in the positive control. Evaluation of antimutagenicity was done as follows: ‘strong’ > 40%, ‘moderate’ between 25–40% and ‘weak’ when the inhibition rate is less than 25% (Negi et al., 2003).

3.2.5.2 Cytokinesis Block Micronucleus Assay (CBMN assay)

Micronuclei are the tiny, extranuclear entities originated from fragments of chromosomes or whole chromosomes which lag behind at anaphase during nuclear division. The cytokinesis-block micronucleus (CBMN) assay is one of the standard cytogenetic tests for assessing genotoxicology of chemicals in which scoring of micronuclei (MN) in once divided cultured human and/or mammalian cells recognised by their binucleated (BN) appearance after inhibition of cytokinesis by cytochalasin (Fenech, 2006).

Procedure

The HepG2 cells (2.5×10^5 cells) were seeded in each culture flask with DMEM medium and incubated for 24 hours. Post incubation, the cells were treated with the test chemicals such as cyclophosphamide (15 µg/ ml) or plant extracts (100 µg/ml) for 3 hours (concentrations are selected based on the cytotoxicity data) for testing its genotoxic effects.

For testing antigenotoxic activity, two types of treatment patterns were adopted such as co-treatment where administration of cyclophosphamide simultaneously with the respective samples and post treatment where cyclophosphamide addition, followed by its removal after 3 hours which was then followed by the addition of plant extracts for 3 hours.

Freshly prepared Cytochalasin B solution was added to cell cultures at a final concentration of 3 µg/ml. Cells were treated for additional 24 hours and then harvested and washed in PBS, resuspended (approximately 5×10^6 cells/ml) and spread onto glass slides (20 µl of cell suspension per slide). After air-drying, the cells were fixed twice using methanol/glacial acetic acid (6:1) for 10 min and

stained with 5% Giemsa solution and mounted with Canadian balsam (Johnson & Varghese, 2024).

By using a light microscope, 1000 binucleated cells were assessed for each treatment. Three biological replicates for each sample were maintained with three technical replicates (slides) each and the percentage of micronuclei were calculated. 500 cells per treatment were scored for the presence of one, two or more than two nuclei in order to calculate Cytokinesis Block Proliferation Index (CBPI).

$$\text{CBPI} = \frac{[1\text{N} + (2 \times 2\text{N}) + (3 \times > 2\text{N})]}{\text{TC}}$$

Where 1N is the number of cells with one nucleus, 2N with two nuclei, >2N with more than two nuclei and TC is the total number of cells examined.

Percentage cytostasis was calculated from CBPI value with the formula

$$\%\ \text{cytostasis} = 100 - 100 \times \frac{CBPIt - 1}{\text{CBPIc} - 1}$$

Where t and c are treated and control samples, respectively

3.2.6. Free radical scavenging assays

The antioxidant or free radical scavenging potentials of plant extracts were tested by using the following protocols.

3.2.6.1. DPPH radical scavenging Assay

The DPPH radical scavenging potential of extracts was measured according to the protocol of Blois, 1958.

To the 1.5ml (30mM) methanolic solution of DPPH (2, 2-diphenyl-1-picryl - hydrazyl-hydrate), 10 µl of extracts at various concentrations (25 µg/ml to 100 µg/ml) and 1.49 ml of methanol were added. After 30 minutes of incubation, discoloration of purple colour was measured at 518 nm. Percentage DPPH scavenging was calculated using this equation.

$$\text{Percentage Scavenging of DPPH radical} = \frac{(\text{A0} - \text{As})}{\text{A0}}$$

Where, A0 is the absorbance of control and As is the absorbance of the sample.

3.2.6.2. ABTS radical scavenging Assay

The ABTS radical scavenging potential of extracts was measured according to the protocol of Re et al., 1999.

ABTS (2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) solution (7 mM) was mixed with 2.45 mM ammonium persulphate (1:1 v/v) and kept at dark for 8-10 hours for generating ABTS radical. This solution was diluted using ethanol to an absorbance of 0.700 at 734 nm. 10 µl of extract at various concentrations (25 µg/ml to 100 µg/ml) was mixed with 2990 µl of radical solution and incubated for 30 minutes. Absorbance was measured at 734 nm. Percentage ABTS radical scavenging was calculated using this equation.

$$\text{Percentage Scavenging of ABTS radical} = \frac{(\text{A0} - \text{As})}{\text{A0}}$$

Where, A0 is the absorbance of control and As is the absorbance of the sample.

3.2.6.3. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging potential of extracts was measured according to the protocol of Halliwell et al., 1987.

20 µl of plant extracts at different concentrations was mixed with 0.1 ml of 2-deoxyribose (2.8 mM), 0.1 ml of ferric chloride (0.1mM), 0.1 ml of ascorbic acid (0.1mM), 0.1 ml of EDTA (0.1mM), 0.1 ml of H_2O_2 (0.1mM) and make up to 1 ml using KH_2PO_4-KOH buffer (20mM, pH 7.4). This mixture was mixed well and incubated for 60 minutes at room temperature. Post incubation, 1 ml of 1% TBA and 1 ml of 28% TCA were added and all the tubes were kept in a boiling water bath for 30 minutes. The absorbance of the resulting supernatant

was measured by a spectrophotometer at 535 nm. The percentage of hydroxyl radical scavenging was evaluated using this equation.

$$\%\ \text{Scavenging of hydroxyl radical} = \frac{(A0\ -\ As)}{A0}$$

Where, A0 is the absorbance of control and As is the absorbance of the sample.

3.2.6.4. Lipid Peroxidation Assay

A modified form of Thiobarbituric acid reactive species (TBARS) assay using egg yolk homogenate as lipid source was used to calculate the ability of the plant extracts to inhibit the lipid peroxidation (Kishida et al., 1993). To 500 µl of egg homogenate (10% v/v in PBS), 100 µl of plant extract at various concentations dissolved in DMSO was added and made upto 1 ml using distilled water. To this, 50 µl of $FeSO_4$ (0.074 M) and 20 µl of ascorbic acid (0.1 M) was added and incubated for 1 hour at 37 ^{o}C to induce lipid peroxidation. Thereafter, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA in 1.1 % w/v SDS were added and heated to 95 ^{o}C for 60 minutes. Then allowed the mixture to cool and 5 ml of butanol was added. Then centrifuged at 3000 rpm for 10 minutes. Absorbance of the organic layer was read at 532 nm. Percentage inhibition of lipid peroxidation was calculated using this equation.

$$\text{Percentage inhibition}\ =\ \frac{(A0\ -\ As\,)}{A0}$$

Where, A0 is the absorbance of control and As is the absorbance of the sample.

3.2.7. EFFECT OF PLANT EXTRACTS ON CELL PROLIFERATION, APOPTOSIS AND CELL CYCLE

3.2.7.1. a. Cell death analysis using Trypan blue dye exclusion assay

Trypan blue dye cannot penetrate into the cytoplasm of live cells due to the presence of their intact plasma membrane. Whereas the dye can easily penetrate

through the porous membrane of dead cells since they lost its integrity. Thus this dye is capable of imparting blue colour to the dead cells exclusively. Number of dead cells in the population was counted using the haemocytometer to calculate the cytotoxicity (Strober, 2001).

Procedure

Trypan blue dye exclusion assay was used for the short term cytotoxicity analysis of ethyl acetate, ethanol and water extracts of the plants. The DLA cells were aspirated from the peritoneal cavity of tumour bearing mice. Viable cell suspension (1×10^6 cells in 0.1 ml) were added to tubes containing different concentrations of the extracts and the volume was made up to 1.0 ml using phosphate buffered saline (PBS). The assay mixtures were incubated for 3 hours at 37 ^{o}C. Post incubation, 0.1 ml of 1% trypan blue solution was added and kept for 2 minutes before loading to a haemocytometer for counting (Johnson & Varghese, 2023a). The numbers of stained (dead) and unstained (live) cells were counted and percentage cytotoxicity was calculated as:

$$\%\ \text{cytotoxicity} = \frac{\text{Number of dead cells}}{\text{Number of total cells}} \times 100$$

IC_{50} values were calculated using SPSS software.

3.2.7.1. b. Trypan blue dye exclusion assay for determining Ferroptosis

Detection of iron content in tumour cells is a way to detect ferroptosis. Restraining of cytotoxicity induced by cytotoxic drugs upon the co-treatment with iron chelators indicates the role of iron in the induction of cell death (Wang et al., 2022).

Ferroptotic potential of selected extracts was tested with trypan blue dye exclusion assay as mentioned above with some modifications in the treatment pattern. Here, the extracts were treated simultaneously with deferoxamine, an iron

chelating compound. The IC_{50} values for the co-treatment obtained were compared with those values obtained when plant extract alone was treated.

3.2.7.2. Cell proliferation assay

YAC-1 cells (1×10^5) from log phase cultures were seeded per well of 6-well flat-bottom culture plate in 1000 µl of RPMI medium supplemented with 10% foetal bovine serum. Cells were treated with the plant extracts (5, 10 and 15 µg/ml concentrations dissolved in DMSO) for defined time intervals (12, 24 and 48 h). After the treatment, the cell suspension was centrifuged at 5000 rpm for three minutes and the pellet was resuspended in 1ml of PBS. Trypan blue staining was done and the number of dead and live cells were counted(Johnson & Varghese, 2023a).

3.2.7.3. MTT Assay

Cytotoxicity and cell viability assessment of plant extracts in $HepG_2$ cells were carried out using MTT assay. This is a colorimetric assay based on the reduction of a yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT) to purple coloured formazan crystals by metabolically active cells. The viable cells reduce the MTT to formazan since they contain NAD (P) H-dependent oxidoreductase enzymes (Mosmann, 1983). The insoluble formazan crystals are dissolved using a solubilizing solution and the absorbance at 570 nm of the resulting purple coloured solution is measured using an ELISA plate reader.

Initially, $HepG_2$ cells (2500 cells/well) were seeded on 96 well plates and incubated at 37 °C (95° humidity & 5% CO_2) for 24 hours. The test samples were prepared in DMEM media and sterilised using 0.2 µm Millipore syringe filter and diluted to appropriate concentrations. Then the samples were added to the wells

containing cells at final concentrations of 0 (vehicle only), 6.25, 12.5, 25, 50, 100 µg/ml respectively and incubated for 24 hours. Post incubation, the media from the wells were aspirated and discarded. 100 µl of 0.5 mg/ml MTT solution in PBS was added to each wells and were further incubated for 2 hours for the development of formazan crystals. The supernatant was collected and 100 µl DMSO were added. The absorbance at 570 nm was measured. Two wells per plate without cells kept as blank.

The cell viability was calculated using the following formula.

$$\text{Percentage of cell viability} = \frac{\text{Average absorbance of treated}}{\text{Average absorbance of control}} \times 100$$

3.2.7.4. Cell cycle analysis

Flow cytometry technique was used to analyse the cell cycle pattern by taking DNA content into account. In this technique light scattering and fluorescence emission, proportional to DNA content is detected by using specific fluorescent probe-labelled cells as they pass through a laser beam (Jayat & Ratinaud, 1993).

Procedure

Approximately 1×10^6 YAC-1 cells were seeded in 6-well plates and incubated overnight. Afterwards, cells were treated with plant extracts at 5 µg/ml or 10 µg/ml concentrations and incubated further for 24 hours. The cells were then washed using PBS by centrifugation at 5000 rpm for 5 minutes and were resuspended in Hoechst stain (5 µg/ml) for 15-20 minutes. It was washed again with PBS by centrifugation and the pellets were resuspended in 250µl culture medium and analysed by flow cytometry.

3.2.7.5. Apoptosis analysis

Annexin V- propidium iodide staining followed by flow cytometric analysis was used to study the apoptosis inducing potentials of the plant extracts. One of the

earliest events taking place in cells experiencing apoptotic cascade is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Annexin V can reversibly bind to phosphatidylserine residues in the presence of divalent calcium ions. Propidium iodide is a fluorescent agent which can stain DNA. Live cells cannot take up PI due to their intact cell and nuclear membranes. In the cells experiencing necrosis and later stages of apoptosis which have leaky DNA content PI can penetrate through the cell membrane and stains DNA. Thus the co-treatment of Annexin V tagged with a fluorescent dye called FITC (Fluorescein isothiocyanate) and PI followed by flow cytometric analysis can be used to discriminate the live (Annexin V^- ; PI^-), early apoptotic (Annexin V^+ ; PI), late apoptotic (Annexin V^+ ; PI^+) and necrotic (Annexin V^- ; PI^+) cells in treated cell population (Lakshmanan & Batra, 2013).

Procedure

The YAC-1 cells (1×10^6) were seeded in a T25 culture flask and treated with various concentrations of plant extracts. After 24 hours of treatment, the spent medium along with the cells were collected and washed twice with PBS by centrifugation (670×g, 5 minutes) and resuspended the pellets in PBS (400 µl). Treated cells (400 µl) were then mixed with 100 µl of incubation buffer with 2 µl of Annexin (1 mg/ml) and 2 µl of propidium iodide (1 mg/ml). After 10 minutes, the cells were analysed using flow cytometry (Johnson & Varghese, 2023a).

3.2.8. EVALUATION OF ACUTE AND SUBACUTE TOXICITY AND ANTITUMOR POTENTIALS OF PLANT EXTRACTS USING MICE MODEL

3.2.8.1. Acute toxicity

Oral Acute toxicity evaluation of plant extracts was done in mice model by following OECD (Organisation for Economic Co-operation and Development) guideline number 423 (OECD, 2002). Female Swiss albino mice completed 14 days of acclimatisation, were grouped (n=6) as follows.

Group I: Vehicle control (sunflower oil)

Group II: VCEA (2000 mg/kg body weight)

Group III: SDEA (2000 mg/kg body weight)

All the mice were fasted overnight prior to the oral administration of the drug/vehicle. The animals were then observed continuously for 1 hour and intermittently for 4 hours and thereafter once every 24 hours for the next 14 days. Clinical observations such as mortality, behavioural changes and any other abnormalities were noted during this study period. Body weight of animals was measured weekly. The amount of food and water consumed per day was also recorded during the entire study period. On the 15^{th} day, the animals under this study were euthanized and the necropsy was done (Johnson & Varghese, 2023b).

3.2.8.2. Subacute toxicity

Sub-acute toxicity determination was conducted according to the OECD guideline number 407 using both male and female Swiss albino mice (OECD, 2008). The animals were weighed and grouped (n=5) as follows:

Group I a: Untreated male

Group II a: Vehicle control male (sunflower oil)

Group III a: Male mice receiving VCEA (50 mg/kg body weight)

Group IV a: Male mice receiving VCEA (100 mg/kg body weight)

Group V a: Male mice receiving SDEA (100 mg/kg body weight)

Group VI a: Male mice receiving SDEA (200 mg/kg body weight)

Group I b: Untreated female

Group II b: Vehicle control female (sunflower oil)

Group III b: Female mice receiving VCEA (50 mg/kg body weight)

Group IV b: Female mice receiving VCEA (100 mg/kg body weight)

Group V b: Female mice receiving SDEA (100 mg/kg body weight)

Group VI b: Female mice receiving SDEA (200 mg/kg body weight)

Drug was administered orally consecutively for 28 days using an oral gavage. Body weight, food and water consumption per group of animals were noted on every third day during the study period. On the 28th day after overnight fasting the animals were euthanized. Haematological and biochemical parameters were analysed using the blood and serum samples collected immediately from the animals. Vital organs were carefully isolated, weighed and subjected for histopathological analysis (Johnson & Varghese, 2023b).

3.2.8.2.1 Haematological parameters

i. Estimation of Haemoglobin

Principle

The haemoglobin is turned to cyanmethemoglobin in the presence of potassium ferricyanide and potassium cyanide. The absorbance of cyanmethemoglobin is proportional to the concentration of haemoglobin (Drabkin & Austin, 1932).

Procedure

The reagents used were procured from Agappe diagnostic kits. 20 µl of fresh blood collected in heparinized tube was mixed with 5 ml of Drabkin's reagent and incubated for 5 minutes at room temperature. It was followed by the measurement of absorbance at 545 nm against reagent blank. The concentration of Hb was calculated by using the following formula.

$$\text{Hb (gm/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times N \times 0.251$$

Where N= concentration of standard (60 mg/dl)

ii. Estimation of red blood cells (RBC)

Principle

Blood sample was diluted 200 times using an RBC diluting fluid enabling the fixation of the RBC and thus preventing haemolysis. RBC count can be estimated using a haemocytometer after dilution and the values can be represented as the number of RBC/cu mm of whole blood (Cheesbrough & McArthur, 1976).

Procedure

The blood was drawn using an RBC pipette up to the mark of 0.5 and diluted up to the mark of 101 using RBC diluting fluid, Hayem's solution [Mercuric chloride (0.25 g), Sodium sulphate (2.50 g) and Sodium chloride (0.50 g) dissolved in 100 ml distilled water]. It was mixed by rotating the RBC pipette between palms for a few minutes. The Neubauer counting chamber was charged with 4-5 drops of diluted blood from the RBC pipette without the formation of any air bubbles and kept for 3-4 minutes. The red blood cells were then counted using a light microscope (40× magnification). The RBC count was calculated using the formula:

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

Here,

Dilution factor = 200

Depth of counting chamber = 0.1mm

Area counted = 5/25

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

iii. Estimation of total white blood cell count (TC)

Principle

Treatment of blood using Turk's fluid results in the lysis of RBCs due to the presence of acetic acid and leaves the WBCs intact which will take up the colour of crystal violet present in this fluid (Cheesbrough & McArthur, 1976).

Procedure

Blood was drawn up to the mark of 0.5 of a WBC pipette and diluted up to the mark of 11 using Turk's fluid. The solution was mixed well by rotating the pipette between palms and keeping it for a few minutes to allow the cells to settle. The Neubauer counting chamber was then charged with a few drops of diluted blood solution deprived of air bubbles and after 3-4 minutes, the white blood cells in the four corner squares of the counting chamber were counted using a light microscope (10× magnification).

The total WBC count was estimated using the formula:

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

iv. Estimation of differential leukocyte count (DC)

Principle

Blood smear was prepared and stained with Leishman stain in order to identify percent different kinds of leukocytes. Neutrophils appear as a purple-coloured nucleus and pink cytoplasm. Eosinophils are seen as pink coloured

cytoplasm and purple coloured, dumbbell shaped nucleus. Basophils appear as purple-coloured nucleus with dark blue granules. Monocytes have a purple-coloured, horseshoe shaped nucleus and pink cytoplasm. Lymphocytes have dark blue circular nuclei with light blue cytoplasm. Platelets can be observed as violet-coloured granules (Wintrobe and Greer, 2009).

Procedure

A thin blood smear was prepared from a drop of blood and air dried. It was then stained using Leishman stain and kept for 2-3 minutes. The stain was diluted with an equal amount of distilled water and kept for 2-3 minutes. Excess stains were removed from the slide by washing and air dried. The WBCs were observed and counted by moving the slides in a zig-zag fashion under a light microscope at 40× magnification.

v. Estimation of platelet count (PLT)

Principle

1% ammonium oxalate solution (isotonic dilution fluid) can exclusively lyse the erythrocytes leaving the platelets intact. Hence platelets can be easily counted with the help of a Neubauer counting chamber and a light microscope (Cheesbrough & McArthur, 1976).

Procedure

RBC pipette was used to draw up the blood and 1:200 dilution of blood was prepared by adding 1% ammonium oxalate (dilution fluid). After gentle mixing for about 2 minutes, it was charged to the Neubauer's counting chamber and observed under 40× magnification using a light microscope. The platelet count was estimated using the following formula:

Number of platelets per mm^3 = N × 10000 Where N = Total number of cells counted.

vi. Estimation of packed cell volume (PCV)

Principle

Anticoagulated blood taken in Wintrobe Micro haematocrit tube (Wintrobe tube) when subjected to centrifugation at a moderate speed results in the sedimentation of erythrocytes at the bottom of the tube. This sedimented part is considered for determining packed cell volume or as haematocrit (Wintrobe and Greer, 2009).

Procedure

The anti-coagulated blood was taken in the Wintrobe tube with the help of a Pasteur pipette. The tubes were then centrifuged at 2500 g for about 30 minutes after thorough mixing of the blood sample. The length of the erythrocyte cell column was measured and the percentage of erythrocyte cell column to the total volume of blood sample was estimated.

vii. Estimation of erythrocyte sedimentation rate (ESR)

Principle

The anti-coagulated blood in Westergren's tube, kept undisturbed in a vertical position is used to note the level of RBCs. The ESR was expressed as the distance travelled by the RBC column (mm/h) (Westergren, 1957).

Procedure

1.6 ml of blood was mixed with 0.4 ml of 3.8% sodium citrate solution and mixed thoroughly. The Westergren's ESR tube was filled with this solution up to the level marked as zero and maintained in vertical position. The level was noted

after 30 minutes, 1 hour and 2 hours after filling the tube. The ESR value of RBCs was calculated as the distance travelled (mm) per 1 hour.

3.2.8.2.2. Biochemical parameters

a. Liver function analysis

The blood collected through cardiac puncture was transferred to Eppendorf tubes and centrifuged at 5000 rpm for 10 minutes and separated the serum. This serum obtained was used to analyse the liver function marker enzymes such as SGOT, SGPT and ALP. Alongside, the amount of bilirubin, albumin, globulin and total protein was also estimated.

i. The Serum glutamate oxaloacetic transaminase (SGOT) activity

Principle

The SGOT catalyses the transamination reaction in which L-aspartate and αketoglutarate gets converted into oxaloacetate and L-glutamate. Oxaloacetate, in the presence of malate dehydrogenase, reacts with NADH and H^+ to form NAD^+ and L-malate. The rate of oxidation of NADH to NAD is measured spectrophotometrically at 340 nm and the change in absorbance is directly proportional to the activity of enzyme (Bergmeyer et al., 1976; Thefeld et al., 1974)

Procedure

The SGOT reagent kit was obtained from Agappe Diagnostics Ltd. The kit contained two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 include Tris buffer having pH 7.8 (88 mmol/l), L-Aspartate (260 mmol/l), MDH (>900 U/l) and LDH (>1500 U/l). Similarly, the R2 include 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 solution remains stable for about one month if kept at 2-8ºC. During assay 1000 µl of working reagent and 100 µl of

serum samples were mixed well and incubated for 1 min at 37 °C. Post incubation, the absorbance was measured at 340 nm keeping 1 minute interval for 3 minutes.

The SGOT activity was estimated using the formula:

$$\text{SGOT activity (U/l)} = (\Delta\text{OD/ min}) \times 1745$$

ii. Serum glutamate pyruvate transaminase (SGPT)

Principle

The SGPT catalyses the transamination reaction L-alanine and αketoglutarate into pyruvate and L-glutamate. In the presence of lactate dehydrogenase, the pyruvate reacts with NADH and H^+ into lactate and NAD^+. The rate of oxidation of NADH to NAD^+ is measured spectrophotometrically at 340 nm and the change in absorbance is directly proportional to the activity of enzyme (Thefeld et al., 1974).

Procedure

The SGPT reagent kit was procured from Agappe Diagnostics Ltd. The kit contains two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 includes Tris buffer having (110 mmol/l), L- alanine (600 mmol/l) and LDH (>1500 U/l). Similarly, the R2 includes NADH (0.24 mmol/l) and α-ketoglutarate (16 mmol/l). Working reagent was prepared by mixing 4 volumes of R1 and 1 volume of R2. This solution remains stable for about one month if kept at 2-8 ºC. During assay 1000 µl of working reagent and 100 µl of serum samples were mixed well and incubated for 1 min at 37 °C. Post incubation, the absorbance was measured at 340 nm keeping 1 min interval for 3 min.

The SGPT activity was estimated using the formula:

$$\text{SGPT activity (U/l)} = (\Delta\text{OD/ min}) \times 1746$$

iii. Alkaline phosphatase (ALP) activity

Principle

The ALP enzyme catalyses the conversion of p-Nitrophenyl phosphate into p-Nitrophenol and inorganic phosphate at pH 10.4. The change in absorbance at 405 nm is directly proportional to the activity of enzyme (Schlebusch et al., 1974)

Procedure

The ALP reagent kit was obtained from Agappe Diagnostics Ltd. The kit includes two reagents, namely reagent 1 (R1) and reagent 2 (R2). Here, the R1 contains the diethanolamine buffer having pH 10.2 (125 mmol/l) and magnesium chloride (0.625 mmol/l). R2 contains p-nitrophenyl phosphate (50 mmol/l). Working reagent was prepared by mixing 4 volumes of R1 and 1 volume of R2. This solution remains stable for about one month if kept at 2-8 ºC. During assay 1000 µl of working reagent and 20 µl of serum samples were mixed well and incubated for 1 min at 37 °C. Post incubation, the absorbance was measured at 340 nm keeping 1 min interval for 3 min.

The ALP activity was estimated using the formula:

$$\text{ALP activity (U/l)} = (\Delta\text{OD/ min}) \times 2750$$

iv. Total Bilirubin

Principle

The sulfanilic acid and sodium nitrite react to form diazotized sulfanilic acid. This diazotized sulfanilic acid acts on bilirubin in the presence of TAB (diazo reagent) to form a pink coloured complex called azobilirubin. The colour intensity can be measured spectrophotometrically at 546 nm (Walters & Gerarde, 1970).

Procedure

To 50 µl of serum, 20 µl total bilirubin activator and 1 ml of bilirubin reagent (28.9 mmol/l sulfanilic acid and 9 mmol/l TAB) provided in the test kit (Agappe Diagnostics Ltd) was added, mixed well and incubated at room

temperature for 5 min. This solution without serum was kept as blank. After incubation, the absorbance was determined at 546 nm.

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

$$\text{Total bilirubin concentration (mg/dl)} = \text{OD of the test} - \text{OD of blank} \times 25$$

v. Total protein estimation

Principle

Total protein estimation was done based on the principle of Biuret reaction (copper salt in an alkaline medium). Protein in the serum sample forms a blue coloured complex when treated with cupric ions in alkaline solution. The intensity of the blue colour developed is proportional to the total protein concentration.

Procedure

Total proteins present in the serum was estimated using the diagnostic kit purchased from Agappe Diagnostics Ltd. 10 µl of serum or standard was mixed with 1 ml of reagent solution in separate tubes and incubated for 5 min at room temperature. Absorbance was measured within 60 sec at 546 nm against the blank.

$$\text{Total Protein Concentration (gm/dl)} = (\text{Absorbance of test} / \text{Absorbance of standard}) \times 6$$

Where 6 gm/dl is the concentration of standard provided with the kit.

vi. Estimation of serum albumin

Albumin was determined based on its reaction with bromocresol green. Albumin binds with Bromocresol Green dye in a buffered medium and converted into a green coloured complex. The intensity of the colour is directly proportional to the amount of albumin.

Procedure

Quantity of albumin present in the serum was estimated using the diagnostic kit purchased from Agappe Diagnostics Ltd. 10 µl of serum or standard was mixed

with 1 ml of reagent solution in separate tubes and incubated for 1 minute at room temperature. Absorbance was measured at 630 nm against the blank.

$$\text{Albumin Concentration (gm/dl)} = (\text{Absorbance of test} / \text{Absorbance of standard}) \times 4$$

Where 4 gm/dl is the concentration of standard provided with the kit.

vii. Estimation of serum globulin

Serum globulin is estimated by subtracting the amount of albumin from the estimated total protein content in the serum.

b. Renal function

Commercially available diagnostic kits were used to analyse the kidney function parameters in the serum.

i. Quantification of serum urea

Principle

The urease enzyme catalyses the conversion of urea into carbon dioxide and ammonia. In this test, ammonium reacts with α-ketoglutarate and NADH to form glutamate and NAD^+. The oxidation of NADH to NAD^+ is proportional to the concentration of urea present in the serum. The absorbance is measured at 340 nm (Young et al., 1975).

Procedure

The diagnostic kit was procured from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. The working reagent was prepared by combining 4 volumes BUN reagent and 1 volume of urease. After that, 1 ml of working reagent and 10 µl of serum was shaken well at 37 ºC and absorbance was measured at 340 nm. The initial absorbance was taken after 30s (A1) and final absorbance after 60s (A2). The change in absorbance (ΔA) was calculated by subtracting A2 from A1. The same

experiment was repeated with standard obtained with the assay kit. The concentration of urea present in the sample was calculated using the formula:

$$\text{Urea (mg/dl)} = (\Delta AT/\Delta AS) \times 50$$

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

ii. Estimation of serum creatinine

Principle

The picric acid changes into sodium picrate in alkaline condition. This sodium picrate reacts with creatinine to form an orange-coloured creatinine picrate complex. This complex has maximum absorbance at the wavelength 520 nm. (Bonsnes & Taussky, 1945; Toro & Ackermann, 1975).

Procedure

The diagnostic kit for testing creatinine was obtained from Euro Diagnostic Systems Pvt. Ltd., Chennai, India. The kit includes two reagents: R1 - picric acid reagent and R2 - alkaline buffer. The working reagent was prepared by mixing equal volume of R1 and R2 reagents. After that, 1 ml of working reagent and 50 µl of serum was mixed thoroughly and absorbance was measured at 520 nm. The initial absorbance was taken after 30s (A0) and final absorbance after 90s (A1). The change in absorbance (ΔA) was obtained by subtracting A1 from A0. The same experiment was repeated with creatinine aqueous standard obtained with the assay kit. The concentration of creatinine present in the serum was calculated using the formula:

$$\text{Creatinine (mg/dl)} = (\Delta AT/\Delta AS) \times 2$$

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

c. Lipid profile

i. Estimation of total cholesterol

Principle

Cholesterol esters in serum are hydrolysed to free cholesterol by the enzyme called cholesterol esterase. Another enzyme called cholesterol oxidase can oxidise these free cholesterol into H_2O_2 and 4-cholesterol-3-one which in the presence of peroxidase couples with 4-aminoantipyrine and phenol to form a red quinone. The intensity of colour can be measured at 505 nm to calculate total cholesterol (Young, 1997).

Procedure

The cholesterol reagent kit consists of 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). 1 ml of the reagent was added to 10 µl of serum. This solution was mixed thoroughly and incubated for 5 min at 37 ºC. Post incubation, the absorbance was measured at 505 nm. The same experiment was done with standard delivered with the assay kit. The total cholesterol in the serum was calculated using the formula:

$$\text{Total cholesterol (mg/dl)} = (\text{Absorbance of sample}/\text{Absorbance of standard}) \times 200$$

ii. Estimation of triglycerides

Principle

Lipoprotein lipase is incubated with serum triglycerides yielding glycerol and fatty acids. This glycerol inturn gets phosphorylated by glycerol kinase into glycerol-3-phosphate. The enzyme glycerol phosphate dehydrogenase converts glycerol-3-phosphate into dihydroxyacetone phosphate (DHAP) and H_2O_2. The

peroxidase enzyme catalyses the reaction of H_2O_2 with 4-aminophenazone (4-AP) and p-chlorophenol to form a red coloured quinone. The absorbance of the final compound is determined at 505 nm. The intensity of quinone is directly proportional to the amount of triglycerides in the serum.

Procedure

The triglycerides present in the serum were estimated by the GPO-POD method. Approximately 1 ml of reagent in the diagnostic kit was added to 10 µl of serum and incubated for about 5 min at 20 ºC. Post incubation, the absorbance was obtained at 505 nm. The same experiment was done using the standard delivered with the assay kit. (The reagent contains 2 mmol/l p-chlorophenol, 50 mmol/l GOOD (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 serum were calculated using the formula:

$$\text{Total triglycerides (mg/dl)} = (\text{Absorbance of sample}/\text{Absorbance of standard}) \times 200$$

iii. HDL cholesterol (HDLc)

Principle

The detergent present in the diagnostic kit solubilizes the HDL cholesterol in the serum. The released HDLc then reacts with the enzymes such as cholesterol esterase and cholesterol oxidase and finally with chromogens to develop a colour proportional to the amount of HDLc which can be measured using the colorimetric method. Meanwhile the non-HDL lipoproteins and chylomicrons will be absorbed by the detergents. Thus their inhibition is overruled (Naito, 1985).

Procedure

To three test tubes labelled as, sample, calibrator and blank, R1 solution (450 µl) was added. Following that, 10 µl of serum and calibrator (lyophilized

human serum, 39.2 mg/dl) were added to the sample and calibrator tubes respectively. The solutions were mixed well and incubated at 37 ^{o}C for 5 min. Post incubation, 150 µl of R2 solution was added to all test tubes, mixed well and incubated at 37 ^{o}C for 5 min. The absorbance was determined at 600 nm against the reagent blank. The R1 solution consists of <1000 U/l cholesterol oxidase and <1 mMDSBmT. The R2 solution consists of <1 mM 4- aminoantipyrine, <1500 U/l cholesterol esterase, <1300 U/l peroxidase <3000 U/l ascorbic oxidase and <2% detergent. The HDL cholesterol in the sample was estimated using the formula:

$$\text{HDLc (mg/dl)} = (\Delta A \text{ sample} / \Delta A \text{ calibrator}) \times \text{concentration of calibrator}$$

iv. LDL cholesterol (LDLc)

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

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

v. VLDL cholesterol (VLDLc)

LDL cholesterol in the serum was calculated using the formula given below:

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

d. Serum electrolytes

i. Determination of sodium and potassium

Principle

Flame photometry method is used for the estimation of sodium and potassium (Deal, 1954). Alkali metal in a solution when administered into non-luminous flame undergo ionisation and produce light at characteristic wavelength (different for Na^{+} and K^{+})as they reached to the ground state which was recorded by the photodetector in the flame photometer and the intensity of emitted light is directly proportional to the concentration of alkali.

Procedure

Sodium (1000 mEq/l) and potassium (100 mEq/l) stock solutions were prepared prior to the experiment. From the stock solution, working standards such as S1 (120/2 mEq/l), S2 (140/4 mEq/l) and S3 (160/6 mEq/l) were prepared. After that 100 µl of serum to be tested and working standards (S1, S2 and S3) were added to different tubes. About 10 ml of distilled water was also added to all tubes, mixed thoroughly and subjected for flame photometry. The concentration of the sample was calculated from the calibration curve.

ii. Determination of chloride ion

Principle

The estimation of chloride ion was done using electrolyte diagnostic reagent kit and photometer equipped system (Levinson, 1976; Schoenfeld & Lewellen, 1964). The chloride ions react with a solution containing mercuric thiocyanate and ferric nitrate to form ferric thiocyanate (red in colour) and mercuric chloride. The intensity of the red colour formed during this reaction is directly proportional to the quantity of chloride ion present in the serum.

Procedure

10 µl of serum was mixed with the reagent (1 ml) in the diagnostic kit and the absorbance was measured at 505 nm against the blank in a Photometer 4010 system. A standard solution was also prepared using chloride provided with the diagnostic kit. The concentration of chloride ions in the serum was estimated using the following formula:

$$\text{Chloride (mmol/l)} = (\text{Absorbance of sample/ Absorbance of standard}) \times 100$$

3.2.8.2.3. Relative organ weight

The animals were euthanized using CO_2 chamber after each treatment periods. Vital organs such as heart, brain, liver, stomach, intestine, lungs, kidney, spleen, testes and ovary from the euthanized animals, both test and control groups, were dissected and washed in 0.9% saline. After wiping, using a clean filter paper, the organs were weighed using an electronic weighing balance and relative organ weight was calculated using the following formula (Peter et al., 2018).

$$\text{Relative organ weight (\%)} = (\text{organ weight})/(\text{body weight}) \times 100$$

3.2.8.2.4. Histopathological analysis

Post sacrifice, a small portion of the vital organs or tumour was immediately taken from the animals, washed in 0.9% saline and fixed in 10% formaldehyde. After several treatments for dehydration in alcohol and clearing in xylene, the tissues were embedded in paraffin and were sectioned (4 μm thickness) using a microtome and stained with haematoxylin and eosin to observe under the light microscope (Leica DM 500). Photomicrographs were captured using the LAZ software at the magnification of 40× (Johnson & Varghese, 2023b).

3.2.9. ANTITUMOR STUDY USING DLA INDUCED SOLID TUMOUR MODEL IN MICE

Male Swiss albino mice of similar body weight and age were grouped (n = six animals) as follows:

Group I: untreated control

Group II: Vehicle control (Sunflower oil)

Group III: positive control (Cyclophosphamide 10 mg/kg b. wt.)

Group IV: VCEA 25 mg/kg b. wt.

Group V: VCEA 50 mg/kg b. wt.

Group IV: SDEA 50 mg/kg b. wt.

Group IV: SDEA 100 mg/kg b. wt.

DLA cells were aspirated from the peritoneal cavity of the tumour-bearing stock mice and nearly 1×10^6 DLA cells were injected intramuscularly into the left hind limb of the experimental mice to develop solid tumours. Drug treatment was started 24 hours after inoculation using an oral gavage and continued for 10 consecutive days. The radii of developing tumours were measured using vernier callipers from 7th day and continued on every 3rd day until 31st day. Tumour volume was estimated using the formula,

$$V = 4/3\ \pi\ r_1^{\,2}\ r_2$$

Where 'r_1' and 'r_2' represent the major and minor diameter, respectively.

On 31st day the animals were euthanized and tumour tissue was excised for histopathological examination through Haematoxylin and Eosin staining. A part of the tissue was used for the apoptosis analysis through TUNEL staining (Johnson & Varghese, 2023b).

3.2.9.1. TUNEL staining

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) staining was done using Tunel enzyme and Tunel Label mix containing fluorescein dUTP and dNTPs (Roche-Sigma Aldrich). Paraffin embedded tissue sections were dewaxed and rehydrated according to standard protocols, such as by heating at 60 °C, followed by washing in xylene and rehydration through a graded series of ethanol and double-distilled water. The slides of tumour tissues were immersed in 0.1M Tris-HCl containing 3% BSA and 20% normal bovine serum for 30 min and rinsed twice with PBS. 50 µl of Tunel mixture (45 µl Tunel label +5 µl Tunel enzyme) was applied to the tissues and incubated at 37 ^{o}C for one hour in the dark.

Slides were then rinsed using PBS for 5 min and 10 µl of Propidium Iodide (10 mg/ml) was added and washed in PBS for 5 min. Analysis of the samples for the detection of fluorescence were made after placing a drop of PBS under Fluorescent Cell Imager (BIO RAD) and images were captured at 100× magnification.

3.2.10. *IN SILICO* STUDIES USING PHYTOCONSTITUENTS IDENTIFIED FROM THE PLANTS UNDER STUDY.

3.2.10.1. Molecular docking studies

Docking studies were performed using AutoDockVina, an open source program designed and developed by Dr. Oleg Trott (Trott & Olson, 2010). Vina is a docking software run with the aid of AutoDock tools (ADT), PyMol visualisation software and Discovery studio.

3.2.10.2. Preparation of receptor proteins and ligands for docking

The chemical structures of receptors and ligands in specified format is required for conducting docking studies. The three dimensional structure of receptors (proteins) derived from crystallographic techniques were downloaded in .pdb format from RSCB-PDB (Research Collaboratory for Structural Bioinformatics - Protein Data Bank). Similarly the ligand structures (phytoconstituents and drugs) were downloaded in .sdf or .mol formats from PubChem database.

1) Protein name – 1EJ9 (PDB-ID) 2.6 Aº Resolution

2) Protein name – 6WBV (PDB-ID) 2.5 Aº Resolution

The downloaded .pdb files of proteins were then visualized using PyMol visualization software. Water molecules, unwanted bound ligands excluding the co-factors were removed. Polypeptide chains that are functionally relevant for the receptor-ligand interaction were retained and hydrogen atoms were added as part of

preparation of proteins for docking analysis. Open Babel software was used to convert the ligands (downloaded from PubChem) in .sdf file or .mol file format into .pdb. Then both .pdb files (protein and ligand) were converted into .pdbqt file format using AutoDock Tools 1.5.6 (ADT MGL tools) and saved in a working folder.

3.2.10.3. Finding the active site of the protein

The 'active site' coordinates of the receptor were acquired by uploading the protein.pdb file in Biovia's Discovery Studio Visualizer. This software can display and define all possible binding sites of the receptor. Generally the first one displayed is the most visible and considered as the favourable one. The active sites refers to binding site (amino acid residues forming temporary bonds with the substrate) and catalytic site (residues that catalyse a reaction of that substrate). Prior knowledge about binding site of proteins could increase the docking efficiency. Pre-calculated grid maps are required for autodock and it is considered that grid stores the potential energy generated from the receptor interaction and it necessarily surround the region of interest such as active sites (Forli et al. 2016).

3.2.10.4. Vinaconfig file generation

Grid box parameters include coordinates of the centre of box x, y, z and size x, y, z with respect to the receptor protein. The input configuration file must contain receptor.pdbqt, ligand.pdbqt, output.pdbqt

3.2.10.5. Molecular docking

Autodock vina results are attained as 'affinity' or 'the docking score' expressed in kcal/mol. The receptor is kept rigid and the ligand as flexible while docking simulation. 'POSE' is defined as the orientation or geometry of the ligand within the binding site of protein. Algorithm searches helped to visualise such

different poses and to determine the preferred orientation. The strength of binding interactions were finally predicted using scoring functions. The results of docking are obtained as binding energy/affinity for each pose in kcal/mol with RMSD values (root mean square deviation). The first pose having least binding energy with zero rmsd value is selected.

3.2.10.6. Analysis of docking results

The output file obtained after docking was then opened in Biovia's Discovery Studio for detailed analysis. Two dimensional as well as the three dimensional images of receptor ligand interaction can be visualised and saved as separate image files. Redocking with ligands co-crystallized with the same receptors is also necessary for validating the docking calculations.

3.2.11. Statistical analysis

Data are expressed as Mean ± SEM. Significance of results was calculated by analyzing the *p*-value using Student's t-test. The IC_{50} was estimated using SPSS software. Results having $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ were considered statistically significant.

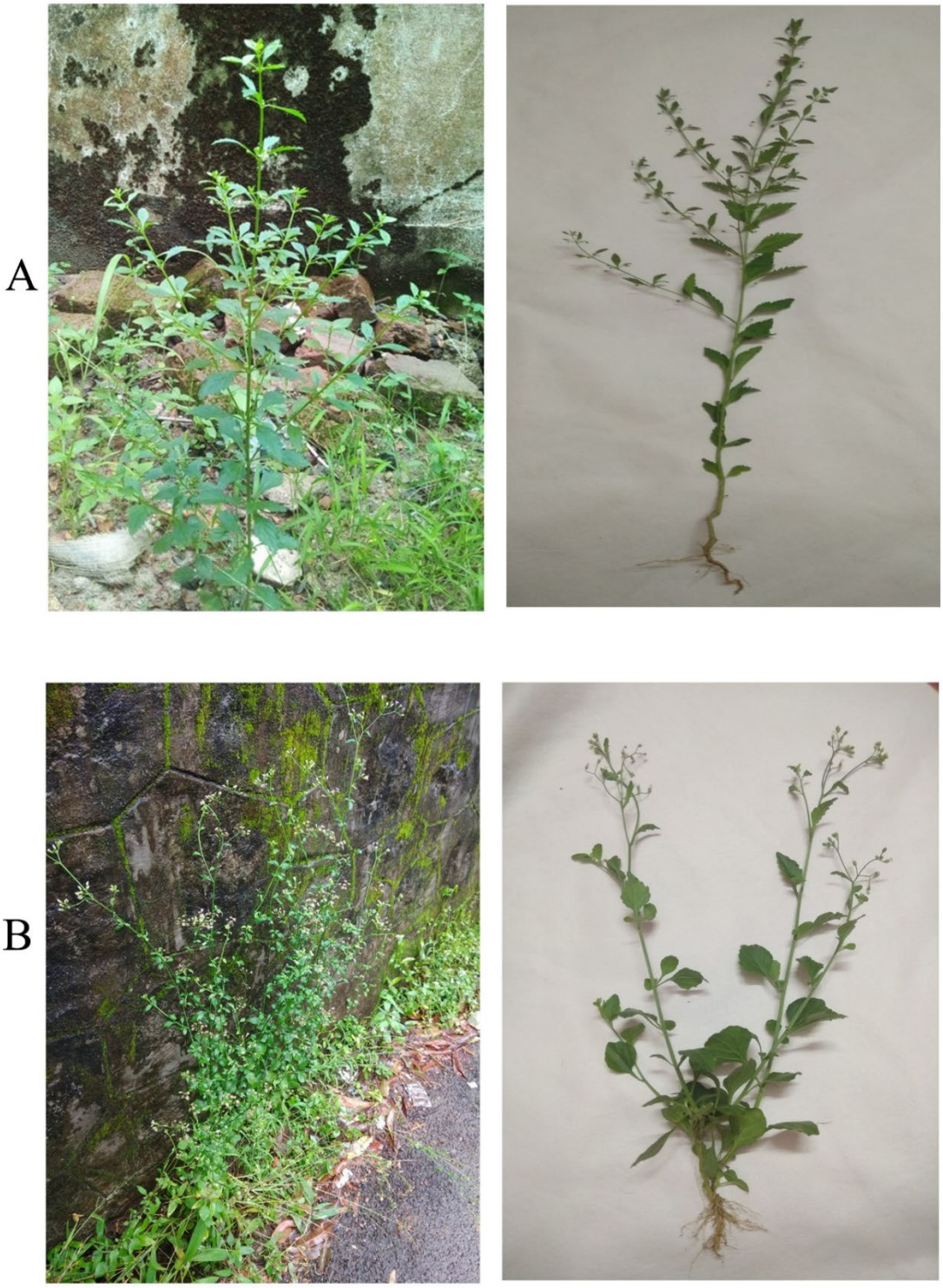


Figure 3.1: Images of Plants under investigation.
A – *Scoparia dulcis*; B – *Vernonia cinerea*

CHAPTER 4

Phytochemical screening of *S. dulcis* & *V. cinerea* extracts

4.1. Introduction

Plants are a sources of diverse bioactive compounds. Plants produce certain non-nutritive secondary metabolites as part of their defence mechanisms against pathogens, herbivores and stressful environmental conditions which are collectively called as phytochemicals (Prakash, 2020). Plants having pharmacologically active secondary metabolites are generally referred to as medicinal plants. Phytochemicals have been used in pharmaceutical studies due to their positive impacts on treating many chronic and severe diseases including cancer (Naczk & Shahidi, 2006). The major groups of these phytochemicals include terpenoids, alkaloids, phytosterols, tannins, saponins and phenolic compounds including flavonoids. Phytochemicals exhibit a number of pharmacological properties like anti-microbial (antibacterial, antiviral, antifungal), anti-oxidant, anti-cancer, wound healing, anti-metabolic diseases (obesity, diabetes, cardiovascular diseases, non-alcoholic fatty liver diseases) activities (Bansal & Priyadarsini, 2022). The essential steps involved in the utilisation of biologically active compounds or the standardised extracts from traditional medicinal plants into modern medicine are extraction, pharmacological screening, isolation and characterization of bioactive constituents, toxicological evaluations and clinical trials (Sasidharan et al., 2011).

Extracting the biologically active ingredients from the medicinal plants is an important step in the preparation of herbal medicine and for experimental purposes. Various solvents are used in the extraction of phytochemicals based on their polarity. Most commonly used solvents are hexane, chloroform, ethyl acetate,

acetone, ethanol, methanol and water. Various methods are used for extracting the phytochemicals. This includes maceration, digestion, decoction, infusion, percolation and soxhlet extraction and certain sophisticated extraction techniques which can extract the phytochemicals more efficiently within a short time span like ultrasound- assisted extraction, microwave assisted extraction and accelerated solvent extraction (Feng et al., 2019). Accelerated solvent extractor uses high pressure in order to efficiently extract all the biomolecules with minimum solvent consumption and reduced time. This elevated pressure also elevates the normal boiling point of solvents and the rate of extraction (Baskar et al., 2019).

Identifying the bioactive constituents which are contributing to the therapeutic potentials of plant extracts are helpful in the search for new drug leads. Chromatographic techniques are often used in the chemical characterization of phytoconstituents in the plant extracts. Since the crude plant extracts contain a large number of bioactive compounds, high or ultra- performance liquid chromatography having high resolving power coupled with mass spectrometry facilitates rapid and accurate identification of chemical constituents from the complex botanical extracts (Ye et al., 2007). Gas chromatography with mass spectrometry is another powerful technique which can be used in validation for qualitative and quantitative investigation of volatile and thermally stable phytocompounds (Bakir et al., 2020; Boğa et al., 2016).

The current chapter deals with the extraction and analysis of phytochemicals present in *S. dulcis* and *V. cinerea.* The whole plant parts were extracted in ethyl acetate, ethanol and water separately and were subjected for both qualitative and quantitative assessment of various classes of phytoconstituents. The volatile constituents in these extracts were analysed by GC-MS method. Further, LC-MS

was also used for the identification of biomolecules abundantly present in the ethyl acetate extract since it was found to be more biologically active in the whole study.

4.2. Methodology

4.2.1. Collection and authentication of plant materials

The detailed methodology regarding the collection and authentication of *S. dulcis* and *V. cinerea* are described in the Methods section 3.2.1.

4.2.2. Extraction

The whole plant parts of *S. dulcis* and *V. cinerea* were extracted in ethyl acetate, ethanol and water using an accelerated solvent extractor (Dionex ASE 150, Thermo Scientific) as per the procedure detailed in Methods section 3.2.2. After extraction, the solvents for the extracts were evaporated using a rotary evaporator to get the dried 'crude' to be used for further studies.

4.2.3. Preliminary Phytochemical Analysis - qualitative

Qualitative phytochemical screening of all the extracts were conducted to identify the presence of secondary metabolites such as alkaloids, polyphenols, flavonoids, tannins, saponins, terpenoids and phytosterols according to standard protocols. Detailed methodology used for qualitative phytochemical analysis is described in Methods section 3.2.3.1.

4.2.4. Preliminary Phytochemical Analysis - quantitative

Estimation of Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Total Terpenoid Content (TTC) was done by following the procedures mentioned in Methods section 3.2.3.2.

4.2.5. Identification of phytocompounds by Gas chromatography - mass spectrometric analysis

Gas chromatography - mass spectrometric analysis (GC-MS) of the extracts were done according to the standard procedures explained in Methods section 3.2.4.i. The identification of compounds was done by comparing the mass spectra of peaks having more area in the chromatogram with the mass spectra of those compounds available in NIST 11 and WILEY 8 libraries.

4.2.6. Identification of phytocompounds by Liquid chromatography - mass spectrometric analysis

Ultra performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-Q-ToF-MS) was used for the chromatographic separation and detection of biomolecules present in the ethyl acetate extract of *S. dulcis* and *V. cinerea*. The detailed procedures are explained in Methods section 3.2.4.ii. MassLynx software (4.1) was used for managing the instrument and also for data acquisition and analysis.

4.3. Results

4.3.1. Extraction and preliminary qualitative phytochemical analysis

Ethyl acetate, ethanol and water were the solvents selected for extraction. Among these solvents, ethyl acetate is the least polar and water being the most polar. The extraction yield was found to be highest for water extracts and lowest for ethyl acetate extracts in both the plants studied (Table 4.1). The qualitative phytochemical analysis showed the presence of different classes of plant secondary metabolites in the various extracts as depicted in table 4.2. Phenolic compounds were found to be present in all six extracts. Terpenoids and phytosterols were present in ethyl acetate

and ethanol extracts of both plants. Alkaloids were not present in the ethyl acetate extracts of both plants.

4.3.2. Quantitative estimation of phytochemicals

Total phenol content was found to be more in the SDEA (47.49 ± 1.99 mg GAE/g) and SDET (41.29 ± 1.25 mg GAE/g) extracts of *S. dulcis* (Table 4.3). Similarly, a high flavonoid content was also observed with SDEA (30.75 ± 0.25 mg QCE/g) and SDET (26.17 ± 1.04 mg QCE/g) extracts. Total terpenoid content was found to be highest in SDEA (55.5 ± 0.66%) when compared to other extracts of *S. dulcis.* In the case of *V. cinerea*, the VCET extract showed highest phenolic content with a TPC value of 45.33 ± 2.52 mg GAE/g, followed by VCEA (40.00±2.65 mg GAE/g). Whereas, TFC and TTC were found to be highest (29.1 2± 0.68 mg QCE /g and 67.66 ± 2.51% respectively) in VCEA compared to other extracts of *V. cinerea*.

4.3.3. Gas chromatography - mass spectrometric analysis

The GC–MS chromatogram of *S. dulcis* and *V. cinerea* extracts were analysed by relating to the known compounds library in the NIST 11 and WILEY 8 databases. The volatile phytocompounds detected (showing ≥ 1.5 % abundance) in the extracts of *S. dulcis* by GC-MS analysis are listed in the order of their abundance in Table 4.4, 4.5 and 4.6. The GC–MS chromatogram of SDEA, SDET and SDWT extracts are also provided (Figure 4.1). Total 17 compounds were identified in the SDEA, of which Phytol seems to be most abundant. Other compounds include Squalene (14.21%), Neophytadiene (10.3%), Dicyclohexylmethane (10.07%), Tetratetracontane (7.22%), Pentatriacontane (6.12%), Beta Caryophyllene (5.57%) and D-Germacrene (4.02%). SDET showed the presence of 10 phytocompounds in GC-MS analysis. Phytol (25.10%) was found to be the most abundantly occurring volatile compound in SDET extract also. Other compounds consists of Cycloartenol

acetate (23.44%), Gamma sitosterol (13.53%), Stigmasta-5, 22-dien-3-ol (10.96%), Neophytadiene (8.50%) and Campesterol (6.42%). Only two compounds namely, 1, 2-Benzenedicarboxylic acid (59.19%) and 2, 3-Dihydro-benzofuran (40.81%) were identified from the SDWT extract. The volatile phytocompounds detected (showing ≥ 1.5 % abundance) from the *V. cinerea* extracts are listed in the order of their abundance (Table 4.7, 4.8 and 4.9). The GC–MS chromatogram of VCEA, VCET and VCWT extracts are also provided (Figure 4.2). Among *V. cinerea* extracts, VCEA showed presence of 6 compounds such as 1-(4-Bromobutyl)-2-piperidinone (27.93%), Beta caryophyllene (13.28%), Caryophyllene oxide (12.99%), Spinacene (10.49%), Nerolidol (10.39%) and Retinal (7.29). Six compound were identified from the VCET extract, of which lupeol (30.70%) was found to be most abundant, followed by Beta amyrin (30.11%), Phytol (18.29%), Stigmasterol (12.59%) and Neophytadiene (5.67%). Only three compounds were identified from the VCWT extracts namely, 1, 2-Benzenedicarboxylic acid (72.83%), 1-(1-Cyclohexen-1-yl) Pyrrolidine (23.25%) and Methyl Palmitate (3.91%).

4.3.4. Liquid chromatography - mass spectrometric analysis

LC-MS analysis of SDEA and VCEA has revealed mass spectral data corresponding to the biomolecules present in them, which allowed their tentative identifications. Compounds identified from SDEA and VCEA extracts along with their m/z values and retention time are depicted in Table 4.10 and 4.11. The total ion chromatogram obtained in the LC-MS analysis of these two extracts are also provided (Figure 4.3 and Figure 4.4). Flavonoids namely Scutellarein, Nepetin, Eupalitin, Tectorigenin, Acacetin, Pectolinarigenin and 5-Demethylnobiletin were found to be present in SDEA. A terpene compound called acevaltrate and

flavonoids such as Genistein, Luteolin, Tectorigenin, Eupalitin and Nevadensin were identified from VCEA extracts.

4.4. Discussion

Proper and timely collection of the plant materials, authentication by experts, adequate drying without compromising the biological properties are the initial steps of plant processing in drug discovery. This is followed by extraction of secondary metabolites using suitable solvents and effective extraction procedures. The solvents for extraction are decided according to the polarity of the solvents from least polar to highly polar water (Abubakar & Haque, 2020). Ethyl acetate, ethanol and water with polarities 0.228, 0.654 and 1.000 respectively were the solvents chosen in this study to extract the phytochemicals from *S. dulcis* and *V. cinerea*. In both plants, maximum yield was obtained in the water extracts, followed by ethanol extracts. Least yield was obtained with ethyl acetate in both plants. The preliminary phytochemical analysis showed the occurrence of phenolic compounds in all the six extracts. Alkaloids seem to be absent in the ethyl acetate (least polar) extracts whereas the terpenoids (generally hydrophobic) were found in both ethyl acetate and ethanol extracts of both plants. In the case of *S. dulcis*, phenolics, flavonoids and terpenoids are quantitatively higher in the ethyl acetate extract among the three extracts tested. In the case of *V. cinerea*, flavonoid content and terpenoid contents were found to be higher in ethyl acetate extract.

GC-MS analysis of *S. dulcis*, indicated the presence of phytol as the most abundant phytoconstituent in both SDEA and SDET extracts. Phytol is a diterpene alcohol having acyclic structures and a constituent of chlorophyll. Its biological activities include antianxiety, cytotoxic, metabolism modulating, antioxidant, antimicrobial, anti-nociceptive, anti-inflammatory, autophagy inducing, proapoptotic

and immune modulating effects (Islam et al., 2018). Another compound having common occurrence in SDEA and SDET extracts was Neophytadiene. It is a terpenoid compound with antibacterial activity and used for treatment of skin diseases, rheumatism and headache (Lalitharani et al., 2010). Two pharmacologically important sesquiterpene compounds identified exclusively from SDEA extract were D germacrene and betabisabolene. Germacrene D possessed antimicrobial activity and insecticidal potentials against mosquitos and aphids (Bruce et al., 2005; Kiran & Devi, 2007). Beta bisabolene is an approved food additive, having balsamic odour, in European countries. It has efficient antitumor and anti-convulsive properties (Orellana-Paucar et al., 2012; Yeo et al., 2016).

LC-MS analysis of *S. dulcis*, revealed many pharmacologically active flavonoid compounds such as Scutellarein, Luteolin, Nepetin, Eupalitin, Tectorigenin, Acacetin, Pectolinarigenin and 5-Demethylnobiletin. Scutellarein is a flavonoid compound reported from *S. dulcis* in earlier studies and possessed good α-glucosidase inhibitory and peroxisome proliferator-activated receptor gamma (PPAR-γ agonistic activities (Liu et al., 2014). Luteolin is a tetrahydroxyflavone with yellow crystalline appearance. Like other flavonoids, Luteolin possesses good antioxidant activity due to their structural features which enhance the donation of hydrogen/electron in order to stabilise free radicals and to chelate transition metals which induce oxidative cell damage. It also showed remarkable anti-inflammatory and anti-allergic responses in many studies. Seelinger et al. (2008) has summarised the anti-inflammatory mechanisms of luteolin in a review article which include suppression of inducible nitric oxygen synthase (iNOS) expression and NO generation, ROS scavenging, blocking of ROS production, stimulation of anti-oxidant enzymes, prevention of leukotriene production and release, inhibition of

pro-inflammatory cytokine expression, blocking of the NFκB pathway, Akt and the mitogen-activated protein kinase (MAPK) pathway, checking of adhesion molecule membrane binding, repression of hyaluronidase and elastase activity, mast cell stabilisation, modulation of cell membrane fluidity and lowering of vascular permeability. Luteolin could penetrate deeper into cell membranes and hence be useful for the treatment of inflammatory and allergic skin diseases as well as for skin protection from solar radiation (Merfort et al., 1994). Moreover, Luteolin is a very efficient anticancer agent against lung, breast, glioblastoma, prostate, colon, and pancreatic cancers (Imran et al., 2019). Nepetin (6-Methoxy derivative of Pentahydroxyflavone 6-Hydroxyluteolin) is a flavone which was not previously reported from this plant yet. Nepetin is also known as Eupafolin and 6-methoxyluteolin. Nepetin showed anti-allergic properties by inhibiting the degranulation of mast cells through PLCγ and Akt signalling pathways and mast cell mediated passive cutaneous anaphylaxis (Ji et al., 2020). In another study demonstrating its anti-inflammatory activity, Nepetin terminated the protein secretion and mRNA expression of IL-6, IL-8 and MCP-1 which was mediated by the inhibitory effect of IL-1β and inactivation of NF-κB and MAPKs in ARPE-19 retinal epithelial cells (Chen et al., 2018). This compound also showed anti proliferative properties *in vitro* (Liu et al., 2015; Militão et al., 2004). Eupalitin, an O-methylated flavonol, is a first time report from S. *dulcis*. Experiments conducted by Kaleem et al. (2016) to determine the anticancer properties of Eupalitin revealed the remarkable potential of this compound in inhibiting the proliferation of PC3 (prostate cancer cell line) and its underlying mechanisms. It was explained from their results that Eupalitin exerted the anticancer mechanism in PC3 cells by inducing apoptosis as the consequences of increase in ROS production, nuclear

condensation, cell cycle arrest and caspase-3 activation. Eupalitin isolated from *Asparagus falcatus* showed anticancer activity in human colorectal cancer cells via caspase 3/7 activation (Ghalib et al., 2013). It also has immunomodulating activities (Pandey et al., 2005). Tectorigenin, an O-methylated isoflavone, is another pharmacologically active compound reporting for the first time, from *S. dulcis*. Pretreatment of tectorigenin prevented the neuroinflammation through downregulation of NF-kB and ERK/JNK pathways in BV-2 microglial cells (Lim et al., 2018). Tectorigenin showed anti-skin damaging effects against UV-B stimulated oxidative stress in human keratinicytes (Noh et al., 2018). This compound also exhibited antitumor activities in a variety of cell lines such as HL-60 (leukemic cell line) and MDA-MB-231, MCF-7 (breast cancer cell lines) (Lee et al., 2001; Zeng et al., 2018b). Acacetin (5, 7-dihydroxy-4-methoxyflavone) is a bioactive flavone having neuroprotective, cardioprotective, anti-inflammatory, antidiabetic, antimicrobial activities and anticancer potentials. This compound could strongly inhibit the activity of several clinically relevant enzymes like topoisomerase 1, glutathione reductase (Boege et al., 1996; Zhang et al., 1997). Acacetin was previously reported from *S. dulcis* by Hayashi et al. (1991). Pectolinarigenin a dimethoxyflavone, was also present in the SDEA extract. It is the aglycone form of Pectolinarin and possesses many biological properties including anti-inflammatory and antidiabetic activities. It showed hepatoprotective properties through the superoxide dismutase (SOD) antioxidant mechanism (Yoo et al., 2008). Anti-inflammatory properties of this compound was revealed in studies using mice models and the inhibitory property on eicosanoid formation in inflammatory lesions was explained as the mechanism behind (Lim et al., 2008). A significant anti-hyperglycemic effect of the mixture containing Pectolinarigenin and Pectolinarin

was observed in an *in vivo* study using diabetic rats (Liao et al., 2010). This compound showed remarkable cytotoxic and antitumor activities in many *in vitro* and *in vivo* studies (Lu et al., 2014; Lu et al., 2016; Wang et al., 2016b). 5-Demethylnobiletin is another phytocompound newly identified from this plant in the present study. It is also a pharmacologically potent compound with chemopreventive and chemotherapeutic properties (Song et al., 2020). In a recent study, it was reported that this compound could attenuate benzopyrene induced colon carcinogenesis through the modulation of TNFα levels and xenobiotic metabolising enzymes (inhibition on CYP_1B_1 and upregulation of UDP-glucuronosyltransferase and glutathione S-transferase) (Chou et al., 2021). A review conducted recently by Ding et al. (2022) summarised the role of 5-Demethylnobiletin for treating diseases like cancer, inflammation related diseases, rheumatoid arthritis and neurodegenerative disorders due to its capability to exert apoptosis, autophagy and anti-inflammatory effects through the regulation of multiple pathways such as JAK2/STAT3, caspase-dependent apoptosis, ROS–AKT/mTOR, MAPK and PKA–CREB signalling.

GC-MS results of VCEA revealed the presence of six volatile compounds namely 1-(4-Bromobutyl)-2-piperidinone, Beta caryophyllene, Caryophyllene oxide, Spinacene, Nerolidol and Retinal as the major constituents. Among them, five compounds namely Dioctyl terephthalate, β-caryophyllene, β-caryophyllene oxide, spinacene and Nerolidol were already reported to have roles in combating cancer cells (Amiel et al., 2012; Dahham et al., 2015; Dong et al., 2021; Gu et al., 2021; Habib & Karim, 2012; Jun et al., 2011; Lee et al., 2000; Nakagawa et al., 1985; Park et al., 2011; Tatman & Mo, 2002). Beta caryophyllene (BCP) is a bicyclic sesquiterpene and belongs to the class of phytocannabinoids with anti-inflammatory,

antibiotic, cytotoxic, antioxidant and local anaesthetic properties (Cho et al., 2007; Galeotti et al., 2001; Pichette et al., 2006; Singh et al., 2006; Tambe et al., 1996). β-caryophyllene oxide (BCPO) is the oxidation derivative of (BCP). Like BCP, BCPO also possesses antinociceptive, anti-inflammatory, and cytotoxic activities (Moghrovyan et al., 2022). There are two types of cannabinoid receptors such as CB_1 and CB_2. BCP exclusively binds with the CB_2 (found on immune cells) and not with CB_1 (found in cells of the central nervous system). So its binding was devoid of the psychoactive side effects associated with the binding of CB_1. BCPO has no affinity with either CB_1 or CB_2 (Fidyt et al., 2016). Nerolidol is a sesquiterpene alcohol with wide range of biological activities including antioxidant, anti-microbial, anti-biofilm, anti-parasitic, insecticidal, anti-ulcer, skin penetration enhancer, anti-tumor, anti-nociceptive and anti-inflammatory properties (Chan et al., 2016).

Ethyl acetate extract of *V. cinerea* possessed many biologically active flavonoids as revealed by the LC-MS analysis such as Genistein, Luteolin, Tectorigenin, Eupalitin, Nevadensin and a terpene compound called acevaltrate. Luteolin, Eupalitin and Tectorigenin were also present in *S. dulcis* ethyl acetate extract. Luteolin was identified from *V. cinerea* in earlier studies (Lai & Wu, 2013) as well. Genistein is an isoflavone and has a chemical structure similar to mammalian oestrogen. Several pharmacological potentials of this compound including protection against cardiovascular diseases, antioxidant, antimicrobial, antidiabetic, anti-inflammatory and anticancer properties were reported in earlier studies (Si and Liu, 2007; Chen et al., 2020b; Sharifi-Rad et al., 2021). In a clinical study conducted by Amanat et al. (2018) using nonalcoholic fatty liver patients, the intake of genistein reduced oxidative and inflammatory indices and insulin resistance. Nevadensin is a biologically active trimethoxyflavone. Its hypotensive,

anti-tubercular, antimicrobial, anti-inflammatory, anti-tumour and anti-cancer activities were reported in many studies (Brahmachari, 2010). It has remarkable expectorant and antitussive potentials by inhibiting cyclooxygenases (Brahmachari et al., 2008). Acevaltrate is a non-glycosylated iridoid compound (a class of cyclopentane pyran monoterpenes). This compound showed significant inhibition against Na^+/K^+-ATPase activity in the rat kidney and brain cells (Bettero et al., 2011). It also possessed the apoptosis inducing potential in myeloma cells by the inhibition of the Otub1/c-Maf axis (Sun et al., 2021b).

The presence of diverse biologically active compounds in these plants obviously points to their promising role in the drug discovery to deal with the prevention and treatment of various chronic diseases which ultimately benefits the wellness of human beings. The combination of compounds in the crude extracts can work together to generate outcomes greater than the effects produced by the individual compounds due to their interactions with each other's performance and the modulation of multiple targets to achieve desirable therapeutic effects.

TABLE 4.1 : YIELD OF EXTRACTION IN VARIOUS SOLVENTS

Name of the plants	Solvent used	Coding	% yield of extraction
Scoparia dulcis	Ethyl acetate	SDEA	6.42 ± 0.38
	Ethanol	SDET	12.27 ± 1.12
	Water	SDWT	17.57 ± 0.60
Vernonia cinerea	Ethyl acetate	VCEA	3.28 ± 0.25
	Ethanol	VCET	10.47 ± 0.21
	Water	VCWT	21.64 ± 0.56

TABLE 4.2: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF PLANT EXTRACTS

Type of phytochemicals	SDEA	SDET	SDWT	VCEA	VCET	VCWT
Alkaloids	-	+	+	-	+	+
Polyphenols	+	+	+	+	+	+
Flavonoids	+	+	-	+	+	+
Tannins	-	-	+	-	-	+
Saponins	-	-	-	-	+	+
Terpenoids	+	+	-	+	+	-
Steroids	+	+	-	+	+	-

+ present, - absent

TABLE 4.3: QUANTITATIVE ESTIMATION OF PHENOLIC, FLAVONOIDS & TERPENOID COMPOUNDS IN THE EXTRACTS

Extracts	Total phenol content (mg GAE/g)	Total flavonoid content (mgQCE/g)	Total terpenoid content (%)
SDEA	47.49 ± 1.99	30.75 ± 0.25	55.5 ± 0.66
SDET	41.29 ± 1.25	26.17 ± 1.04	32.25 ± 1.09
SDWT	8.92 ± 0.52	ND	ND
VCEA	40.00 ± 2.65	29.12 ± 0.68	67.66 ± 2.51
VCET	45.33 ± 2.52	17.25 ± 1.09	15.33 ± 1.52
VCWT	12.33 ± 2.52	4.08 ± 0.38	ND

Data are expressed as mean ± standard deviation ; ND-Not detected

TABLE 4.4: PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF SDEA				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	31.933	21.02	$C_{20}H_{40}O$	Phytol
2	43.175	14.21	$C_{30}H_{50}$	Squalene
3	26.602	10.30	$C_{20}H_{38}$	Neophytadiene
4	23.786	10.07	$C_{13}H_{24}$	Dicyclohexylmethane
5	44.053	7.22	$C_{44}H_{90}$	Tetratetracontane
6	47.233	6.12	$C_{35}H_{72}$	Pentatriacontane
7	17.264	5.57	$C_{15}H_{24}$	Beta Caryophyllene
8	18.794	4.02	$C_{15}H_{24}$	D-Germacrene
9	21.395	2.97	$C_{16}H_{34}$	Hexadecane
10	16.559	2.83	$C_{14}H_{30}$	Tetradecane
11	25.772	2.79	$C_{18}H_{38}$	Octadecane
12	33.268	2.45	$C_{20}H_{30}$	Abietatriene
13	19.486	2.41	$C_{15}H_{24}O$	2,6-Di-T-Butyl-P-Cresol
14	27.476	2.37	$C_{20}H_{40}O$	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
15	18.124	2.32	$C_{15}H_{24}$	Alpha Caryophyllene
16	28.048	1.75	$C_{15}H_{24}$	Beta-bisabolene
17	26.792	1.58	$C_{18}H_{36}O$	Hexahydrofarnesylacetone

TABLE 4.5: PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF SDET				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	22.299	25.10	$C_{20}H_{40}O$	phytol
2	46.544	23.44	$C_{32}H_{52}O_2$	Cycloartenol acetate
3	44.758	13.53	$C_{29}H_{52}O_2$	Gamma sitosterol
4	43.169	10.96	$C_{29}H_{48}O$	Stigmasta-5,22-dien-3-ol
5	16.642	8.50	$C_{20}H_{38}$	Neophytadiene
6	42.369	6.42	$C_{28}H_{48}O$	Campesterol
7	40.149	4.16	$C_{29}H_{50}O_2$	Vitamin E
8	19.453	3.82	$C_{18}H_{36}O_2$	Ethyl palmitate
9	23.345	2.48	$C_{20}H_{36}O_2$	Ethyl linoleate
10	24.005	1.59	$C_{20}H_{40}O_2$	Ethyl stearate

TABLE 4.6: PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF SDWT				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	30.741	59.19	C_8H_6O	1,2-Benzenedicarboxylic acid
2	7.064	40.81	C_8H_8O	2,3-Dihydro-benzofuran

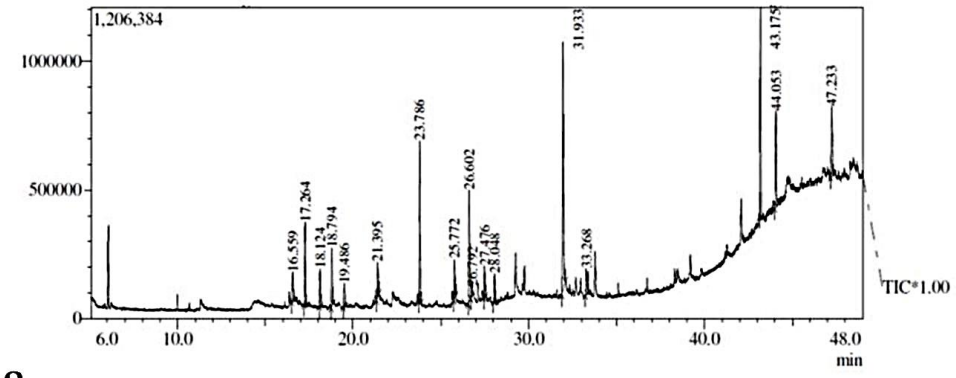
TABLE 4.7 : PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF VCEA				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	23.778	27.93	$C_9H_{16}BrNO$	1-(4-Bromobutyl)-2-piperidinone
2	17.254	13.28	$C_{15}H_{24}$	Beta caryophyllene
3	21.291	12.99	$C_{15}H_{24}O$	Caryophyllene oxide
4	43.174	10.49	$C_{30}H_{50}$	Spinacene
5	32.937	10.39	$C_{15}H_{26}O$	Nerolidol
6	19.157	7.29	$C_{20}H_{28}O$	Retinal (Vit. A)

TABLE 4.8: PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF VCET				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	47.239	30.70	$C_{30}H_{50}O$	Lupeol
2	45.787	30.11	$C_{30}H_{50}O$	Beta amyrin
3	22.300	18.29	$C_{20}H_{40}O$	Phytol
4	43.182	12.59	$C_{29}H_{48}O$	Stigmasterol
5	16.639	5.67	$C_{20}H_{38}$	Neophytadiene
6	19.448	2.65	$C_{18}H_{36}O_2$	Ethyl palmitate

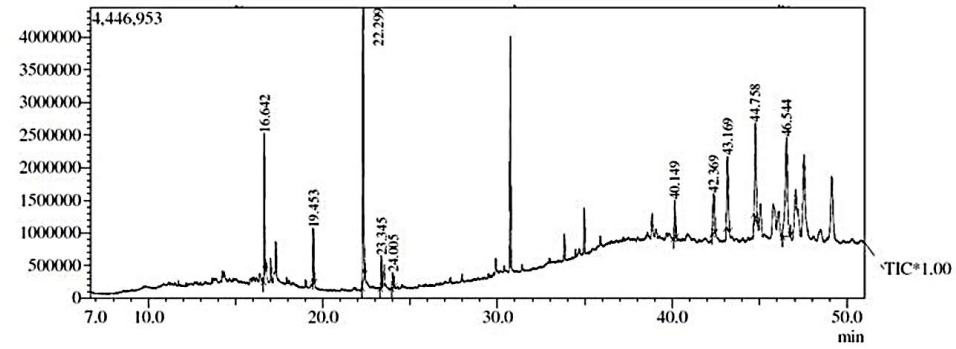
TABLE 4.9: PHYTOCOMPOUNDS IDENTIFIED BY GC-MS ANALYSIS OF VCWT				
SL. No.	Retention Time	Area %	Molecular Formula	Compound Name
1	39.176	72.83	$C_{18}H_{16}O_8$	1,2-Benzenedicarboxylic acid
2	16.943	23.25	$C_{10}H_{17}N$	1-(1-Cyclohexen-1-yl)Pyrrolidine
3	28.330	3.91	$C_{17}H_{34}O_2$	Methyl Palmitate

TABLE 4.10: PHYTOCOMPOUNDS IDENTIFIED BY LC-MS ANALYSIS OF SDEA					
SL. No.	Retention time	Relative abundance (area)	Molecular formula	Compound name	m/z value
1	2.783	14179	$C_8H_7NO_3$	Methyl-6-formyl nicotinate	166
2	3.126	25177	$C_{15}H_{10}O_6$	Scutellarein	287
3	3.371	13343	$C_{15}H_{10}O_6$	Luteolin	287
4	3.362	11667	$C_{16}H_{12}O_7$	Nepetin	317
5	3.579	13468	$C_{17}H_{14}O_7$	Eupalitin	331
6	3.605	85969	$C_{18}H_{16}O_{18}$	2-(2,6-Dihydroxyphenyl)-5-hydroxy-6,7,8-trimethoxy-4H-chromen-4-one	361
7	3.631	7708	$C_{16}H_{12}O_6$	Tectorigenin	301
8	4.293	24464	$C_{16}H_{12}O_5$	Acacetin	285
9	4.353	89606	$C_{17}H_{14}O_6$	Pectolinarigenin	315
10	4.929	39428	$C_{20}H_{20}O_8$	5-Demethylnobiletin	389

TABLE 4.11: PHYTOCOMPOUNDS IDENTIFIED BY LC-MS/MS ANALYSIS OF VCEA					
SL. No.	Retention time	Relative abundance (Area %)	Molecular formula	Compound name	m/z value
1	2.800	7444	$C_8H_7NO_3$	Methyl-6-formyl nicotinate	166
2	4.241	9688	$C_{11}H_{16}O_2$	4-(4-Methoxyphenyl)-2-butanol	181
3	3.031	13549	$C_{11}H_{16}O_3$	Zingerol	197
4	3.648	23210	$C_{15}H_{10}O_5$	Genistein	271
5	3.422	26206	$C_{15}H_{10}O_6$	Luteolin	287
6	3.718	13960	$C_{16}H_{12}O_6$	Tectorigenin	301
7	3.711	6082	$C_{17}H_{14}O_7$	Eupalitin	331
8	4.476	30512	$C_{18}H_{16}O_7$	Nevadensin	345
9	3.979	17101	$C_{23}H_{30}O_{10}$	Diacetyl neosolaniol	467
10	4.187	38287	$C_{24}H_{32}O_{10}$	Acevaltrate	487



a



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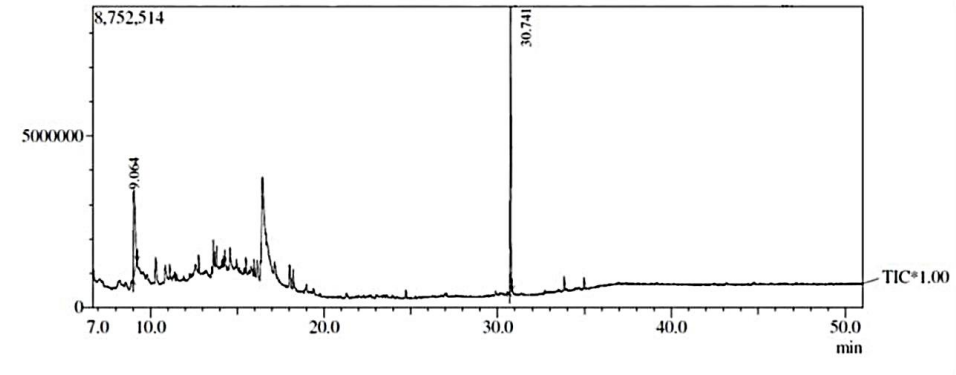

c

Figure 4.1: Gas chromatogram of SDEA (a), SDET (b) and SDWT (c) extracts

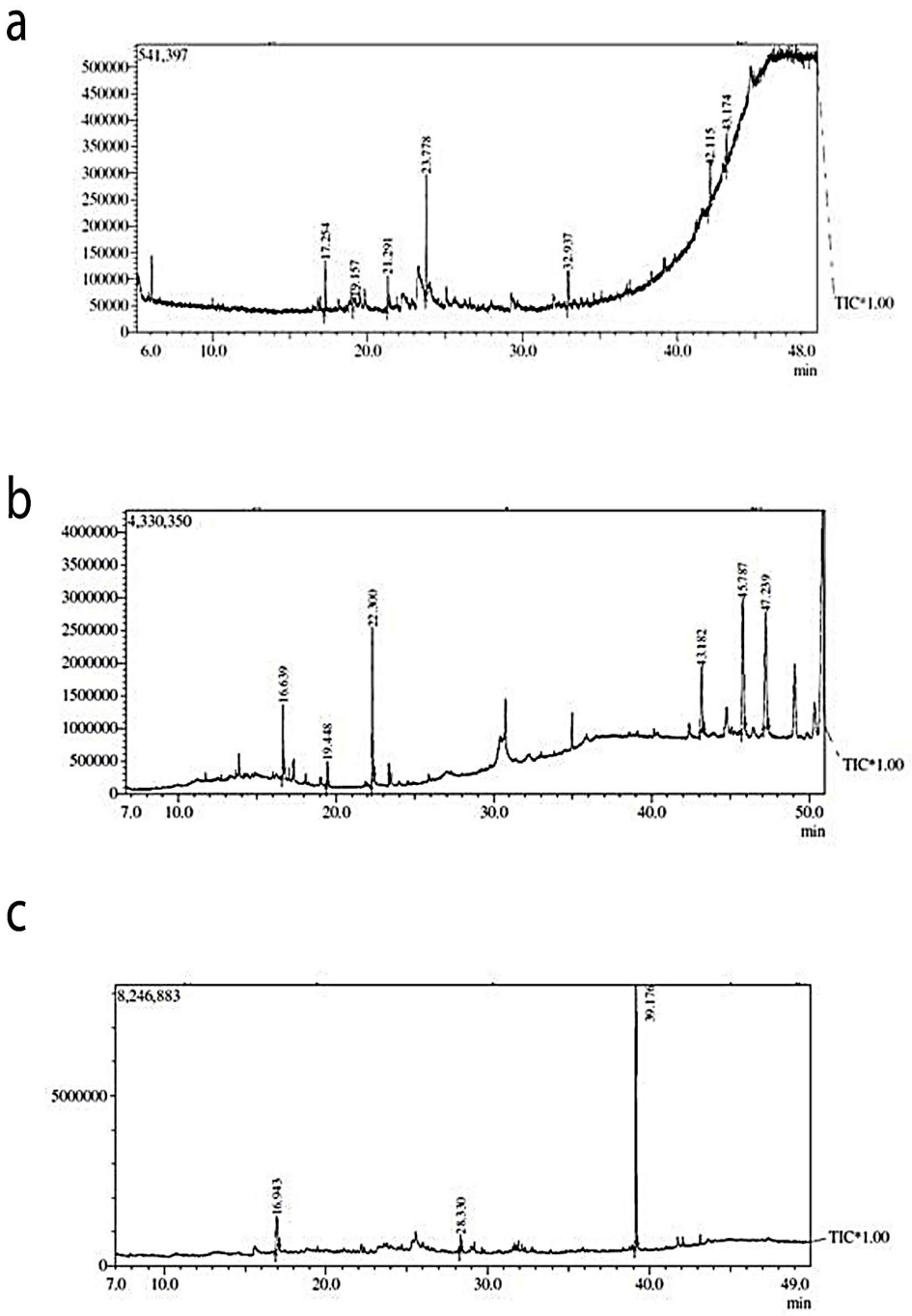

Figure 4.2: Gas chromatogram of VCEA (a), VCET (b) and VCWT (c) extracts

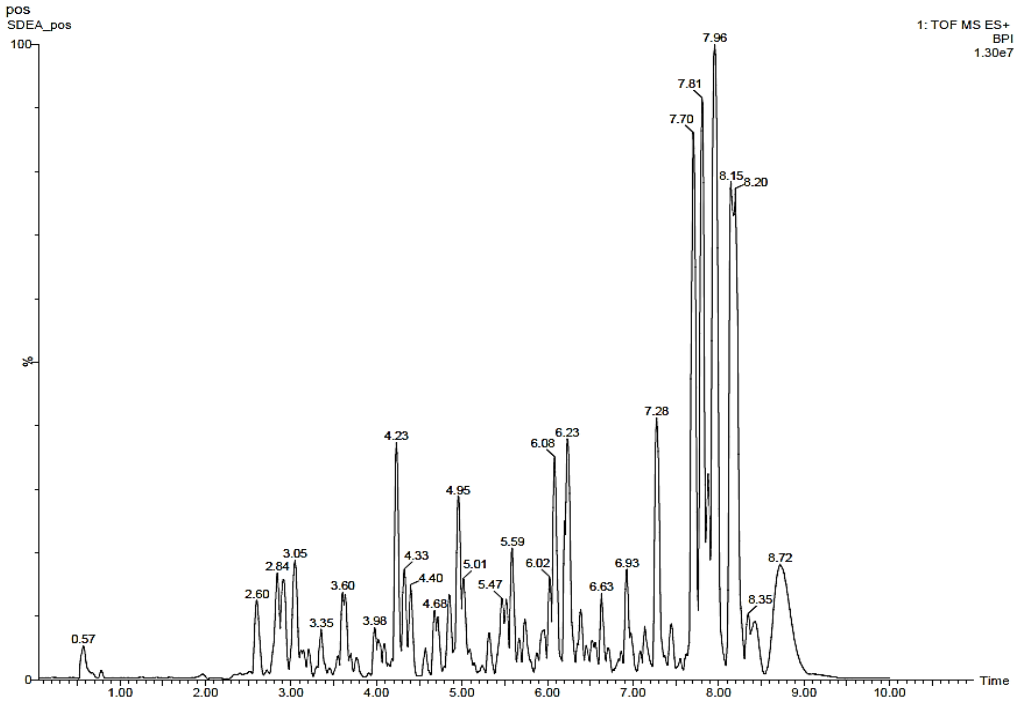

Figure 4.3: Total ion chromatogram obtained in the LC-MS analysis of SDEA

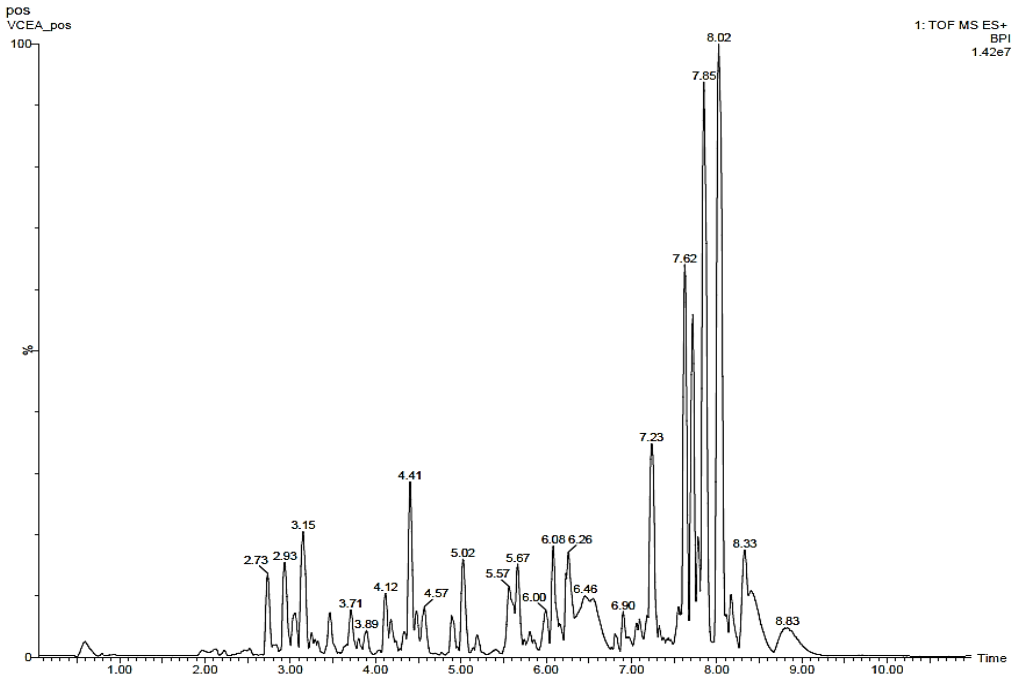

Figure 4.4: Total ion chromatogram obtained in the LC-MS analysis of VCEA

CHAPTER 5

Genotoxic/genoprotective assessment of *S. dulcis* and *V. cinerea*

5.1. Introduction

Cells sustain genomic integrity by different kinds of checkpoints and repair modalities. Any defects in these mechanisms predominantly due to gene mutations, DNA strand breaks and chromosomal aberrations on the regulators of the cell division and tumour suppression results in genetic instability which is often found in association with the pathological conditions such as ageing, carcinogenesis, metabolic syndromes, cardiovascular diseases, immune deficiencies and neurodegenerative diseases (Yao & Dai, 2014; Georgoulis et al., 2017). In cancer patients undergoing chemotherapy using genotoxic agents like cyclophosphamide, the high levels of induced DNA damages leads to the generation of secondary pathological conditions including oxidative stress, inflammatory response and multiple organ toxicity and often results in secondary tumour formation (Swift & Golsteyn, 2014). Exploring the pharmaceuticals having genoprotective or genome-stabilzing properties will help to overcome the adversities associated with the genome instability and such agents can also be referred to as chemopreventive agents.

Chemopreventive drugs generally act as blocking agents which hinders the interaction of genotoxic agents with DNA thereby preventing tumour initiation or as suppressive agents which interfere with the signal transduction pathways, thus suppressing tumour promotion and progression (Steward & Brown, 2013). Plants used in traditional medicines are the promising sources of bioactive substances

which can maintain the genomic stability and protect the genes from genotoxic action of xenobiotics by various mechanisms (Koklesova et al., 2020). Some genotoxic agents induce DNA damage through the generation of free radicals either directly or during metabolic activation in the liver. Phytochemicals with antioxidant potentials or those which can modulate liver detoxification enzymes can effectively protect genes from such agents.

Scoparia dulcis L. is a traditional anti-diabetic plant found in India. The antioxidant action of this plant offers protection from the long term complications by scavenging the free radical generation during diabetes (Latha et al., 2004). *Vernonia cinerea* (L.) Less is an important medicinal plant used in Ayurveda recommended for removing toxins from blood. This plant is used as the blood purifier and general tonic in the Unani system of medicine also (Zakir et al., 2020). The present chapter deals with the assessment of the genoprotective prospects of these plants against potential genotoxic agents.

5.2. Methodology

5.2.1. Collection of plant materials and extraction

The methodology used for the collection and extraction is detailed in section 3.2.1 and 3.2.2 of chapter 3.

5.2.2. Evaluation of mutagenic and antimutagenic potentials of plant extracts using Ames assay

Ames *Salmonella*/microsome assay (bacterial reverse mutation assay) with standard pre incubation procedure was used to test the mutagenic/ antimutagenic potential of plant extracts using *Salmonella typhimurium* TA100 strains which carries the mutations in the histidine operon in the category of base pair substitution.

The procedure used for this assay is explained in the methodology section 3.2.5.1 of chapter 3.

5.2.3. MTT assay using HepG2 cell lines

The procedure for MTT assay used for testing the cell viability is explained in section 3.2.7.3 of chapter 3.

5.2.4. Cytokinesis Block Micronucleus Assay (CBMN assay)

The genotoxic effects of plant extracts was assessed using the CBMN assay. The genoprotective or antigenotoxic potentials of plant extracts was analysed using the same assay. The methodology used for conducting this assay is provided in the section 3.2.5.2 of chapter 3.

5.2.5. *In vitro* Free radical Scavenging Assays

The assays such as DPPH radical assay, ABTS radical assay, hydroxyl radical assay, Lipid peroxides scavenging assay were conducted according to the methods described in method section 3.2.6.

5.2.6. Statistical analysis

The data obtained are expressed as mean ± SEM. The values were statistically tested using Student's t test in Microsoft Excel. p value $<$ 0.05 were considered as significant.

5.3. Results

5.3.1. Evaluation of mutagenic and antimutagenic potentials of plant extracts

The results from mutagenicity screening using Ames assay showed that the ethyl acetate, ethanol and water extracts (50 µg/ml and 100 µg/ml) from both the plants under study did not increase the number of revertant colonies significantly in *Salmonella* TA100 strains when compared with the vehicle control plates (Figure 5.1 & 5.2). Whereas the Sodium azide, a potential direct acting mutagenic compound used as positive control in this study, induced 767.67 ± 76.00 revertant

colonies which was significantly higher than that of vehicle control. This test was followed by the assessment of antimutagenic potential at the same doses of plant extracts in the presence of Sodium Azide. *S. dulcis* ethyl acetate extract (SDEA) showed strong antimutagenic potentials (58.32% and 72.95% inhibition at the doses of 50 µg/ml and 100 µg/ml respectively) than ethanol and water extracts. Ethyl acetate extract of *V. cinerea* (VCEA) inhibited the induction of mutations by 57.71% at 100 µg/ml, whereas the lower dose (50 µg/ml) expressed only a slight antimutagenic effect. Both ethanol extract (VCET) and water extract (VCWT) of this plant were not inhibiting the mutagenesis (Table 5.1 & Figure 5.3)

5.3.2. Cell viability test using $HepG_2$ cell line

Cell viability of ethyl acetate, ethanol and water extracts of the plants under study in $HepG_2$ cells after 24 hours of treatment was determined using MTT assay. The IC_{50} value of cyclophosphamide was found to be 35.89 µg/ml. The IC_{50} value of all the plant extracts tested were greater than 100 µg/ml (Figure 5.4).

5.3.3. Evaluation of genotoxic and antigenotoxic potentials of plant extracts

The number of micronuclei in 1000 binucleated cells were counted to determine the genotoxic potentials of plant extracts. The results showed that Cyclophosphamide, at concentration of 15 µg/ml induced 231.33±4.16 micronuclei in $HepG_2$ cells which was significantly greater than that of vehicle control. The extracts from both the plants did not show any significant increase in the number micronuclei generated (Table 5.2). Figure 5.5 depicts the appearance of mononucleated, binucleated, multinucleated and binucleated cells with micronuclei.

Experiments were conducted to test the antigenotoxic potentials of the plant extracts by analysing the reduction in the number of micronuclei generated when the extracts were co-treated with a known genotoxic agent, cyclophosphamide. Both co-

treatment and post-treatment protocols were employed. All extracts of *S. dulcis* significantly reduced the number of micronuclei formation induced by the Cyclophosphamide in both simultaneous and post-treatment protocols. The reduction in micronuclei generated while treating the cells with SDEA and CP simultaneously is 82.70 ± 0.67% (Table 5.3). SDEA treatment after the incubation of cells with CP resulted in only 38.21 ± 2.46 % inhibition. In the case of SDET and SDWT, the reduction was 59.37 ± 0.15% and 54.88 ± 1.56% respectively in simultaneous treatment. These extracts were also found to be weakly inhibiting the micronuclei formation in the post-treatment method (18.86 ± 0.81% and 10.76 ± 3.66% respectively). Among the extracts of *V. cinerea,* only the ethyl acetate extract expressed a significant reduction in the micronuclei formation (74.63 ± 0.76%) upon simultaneous treatment (Table 5.4). All the three extracts were found to be weak in protecting the cells from micronuclei formation in the post-treatment method.

Cytokinesis block proliferation index (CBPI) and the associated percentage cytostasis of both plant extracts was analysed as part of CBMN assay and the values obtained are shown in table 5.5. The CBPI and cytostasis observed for cyclophosphamide treatment were 1.64 ± 0.03 and 33.03 ± 1.90 % respectively. All the tested plant extracts showed further reduction in CBPI and increase in percentage cytostasis on simultaneous treatment with cyclophosphamide. Among these, the ethyl acetate extracts of both plants expressed a remarkable increase in the cytostasis (53.12 ± 0.58% for SDEA and 53.47 ± 1.74% for VCEA).

5.3.4. Evaluation of free radical scavenging potential of plant extracts

All the solvent extracts showed a concentration dependent increase in scavenging the various types of free radicals**.** Ethyl acetate extract of both plants are least effective in scavenging the free radicals since IC_{50} values of these extracts in

DPPH, ABTS, Hydroxyl and lipid peroxide radical scavenging assay was found to be more than 100 µg/ml. Ethanol extracts of both plants was found to be more effective in scavenging DPPH and hydroxyl radicals. Water extracts of both plants expressed more potential in scavenging ABTS and lipid peroxide radicals (Figure 5.6).

5.4. Discussion

Genomic instability plays a key role in the initiation and progression of tumours. Some biologically active chemical compounds of plant origin are capable of protecting cells from genotoxic events either by prevention or reversal of genetic mutations and chromosomal aberrations. Such compounds can act as good chemopreventive agents. The present study has evaluated the genoprotective potentials of the traditional medicinal plants *S. dulcis* and *V. cinerea* against the action of genotoxic agents in prokaryotic and eukaryotic systems.

Bacterial reverse mutation test detects and revert mutations present in the test strains and restore the functional capability of the bacteria to enable the synthesis of an essential amino acid required for their growth. Such revertant bacteria can be detected by their potential to grow in the absence of the amino acid required by their parent strain (OECD 471, 2020). In this experiment, S. typhimurium TA 100 strains were used to study the effect of extracts on treatment with a potential mutagen called sodium azide. Sodium azide (NaN_3) is a commonly used pesticide and industrial nitrogen gas generator and is a potent mutagen in micro-organisms. Sodium azide under acidic pH is converted into hydrogen azide which can easily penetrate into the cell membrane. Inside the cells, it is converted into an organic metabolite, L- azidoalanine catalysed by the enzyme O-acetylserine sulfhydrylase. This L-azidoalanine is inducing point mutations in DNA (Gruszka et

al., 2012). The present study revealed that any of the crude solvent extracts tested are not capable of eliciting mutations in the TA 100 strain. Interestingly, the observed reduction in the number of revertant colonies while treating the bacterial strains with plant extracts (especially SDEA and VCEA) with sodium azide indicates their strong antimutagenic potentials (Johnson & Varghese, 2024).

Prior to genotoxicity/antigenotoxicity evaluation, the effects of the genotoxic agent (to be used in the experiment) and the plant extracts on viability of $HepG_2$ cells were tested using MTT assay to assure that sufficient cells will reach mitosis during the test. All the extracts tested showed an IC_{50} value greater than 100 µg/ml in the cell viability assay. The *in vitro* CBMN assay was used for testing the plant extracts for imparting genotoxic or genoprotective effects in the eukaryotic system. Appearance of micronuclei in cytokinesis-blocked, binucleated cells was assessed to determine the chromosomal damages. Micronuclei are tiny, extranuclear entities generated from broken chromosomes or whole chromosomes which lag behind during mitotic division and thus not incorporated in the daughter nuclei (Krupina et al., 2021). Cyclophosphamide, a chemotherapeutic pro-drug which requires hepatic cytochrome P-450 reaction to become the active metabolite 4-hydroxycyclophosphamide which penetrates the cell and decomposes to form phosphoramide mustard (anticancer metabolite) and acrolein (toxic metabolite) was used as the positive control in this experiment. Thus, the human hepatoma cell line (HepG2) that retains most of the endogenous metabolic activities of fresh hepatocytes was used in the genotoxicity/antigenotoxicity experiments (Natarajan & Darroudi, 1991). The potential of cyclophosphamide in creating DNA damage was evident in many studies. Samarth et al. (2018) has reported that a significant increase in micronuclei frequency was observed after administration of cyclophosphamide at

5 µg/ml in cultured human lymphocytes. The results of the present study was consistent with their findings in generating micronuclei.

It is noted that the plants used in this study produced negligible amounts of micronuclei when compared with the drug, cyclophosphamide. Additionally, the ethyl acetate extract from both plants were capable of protecting the genes from the toxic effects of this drug. There are various mechanisms of action by which phytochemicals protect genes from mutagens or genotoxic agents which can be broadly classified as desmutagenic actions and bioantimutagenic actions. The desmutagenic actions involve irreversible binding to the genotoxic agent either extracellularly or intracellularly, chemical inactivation by the modulation of liver metabolic enzymes and also the scavenging of free radicals. The bioantimutagenic actions indicate modulation of DNA replication and promoting DNA repair (Słoczyńska et al., 2014). According to the results obtained in the present study, the desmutagenic action can be inferred as the predominant mechanism occurred because of the strong inhibition of micronuclei formation observed when the extract was co-treated with the cyclophosphamide rather than in the post-treatment (Johnson & Varghese, 2024).

It is evident from this study that ethyl acetate extracts of both plants are very weak in scavenging the free radicals compared to other solvent extracts. Hence the possibility of desmutagenic actions of SDEA and VCEA by scavenging the free radicals can be excluded. While analysing the extract constituents, the compounds with reported activities on inducing endogenous antioxidant enzymes are present in SDEA extract. Pectolinarigenin is an example for such compounds since it can trigger antioxidant enzymes through the Nrf2/ARE pathway in $HepG_2$ cells (Shiraiwa et al., 2022). Squalene, which covers the second largest peak in the gas

chromatogram, is another such compound stimulating the genes encoding antioxidant enzymes through the similar pathway (Ibrahim et al., 2021). In a study conducted by Narayan et al. (2010) in Balb/c mice, the administration of squalene at the doses of 1 and 4 mmol/g body weight was found to be effectively reducing the doxorubicin induced genotoxicity formation which was revealed in two genotoxicity assays such as comet assay and micronucleus assay.

Ethyl acetate extract of *V. cinerea* also exhibited significant genoprotective property in the simultaneous treatment pointing towards its desmutagenic action of its phytoconstituents. GC-MS analysis of VCEA showed the presence of some sesquiterpenes like beta caryophyllene, beta caryophyllene oxide and nerolidol. An earlier study evaluating the antimutagenicity of beta caryophyllene using Ames assay showed a strong antimutagenic property (83.9% inhibition) against 2-nitrofluorene (6.40 mg/plate) in *S. typhimurium* TA 100 strain and the chemical inactivation of mutagen by this compound was inferred as the main antimutagenic mechanism (Di Sotto et al., 2008). Beta caryophyllene was also found to be present in SDEA extract. Further, beta caryophyllene oxide and nerolidol could exert the antimutagenic property by modulating liver xenobiotic metabolising enzymes on exposure to genotoxic agents (Lněničková et al., 2018). Genistein could trigger the antioxidant enzymes through AMP-activated protein kinase activation (Park et al., 2010).

Overall, it can be inferred from this study that the phytoconstituents especially the flavonoids and terpenes present in the ethyl acetate extract of both plants act together in blocking the interaction of genotoxic agent with the DNA by chemical inactivation, modulation of xenobiotic metabolising enzymes and by exerting antioxidant effect since more genoprotective effect was observed in

simultaneous treatment protocol when compared with the results obtained from post-treatment protocol. Beta caryophyllene, Luteolin, Eupalitin and Tectorigenin are the compounds present in both SDEA and VCEA. Among the extracts tested, ethyl acetate extract of both plants were shown to have less number of micronuclei generated in the post-treatment experiments. This indicates that some of the compounds in these extracts can reverse the genotoxic event induced by the activated metabolites of cyclophosphamide. This can be achieved mainly by inducing DNA repair processes. Luteolin, a compound commonly present in both SDEA and VCEA. According to a study conducted by Tjioe et al. (2016) luteolin treatment (10 mM) to oral squamous carcinoma cells (OSCC) induces DNA double-strand break repair pathway and cell cycle arrest through ATM phosphorylation and activation of its downstream targets, H2AX and CHK2. Similarly, the genoprotective effect of luteolin by increasing DNA repair activity in Caco-2 cells in addition to their protective effects from oxidative damage was reported by Ramos et al. (2010).

Cytokinesis block proliferation index (CBPI) is a widely used, accurate and relevant index for measuring the effects of cell cycle delay or cytotoxicity (Lorge et al., 2008). The observed reductions in the CBPI and increase in cytostasis of ethyl acetate extracts of both plants when co-treated with CP reflects its ability to interfere with the proliferation of cells with damaged genes which in turn gives sufficient time for apoptotic or repair machinery to act further. Phytochemicals capable of preventing DNA damage together with the targeted cytotoxic effects on tumour cells can play vital roles in the search for new chemotherapeutic drugs or adjuvant to increase the potentials of drugs already in use for cancer treatment (Johnson & Varghese, 2024)

Table 5.1: Antimutagenic effect *Scoparia dulcis* & *Vernonia cinerea* against the mutagenicity induced by Sodium azide to *S. typhimurium* TA 100.

Treatment	No. of revertant	Percentage inhibition (%)
Negative control	166.33 ± 0.88	
Vehicle control (DMSO)	164.00 ± 2.08	
PC (NaN_3 5 µg/plate)	767.67 ± 43.88	
PC + SDEA (50 µg/plate)	320.00 ± 2.31	58.06 ± 2.21^{S}
PC + SDEA (100 µg/plate)	207.67 ± 1.45	72.78 ± 1.47^{S}
PC + SDET (50 µg/plate)	393.67 ± 1.83	48.40 ± 2.83^{S}
PC + SDET (100 µg/plate)	358.33 ± 1.76	53.00 ± 2.81^{S}
PC + SDWT (50 µg/plate)	559.00 ± 2.08	26.71 ± 4.09^{M}
PC + SDWT (100 µg/plate)	513.33 ± 7.26	32.78 ± 3.05^{M}
PC + VCEA (50 µg/plate)	606.67 ± 8.82	20.57 ± 3.55^{W}
PC + VCEA (100 µg/plate)	324.67 ± 5.70	57.33 ± 3.32^{S}
PC + VCET (50 µg/plate)	520.33 ± 1.20	31.77 ± 3.92^{M}
PC + VCET (100 µg/plate)	462.67 ± 6.36	39.42 ± 2.76^{M}
PC + VCWT (50 µg/plate)	663.33 ± 3.06	12.99 ± 5.29^{W}
PC + VCWT (100 µg/plate)	618.67 ± 21.22	18.93 ± 4.84^{W}

Data are presented as the mean ± SEM. NaN_3: Sodium azide. PC: Positive control. NaN_3 (5 µg/ml) is the positive control used for TA100 strain. The symbol (S) represents strong antimutagenic effects and (M) represents moderate antimutagenic effects.

Table 5.2: Genotoxicity determination of plant extracts from *Scoparia dulcis* and *Vernonia cinerea* on the induction of micronuclei in $HepG_2$ cells.	
Treatment	No. of Micronuclei/1000 binucleated cells
Vehicle control (DMSO)	0.33 ± 0.58
Cyclophosphamide (15 µg/ml)	231.33 ± 4.16***
SDEA (50 µg/ml)	5.67 ± 0.33
SDEA (100 µg/ml)	4.00 ± 0.58
SDET (50 µg/ml)	2.67 ± 0.33
SDET (100 µg/ml)	2.00 ± 0.58
SDWT (50 µg/ml)	2.67 ± 0.88
SDWT (100 µg/ml)	2.67 ± 0.33
VCEA (50 µg/ml)	7.33 ± 0.33
VCEA (100 µg/ml)	9.00 ± 0.58
VCET (50 µg/ml)	3.00 ± 1.00
VCET (100 µg/ml)	3.33 ± 0.33
VCWT (50 µg/ml)	1.00 ± 0.58
VCWT (100 µg/ml)	1.33 ± 0.33
Data are represented as mean ± SEM. Comparisons were made between vehicle controls with the treated groups separately. (***) represents statistical significance at $p \leq 0.001$.	

Table 5.3: Antigenotoxic effect of plant extracts from *Scoparia dulcis* L. on the number of micronuclei, cell proliferation and cytostasis when co-administered with a genotoxic drug Cyclophosphamide on $HepG_2$ cells.

Treatment	No. of micronucleated cells/1000 binucleated cells	Percentage reduction in micronuclei (%)
Vehicle control (DMSO)	0.33 ± 0.33	
Positive control (CP 15 µg/ml)	231.33 ± 2.40	
CP + SDEA (SIM)	40.00 ± 1.15***	82.70 ± 0.67
CP + SDEA (POST)	143.00 ± 6.51***	38.21 ± 2.46
CP + SDET (SIM)	94.00 ± 1.15***	59.37 ± 0.15
CP + SDET (POST)	187.67 ± 0.33***	18.86 ± 0.81
CP + SDWT (SIM)	104.33 ± 2.96***	54.88 ± 1.56
CP + SDWT (POST)	206.33 ± 7.31*	10.76 ± 3.66

Data are represented as mean ± SEM. Cyclophosphamide (CP) at 15 µg/ml was used in treatment with plant extracts. (SIM)-Simultaneous treatment; (POST)-post treatment. Plant extract concentration - 100 µg/ml. Comparisons were made between positive controls with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$ and (***) represents statistical significance at $p \leq 0.001$.

Table 5.4: Antigenotoxic effect of plant extracts from *Vernonia cinerea* (L.) Less on the number of micronuclei, cell proliferation and cytostasis when co-administered with a genotoxic drug Cyclophosphamide on $HepG_2$ cells.

Treatments	No. of micronucleated cells/1000 binucleated	Percentage reduction in micronuclei (%)
Vehicle control (DMSO)	0.33 ± 0.58	
Positive control (CP 15µg/ml)	231.33 ± 2.40	
CP + VCEA (SIM)	58.67 ± 1.20***	74.63 ± 0.76
CP + VCEA (POST)	167.67 ± 14.19*	27.55 ± 6.00
CP + VCET (SIM)	192.67 ± 11.10*	16.69 ± 5.00
CP + VCET (POST)	206.00 ± 0.58ns	8.19 ± 4.09
CP + VCWT (SIM)	179.67 ± 13.17*	22.31 ± 5.87
CP + VCWT (POST)	188.67 ± 13.04*	18.34 ± 6.26

Data are represented as mean ± SEM. Cyclophosphamide (CP) at 15 µg/ml was used in treatment with plant extracts. (SIM)-Simultaneous treatment; (POST)-post treatment. Plant extract concentration - 100 µg/ml. Comparisons were made between positive controls with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$ and (***) represents statistical significance at $p \leq 0.001$.

Table 5.5 : Evaluation of cytokinesis block proliferation index (CBPI) and cytostasis of *S.dulcis* and *V. cinerea* extracts upon co-treatment with		
Treatments	CBPI	Percentage cytostasis (%)
Vehicle control (DMSO)	1.96 ± 0.01	
Positive control (CP)15µg/ml)	1.64 ± 0.03	33.03 ± 1.90
CP + SDEA (100 µg/ml)	1.45 ± 0.01	53.12 ± 0.58
CP + SDET (100 µg/ml)	1.52 ± 0.01	46.14 ± 1.56
CP + SDWT (100 µg/ml)	1.60 ± 0.01	37.12 ± 1.54
CP + VCEA (100 µg/ml)	1.45 ± 0.02	53.47 ± 1.74
CP + VCET (100 µg/ml)	1.56 ± 0.01	41.63 ± 1.65
CP+ VCWT (100 µg/ml)	1.49 ± 0.01	48.93 ± 1.22
Data are represented as mean ± SEM. Cyclophosphamide (CP) at 15 µg/ml was used in treatment with plant extracts.		

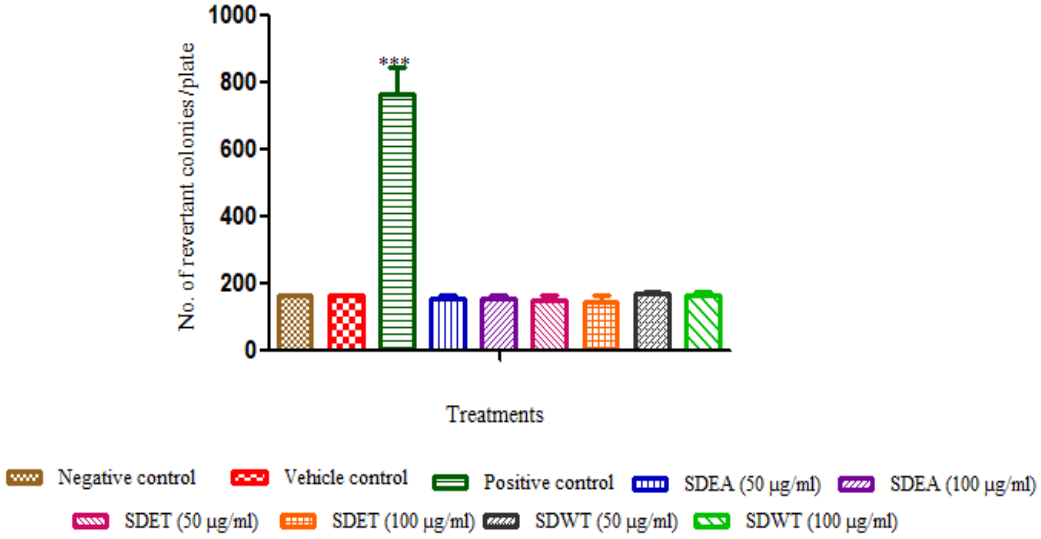

Figure 5.1: Mutagenicity assessment of *Scoparia dulcis* by Ames test using *S. typhimurium* TA 100. Figure represents the number of revertant colonies per each treatment in triplicate plates (mean ± SEM). Comparisons were made between vehicle control with the treated groups separately. The symbol (***) represents statistical significance at p value $p \leq 0.001$

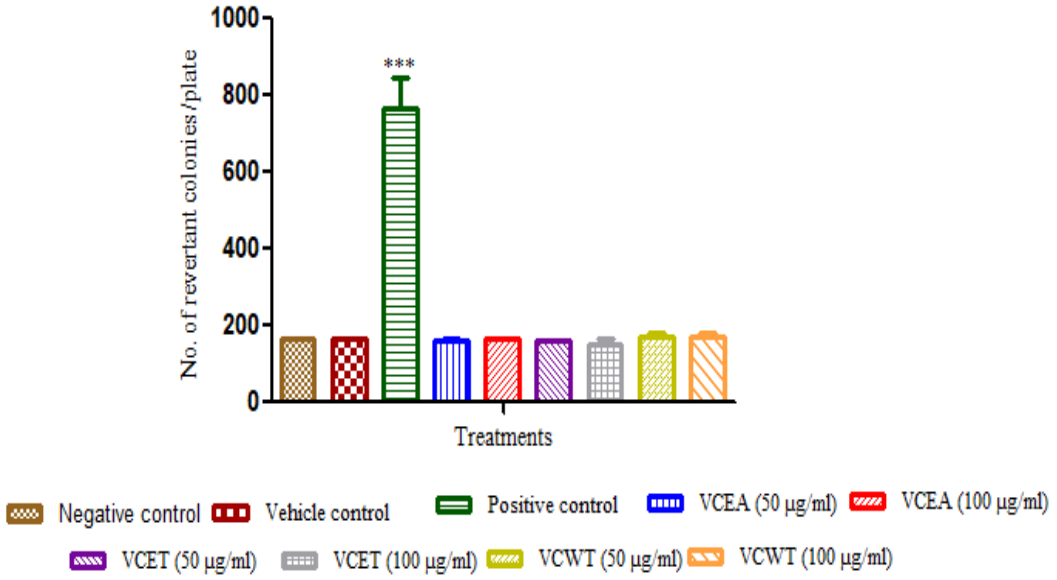

Figure 5.2: Mutagenicity assessment of *Vernonia cinerea* (L.) Less by Ames test using *S. typhimurium* TA 100. Figure represents the number of revertant colonies per each treatment in triplicate plates (mean ± SEM). Comparisons were made between vehicle control with the treated groups separately. The symbol (***) represents statistical significance at p value $p \leq 0.001$

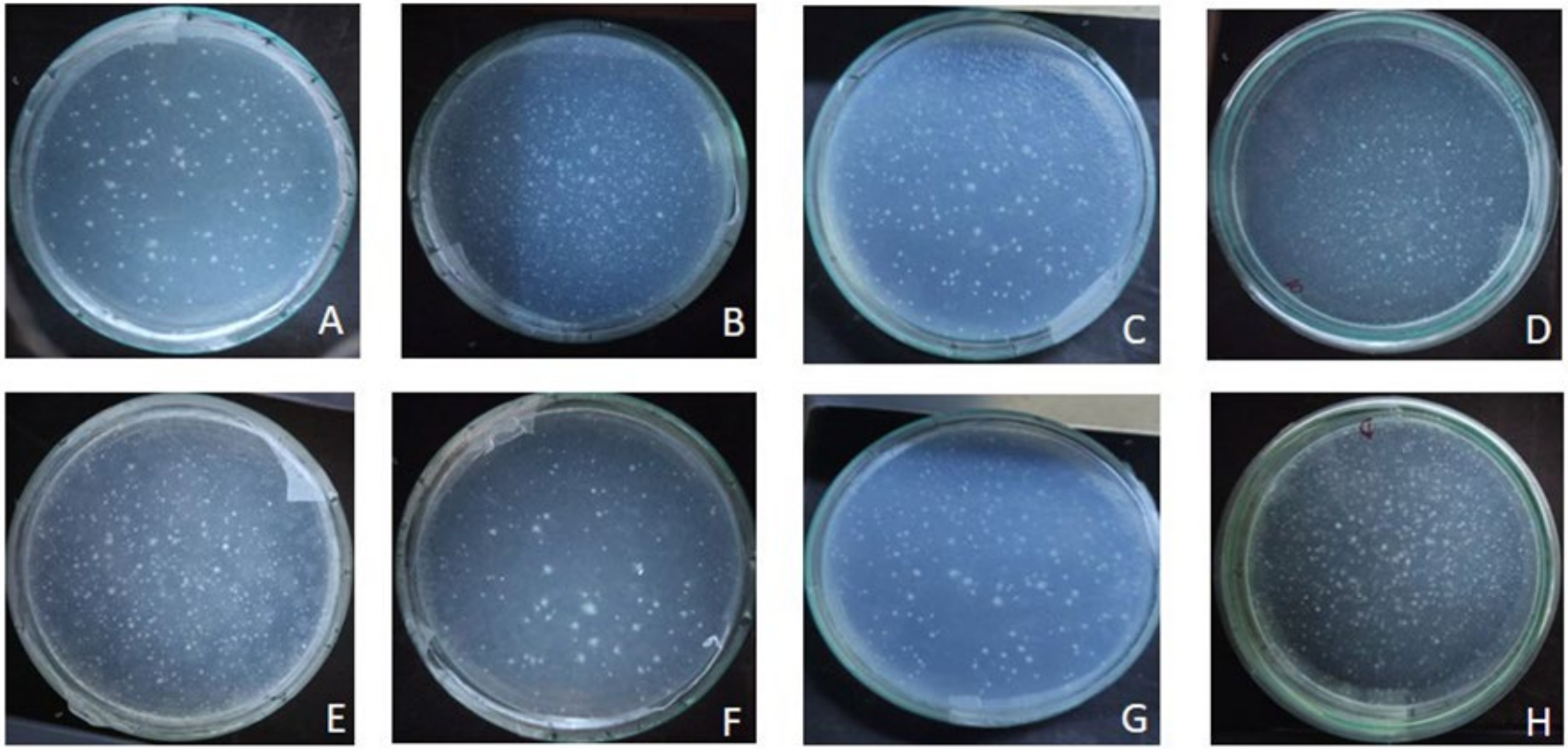

Figure 5.3: Representative images of his+ revertant colonies after 48 h treatment in glucose minimal plate. A - Negative control; B - Positive control (Sodium azide 5 µg/ml); C - VCEA (100 µg/ml); D - VCET (100 µg/ml); E - VCWT (100 µg/ml); F - SDEA (100 µg/ml); G - SDET (100 µg/ml); H - SDWT (100 µg/ml)

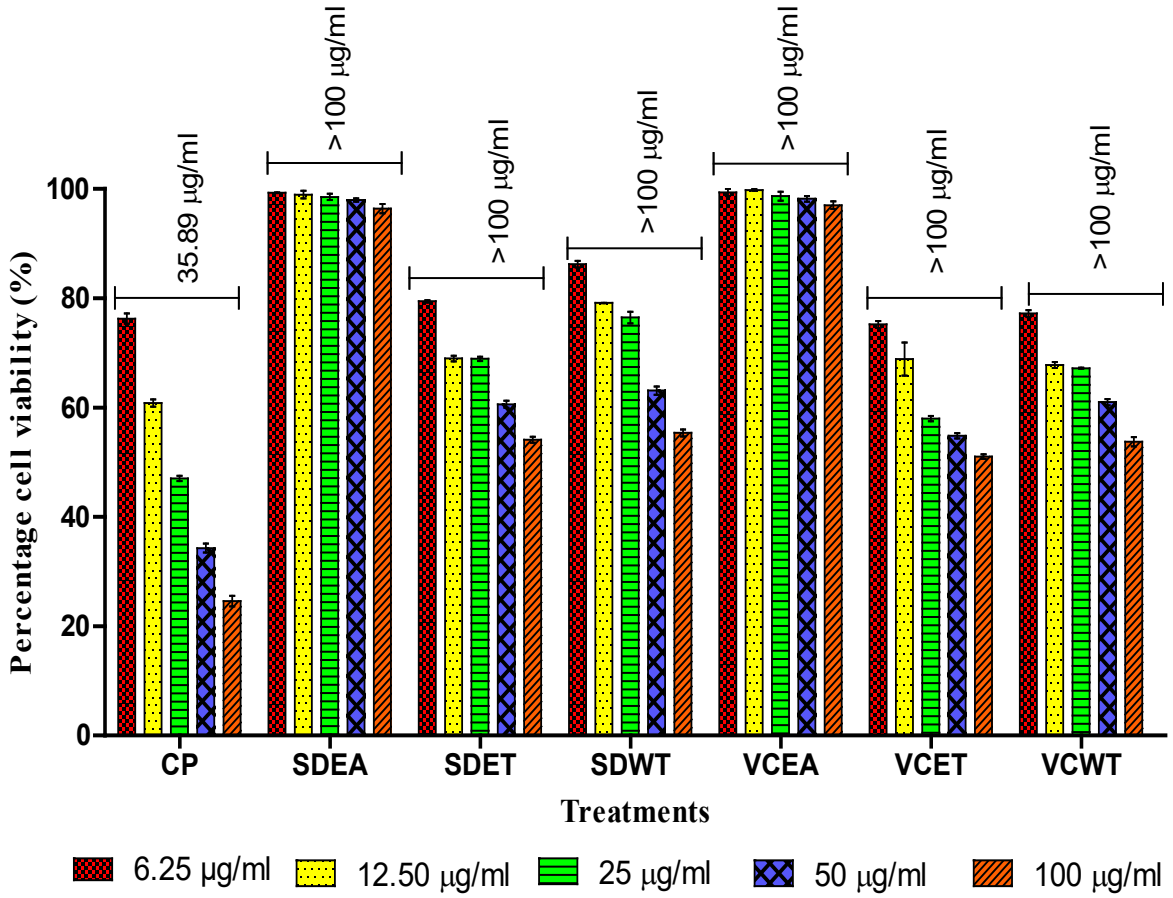

Figure 5.4. Cell viability determination of cyclophosphamide and various plant extracts on $HepG_2$ using MTT assay. IC_{50} value for each group is shown (⊢——⊣)

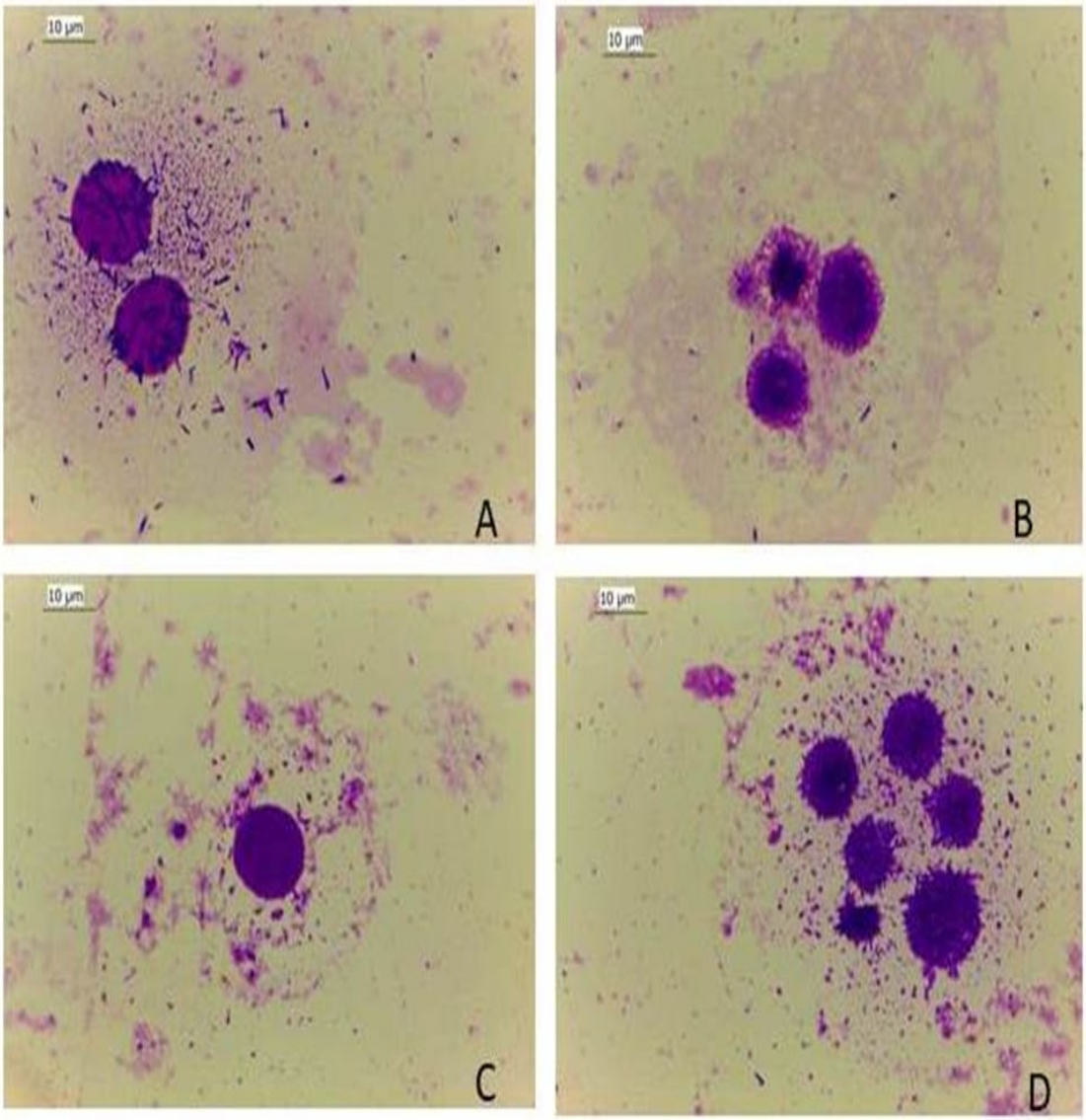

Figure 5.5: Representative images of cell types scored in CBMN assay. A - binucleated cell; B - binucleated cell with Micronucleus; C - mono nucleated cell; D - Multinucleated cell

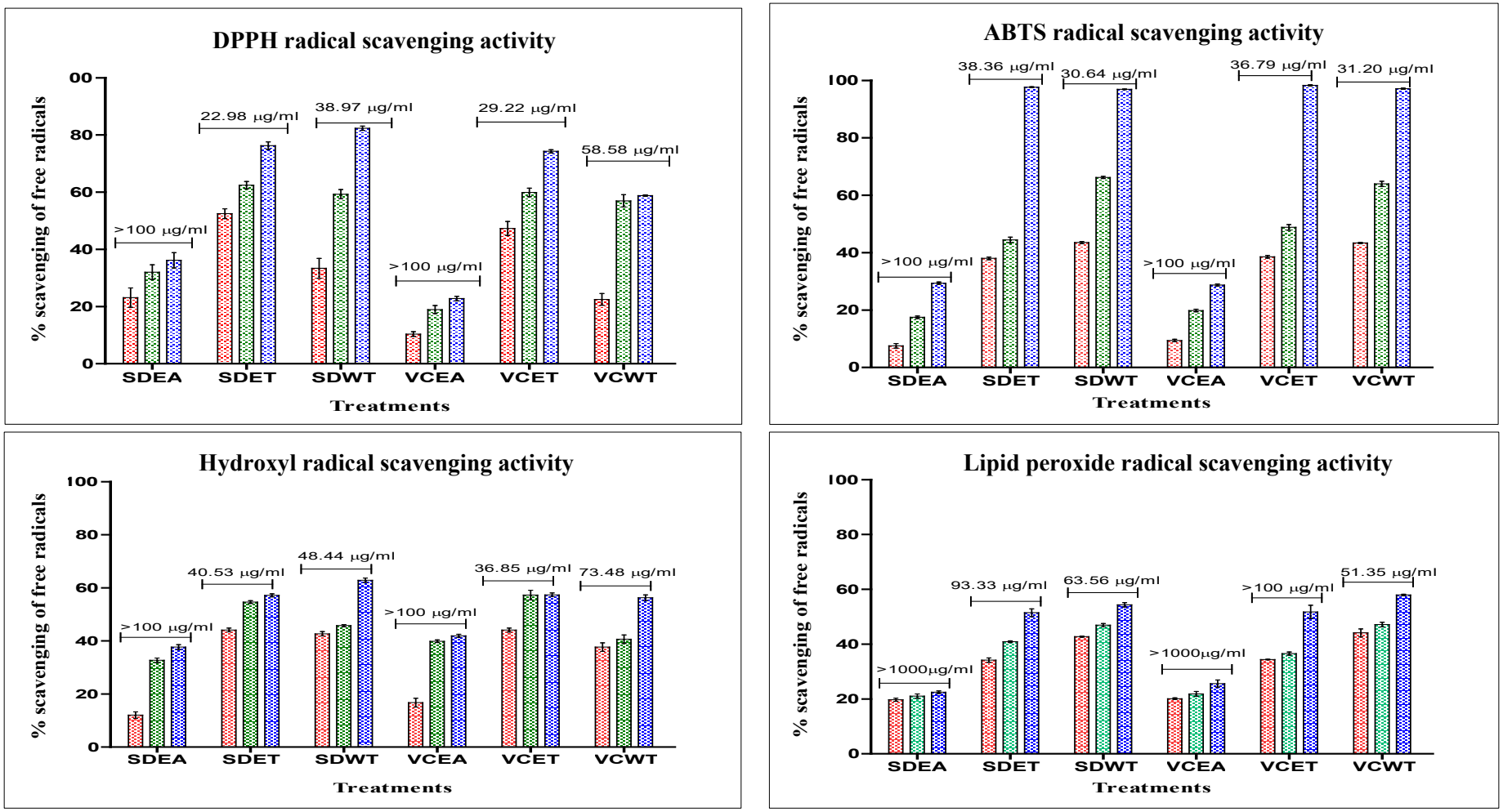

Figure 5.6. Determination of free radical scavenging activity of different solvent extracts from *Scoparia dulcis* and *Vernonia cinerea.*
IC_{50} values for each group is shown (⊢——⊣) 25 (µg/ml) 50 (µg/ml) 100 (µg/ml)

CHAPTER 6

Effect of *S. dulcis* & *V. cinerea* on cell proliferation, apoptosis and cell cycle

6.1. Introduction

Chemotherapeutic drugs used in cancer treatment work by destroying the cancer cells and shrinking or retarding their further proliferation. The enormous chemical diversity and the capability for selective inhibition of proliferation and induction of apoptosis makes the phytochemicals the good drug candidates in the search for new powerful chemotherapeutic agents (Nguyen et al., 2020). Discovering more chemotherapeutic drugs for lymphoma treatment has paramount importance since most of the anti-lymphoma drugs proven by preclinical studies often fail in clinical practice due to the adverse side effect upon its systemic application (Wu et al., 2014).

Since sustained proliferation is a hallmark of cancer cells, the cell based *in vitro* assays for testing cytotoxicity and anti-cell proliferative assays are usually employed for screening the anticancer potential of test molecules. Dye exclusion assays, especially the trypan blue exclusion assay is a simple yet powerful bioassay for the determination of cytotoxicity (Strober, 2001). Evading apoptosis is another important feature of cancer cells. Thus the agents having the potential to induce apoptosis in turn indicates their anticancer potential. Certain biochemical events that occur as part of apoptosis can be considered as the markers in the assays detecting the apoptosis. Flow cytometry is an accurate and automated technique used in the quantitative measurement of apoptosis (Adan et al., 2017). This technique can also be also used for the assessment of cell cycle phases and the

potential of test compounds in triggering any cell cycle arrest at certain phases. Cell cycle inhibitors are another category of anticancer agents since deregulation of cell cycle is another characteristic feature of cancer cells.

This chapter is focussed on the evaluation of cytotoxic potentials of the ethyl acetate, ethanol and water extracts of *Scoparia dulcis* and *Vernonia cinerea* in lymphoma cells. Effect of the potent extract on cell proliferation, cell cycle regulation and apoptosis is also evaluated. Predicting the mechanism of action of each plant constituent using *in silico* approaches is also attempted here.

6.2. Methodology

6.2.1. Extraction

The methodology used for preparation of plant extract was given in the methodology section 3.2.2 of chapter 3.

6.2.2. Cell death analysis

The trypan blue dye exclusion assay was used to test the cytotoxic potentials of extracts on DLA cells. The assay protocol is explained in the section 3.2.7.1.a of chapter 3.

6.2.3. Cell death analysis in the presence of iron chelator

To explore the ferroptotic potential of various extracts, trypan blue dye exclusion assay was repeated with deferoxamine, an iron chelating compound. The protocol is given in section 3.2.7.1.b of chapter 3.

6.2.4. Cell proliferation assay

Long-term cell proliferation assay was done using YAC-1 cells (lymphoma) and the procedure is detailed in the section 3.2.7.2 of chapter 3.

6.2.5. Cell cycle analysis

The potential of plant extracts interfering with the cell cycle were performed using YAC-1 cells. The test procedure is given in section 3.2.7.4 of chapter 3.

6.2.6. Apoptosis analysis

Apoptosis detection using Annexin V- propidium iodide staining and flow cytometric analysis was conducted using YAC-1 cells and the protocol is explained in the section 3.2.7.5 of chapter 3.

6.2.7. Molecular docking analysis

The binding affinity of the phytochemicals identified from active extracts were tested using molecular docking studies against some target proteins. The protocol used for molecular docking analysis is given in section 3.2.10 of chapter 3.

6.2.8. Statistical analysis

All data are expressed as mean ± SEM and tested using Student's t-test (p<0.05) using Microsoft Excel. IC_{50} values were calculated using SPSS software.

6.3. Results

6.3.1. Cytotoxicity analysis

Cytotoxicity screening of various extracts of *S. dulcis* have shown that ethyl acetate extract (SDEA) among others possessed a strong cytotoxic activity to lymphoma cells with an IC_{50} value of 15.41 µg/ml (Table 6.1). Ethanol extract (SDET) is also inducing cytotoxicity on DLA cells with an IC_{50} value of 40.49 µg/ml. But water extract (SDWT) was not found to be cytotoxic to lymphoma cells (IC_{50} value >200). Cytotoxicity assay conducted with *V. cinerea* extracts showed a strong cytotoxicity in the ethyl acetate extract (VCEA) with 96% cytotoxicity at the highest concentration of 200 µg/ml. The IC_{50} value was found to be 61.24 µg/ml for VCEA. Ethanol (VCET) and water (VCWT) extracts expressed least cytotoxicity (IC_{50} value >200). When the lymphoma cells were treated with VCEA

simultaneously with deferoxamine (iron chelator), the IC_{50} value of VCEA was increased from 61.24 µg/ml to a value greater than 200 µg/ml (Table 6.2).

6.3.2. Cell proliferation assay

Trypan blue dye exclusion assay was utilized to find out the effect of SDEA and VCEA on the proliferation of lymphoma cells with longer treatment period such as 12, 24, and 48 hours. SDEA and VCEA (5 µg/ml, 10 µg/ml and 15 µg/ml) inhibited the growth of lymphoma cells both in a concentration and time dependent manner. After 48 hours, vehicle-treated cells were multiplied into $1.95 \pm 0.03 \times 10^5$ from the initial seeding of about $1.05 \pm 0.05 \times 10^5$ cells, whereas significant reduction in the number of viable cells were found at 15 µg/ml of SDEA at this time point (Figure 6.1). VCEA was also found to be significantly retarded the proliferation of YAC-1 cells in concentration and time dependent manner. After 48 hours of incubation, number of viable cells reduced to nearly 16 thousand cells with 15 µg/ml of VCEA at the same time point (Figure 6.2).

6.3.3. Cell cycle analysis

Cell cycle analysis of YAC-1 cells treated with SDEA at concentration of 5 µg/ml and 10 µg/ml for 24 hours using FACS scan revealed accumulation of cells at S and G_2/M phase when compared with the vehicle controls (Table 6.3 and Figure 6.3). Vehicle treatedYAC-1 cell distribution was 40.6% cells at G_1 phase, 2.5% cells at S phase and 6.8 % cells at G_2/M phase, whereas SDEA treatment at 10 µg/ml results in about 8.8% cells at G_1 phase, 20.8% cells at S phase and 28.5 % cells at G_2/M phase. These results clearly showed the remarkable reduction of cell number in G_1 phase and accumulation of cells in S and G_2/M phase upon SDEA treatment. Further in the case of VCEA, G_2/M phase cell cycle arrest was evident from the flow cytometric analysis (Table 6.4 and Figure 6.4). In vehicle control treated cells only

9% cells were seen in the G_2/M phase, whereas, a hike in the number of cells (17%) in this phase can be observed in VCEA treated cells.

6.3.4. Apoptosis evaluation

Flow cytometric analysis after Annexin V-FITC/PI co-staining of the treated cells revealed the distribution of cells that are alive, early apoptotic, late apoptotic and necrotic stages (Table 6.5; 6.6). A dose dependent increase in the number of cells experiencing apoptosis along with the reduction in the number of live cells could be observed in both SDEA and VCEA treated cells. About 92.7 % of live cells were found in vehicle treated cells. While only 54 % of cells can be observed in the SDEA (15 µg/ml) treated cells. Only 2.2 % (1.6 + 0.6) of cells experiencing apoptosis (including both early and late stages) were found in vehicle treated cells. Whereas 35.3% (28.6+6.7) cells experiencing apoptosis can be observed in the SDEA (15 µg/ml) treated cells. In the case of VCEA at 15 µg/ml, a total of 49.1% (45.1+4) cells undergoing apoptosis were found (Figure 6.5 and 6.6).

6.3.5. Molecular docking evaluations

The binding affinity of SDEA constituents to topoisomerase-1 enzyme is given in table 6.7. Pectolinarigenin and Luteolin showed stronger binding affinity (-9.8 kcal/mol) with Topoisomerase- DNA complex. The docking scores of Scutellarein, Acacetin and Nepetin to Topoisomerase bound to DNA were -8.6 kcal/mol, -9.3 kcal/mol and -9.7 kcal/mol respectively (Figure 6.7 and 6.8).

The binding energy of the VCEA constituents to Ferroportin-1 was depicted in table 6.8. Nerolidol and Luteolin showed strong docking scores compared to others. A detailed receptor-ligand interaction map is illustrated in figure 6.9.

6.4. Discussion

Insensitivity to signals which regulates cellular proliferation is a characteristic feature found in most cancer types. There are phytochemicals that enable the cell to restrict or slow down the abnormal cell proliferation through various mechanisms and induce apoptosis. Treatment of YAC-1 lymphoma cells with SDEA extracts resulted in the concentration and time dependent antiproliferative effects on lymphoma cells indicating the potential of SDEA components in interfering the tumour progression. Cell cycle refers to the irreversible sequential events involved in the cell proliferation process and is highly conserved in eukaryotic cells. Transitions from one phase to another are strictly controlled by checkpoint pathways in normal cells and are malfunctioned in many cancer cell types (Mills et al., 2018). Drugs activating cell cycle arrest in cancer cells are an effective approach in cancer therapy. Many phytochemicals exert their antiproliferative effects by regulating the various cell cycle phases leading to cell cycle arrest in tumour cells (Bailon-Moscoso et al., 2017). Here the flow cytometric analysis indicated that SDEA treatment induced S phase and G_2/M phase arrest in lymphoma cells so that the cell gets ample time for appropriate repair machinery to act before entering to mitosis stage (Barkley et al., 2007). If the DNA damage is found to be unrepaired, further signalling mechanisms push those cells to apoptotic cell death (Lo et al., 2015). Apoptosis is accompanied by characteristic morphological changes, heterochromatin condensation, cellular shrinkage, budding and formation of apoptotic bodies. In the present study Annexin V-propidium iodide staining and subsequent flow cytometric analysis confirmed the pro-apoptotic potential of SDEA extract in lymphoma cells.

DNA topoisomerases catalyse the sequential breakage and religation of either single strand (topoisomerase I [topo I]) or both strands of DNA (topoisomerase II [topo II]) to release the excess tension or supercoiling of DNA and help cells to maintain the DNA integrity during replication, transcription, recombination, integration, and chromosomal segregation (McKie et al., 2021). There are two categories of Topoisomerase inhibitors namely, topoisomerase suppressors and topoisomerase poisons. Topoisomerase suppressors directly bind to the enzyme and inhibit its activity leading to the death of tumour cells. Whereas Topoisomerase poisons form a ternary complex with the topoisomerase I-DNA cleavage complex (topo I-DNA) and thus stabilises the complex and forms irreversible DNA double-strand break during replication. Sevaral clinically used chemotherapeutic drugs target Topoisomerases inhibition for their activity. For example, Camptothecin is a well-known anticancer drug isolated from the stem wood of the Chinese tree, *Camptotheca acuminata* that can act as topoisomerase poison and causes S phase specific killing of tumour cells when the ternary complex reach at the DNA replication fork (Liu et al., 2000). Topotecan and Irinotecan are well known camptothecin derivatives clinically used for treatment of ovarian and colorectal cancers respectively (Pommier, 2006).

The role of DNA topoisomerase inhibition in mediating S and G_2/M phase arrest leading to apoptosis have been revealed in a large body of studies. Diphenyl ditelluride, a stable synthetic organotellurium compound, showed antiproliferative effect to Chinese hamster fibroblast (V79) cells due to its interaction with topoisomerase I enzyme and the associated S phase cell cycle arrest leading to apoptosis (Jorge et al., 2015). Liu et al. (2013) found that a synthetic compound named TCH-1030 (9-methoxy-6-(piperazin-1-yl)-11H-indeno [1, 2-c] quinoline-11-

one O-3-(dimethylamino) propyl oxime) triggered S phase arrest and apoptosis which was primarily mediated by topoisomerase I inhibition. Jang et al. (2018) investigated the anticancer mechanism of a compound named MHY440 as its potential in inhibiting topoisomerase I leading to G_2/M phase cell cycle arrest that in turn ultimately triggers apoptosis in gastric cancer cells. Wang et al. (2023) evaluated the antitumor potential of an alkaloid namely Narciclasine, and found that it suppresses the topoisomerase enzyme before the enzyme interacts with DNA unlike the action of camptothecin. An earlier study revealed that Acacetin, a naturally occurring flavone, could bind individually with the covalent Topo I-DNA complex prior to DNA cleavage though it does not interfere with the cleavage. However this ternary complex stabilises the DNA Topo I intermediate and prevents further religation step (Boege et al., 1996).

Here, we analysed the binding affinities of pharmacologically relevant SDEA phytoconstituents with topoisomerase I using molecular docking because of the strong S phase arrest in lymphoma cells on SDEA treatment. Interestingly, the phytoconstituents especially Pectolinarigenin, Scutellarein, Acacetin, Luteolin and Nepetin showed strong binding interaction to the topoisomerase I-DNA complex than with topoisomerase I alone (uncomplexed with DNA) indicating the potential of these compounds to act as topoisomerase poisons. Molecular docking studies conducted by Staker et al. (2002) against the same enzyme revealed that topotecan can act as an intercalating agent since it was found to span across both strands of DNA together with some amino acid interactions. Similarly, such an intercalating potential (interactions with both DNA strands [C and D] as well as aminoacids) of the reference drugs as well as the SDEA phytoconstituents was also noticed in this

study. The binding affinity obtained for the reference drugs were also found to be consistent with similar studies conducted by Madeddu et al. (2022).

Flow cytometric analysis of cells treated with ethyl acetate extract of *V. cinerea* unveiled its potential in exerting G_2/M arrest and apoptosis (Johnson & Varghese, 2023a). Previous studies indicated that generation of intracellular ROS and subsequent DNA damage has roles in induction of G_2/M arrest. Guo et al. (2014) found that G_2/M arrest and apoptosis induced by Cucurbitacin B in K562 leukemia cells was dependent on the intracellular ROS generation. Greenshields et al. (2017) investigated that anti-malarial drug Artesunate induced anti-proliferative effects in ovarian cancer cells through ROS dependent G_2/M arrest. This study revealed the involvement of ferroptosis in Artesunate mediated killing of cancer cells since a significant reduction in cytotoxicity was found on co-treatment with a ferroptosis inhibitor *viz*, ferrostatin-1.

Sperotto et al. (2013) has reported the potential of nerolidol, a sesquiterpene, in the generation of ROS, such as H_2O_2, hydroxyl radical ($OH^{\cdot}$), and hydroperoxides (ROOH) in SOD deficient yeast cells and the induction of single strand breaks. In addition to this, nerolidol downregulated the ATM/Akt pathway and displayed cell cycle arrest at G1 phase in leiomyoma cells (Dong et al., 2021). This effect on leiomyoma was decreased significantly upon ferrostatin-1 treatment which traps peroxyl radicals, leading to the inhibition of ferroptosis. According to Soltan et al. (2019) ROS generation induced by a particular drug (indirubin derivative DKP-071) is suggested to be a useful targeted therapy for cutaneous T cell lymphoma, where ROS can act as a signaling molecule for the induction of apoptosis. Free intracellular iron (Fe^{2+}) catalyzes Fenton reaction and generates hydroxyl radicals which subsequently leads to lipid peroxidation and loss of membrane integrity and

ultimately the ferroptotic cell death. Dixon et al. (2012) investigated that Erastin could increase lipid ROS formation in two hours treatment in NRAS mutant HT-1080 fibrosarcoma cells and subsequent cell death. Treatment of this compound along with an iron chelator (deferoxamine 100 μM) could inhibit this effect suggesting that iron dependent accumulation of ROS might be the mechanism behind this cell death.

The molecular docking analysis we conducted revealed the potentials of VCEA constituents in inducing ferroptosis. The strong interactions of VCEA constituents (Nerolidol and Luteolin) with Ferroportin-1, an iron exporting transmembrane protein. These compounds could strongly bind with the hepcidin binding domain of Ferroportin-1 triggering ferroptosis. Hepcidin is a protein that blocks Ferroportin's action. Thus Nerolidol and Luteolin can probably act as the competitive inhibitor of Ferroportin-1. We repeated the 3 hour cytotoxicity experiments with co-treatment of iron chelator. When the lymphoma cells were treated with *V. cinerea* extracts along with deferoxamine, the cytotoxicity was found to be significantly decreased in VCEA treated cells compared to other extracts indicating the iron mediated cell death induced by VCEA (Johnson & Varghese, 2023a). It is interesting to note in this regard that the ethyl acetate extract possesses very little ROS scavenging potentials when compared with other extracts, as revealed in the previous chapter.

Table 6.1: Short term cytotoxicity screening of the *S. dulcis* extracts against DLA cells						
Extract (µg/ml)	Percentage (%) cell death after 3h treatment					
	SDEA	SDEA + DFX	SDET	SDET + DFX	SDWT	SDWT + DFX
10	48±1.15	46±1.74	20±3.46	18±2.30	0±0.00	0±0.00
20	50±1.74	49±1.15	28±2.30	29±1.74	0±0.00	0±0.00
50	68±2.30	65±0.00	42±1.74	41±4.04	2±0.00	3±1.74
100	100±0.00	100±0.00	72±1.15	71±3.46	6±2.30	8±0.00
200	100±0.00	100±0.00	100±0.00	100±0.00	17±1.74	19±2.30
IC_{50}	15.41	16.42	40.49	41.62	>200	>200
DFX – Deferoxamine (50 µM)						

Table 6.2: Short term cytotoxicity screening of the *V. cinerea* extracts against DLA cells						
Extract (µg/ml)	Percentage (%) cell death after 3h treatment					
	VCEA	VCEA + DFX	VCET	VCET + DFX	VCWT	VCWT + DFX
10	8±1.74	0±0.0	0±0.00	0±0.00	0±0.00	0±0.00
20	17±1.15	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
50	32±2.30	5±1.15	5±1.15	4±0.00	8±1.15	9±1.15
100	60±1.15	7±1.74	11±2.30	14±1.15	14±1.15	14±1.74
200	96±1.74	18±1.15	22±1.74	24±2.30	24±2.30	25±1.15
IC_{50}	61.24	>200	>200	>200	>200	>200
DFX – Deferoxamine (50 µM)						

Table 6.3: Effect of SDEA on YAC-1 cell cycle			
Treatment	Percentage of cells (%)		
	G_1	S	G_2M
Vehicle Control	40.6	2.5	6.8
5 (µg/ml)	23	16	25.3
10(µg/ml)	8.8	20.8	28.5

Table 6.4: Effect of VCEA on YAC-1 cell cycle			
Treatment	Percentage of cells (%)		
	G_1	S	G_2M
Vehicle Control	41	2.7	9
5 (µg/ml)	40	2.2	16.5
10(µg/ml)	40.7	2.4	17

Table 6.5: Effect of SDEA on apoptosis induction				
Treatment	Percentage of cell population (%)			
	Necrotic cells	Apoptotic cells		Live cells
		Early stage	Late stage	
Vehicle Control	5.1	1.6	0.6	92.7
5 (µg/ml)	2	26.4	2.7	68.9
10 (µg/ml)	9.9	22.8	5.5	61.7
15 (µg/ml)	10.7	28.6	6.7	54

Table 6.6: Effect of VCEA on apoptosis induction				
Treatment	Percentage of cell population (%)			
	Necrotic cells	Apoptotic cells		Live cells
		Early stage	Late stage	
Vehicle Control	5.1	1.6	0.6	92.7
5 (µg/ml)	12.4	21.2	5.9	60.4
10 (µg/ml)	3.3	39.9	5.1	51.7
15 (µg/ml)	3	45.1	4	48

Table 6.7: Binding affinity (Kcal/mol) of selected ligands from *S. dulcis* and reference drugs to human Topoisomerase 1 and Ferroportin 1			
Ligands	Binding affinity (Kcal/mol)		
	Topo 1	Topo 1 - DNA	Ferroportin 1
Pectolinarigenin	-7.2	-9.8	-7.7
5-Demethylnobiletin	-6.9	-8.1	-7.4
Scutellarein	-7.4	-8.6	-7.8
Acacetin	-7.1	-9.3	-7.3
Eupalitin	-6.8	-8.5	-7.4
Luteolin	-7.4	-9.8	-7.9
Nepetin	-7.3	-9.7	-7.9
Tectorigenin	-7.3	-8.2	-7.4
Beta Bisabolene	-5.4	-7.0	-6.2
D-Germacrene	-6.4	-6.9	-6.6
Abietatriene	-7.2	-8.3	-7.6
Camptothecin	-7.0	-8.6	-8.7
Topotecan	-6.7	-9.0	-8.6

Table 6.8: Binding affinity (Kcal/mol) of selected ligands from *V. cinerea* and reference drugs to Ferroportin-1			
Ligands	Binding affinity (Kcal/mol)		
	Topo 1	Topo 1 - DNA	Ferroportin 1
Nerolidol	-4.4	-5.3	-7.8
Acevaltrate	-6.8	-8.5	-7.4
Nevadensin	-7.0	-9.2	-7.4
Luteolin	-7.4	-8.6	-7.9
Genistein	-6.9	-8.7	-7.2
Tectorigenin	-7.3	-8.2	-7.4
Zingerol	-4.4	-5.4	-5.6
Eupalitin	-6.8	-8.5	-7.4
Vamifeport	-5.5	-6.2	-7.3

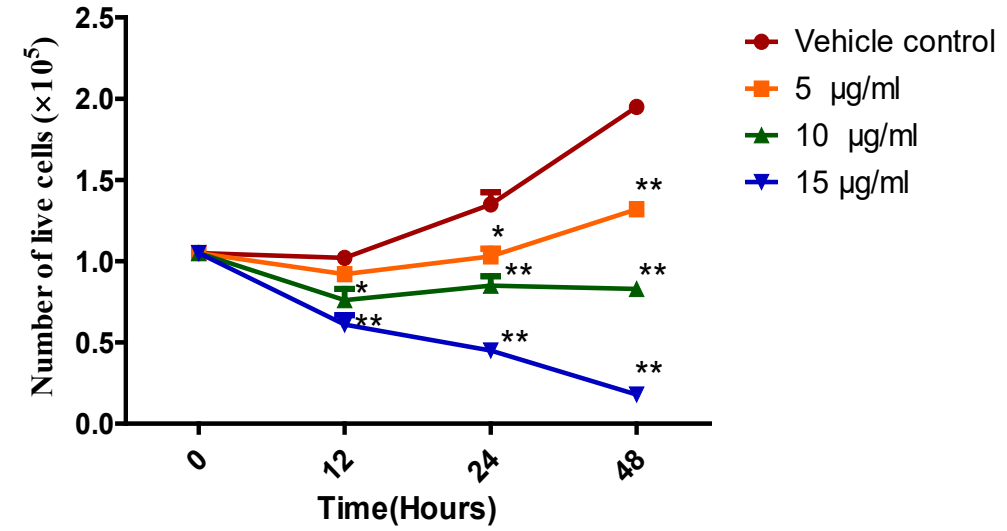

Figure 6.1: Effect of the SDEA on cell proliferation. Graph showing the number of live cells (YAC-1) at three different time points (12, 24 and 48 h) after treatment with SDEA extract (5, 10 and 15µg/ml). All the treated group were separately compared with the vehicle control at each time point. The symbol (*) represents statistical significance at $p \leq 0.05$. The symbol (**) represents statistical significance at $p \leq 0.01$

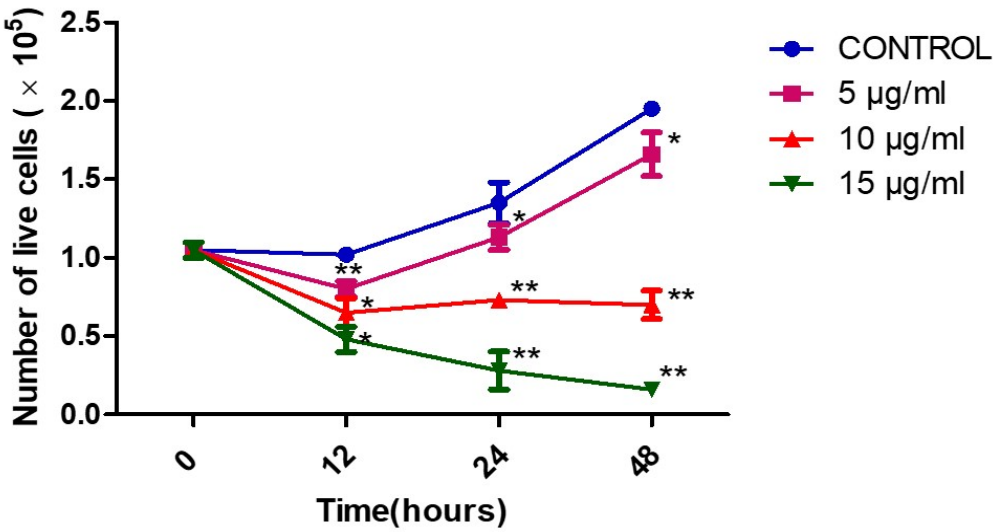

Figure 6.2: Effect of the VCEA on cell proliferation : Graph showing the number of live cells (YAC-1) at three different time points (12, 24 and 48 h) after treatment with VCEA extract (5, 10 and 15µg/ml). All the treated group were separately compared with the vehicle control at each time point. The symbol (*) represents statistical significance at $p \leq 0.05$. The symbol (**) represents statistical significance at $p \leq 0.01$

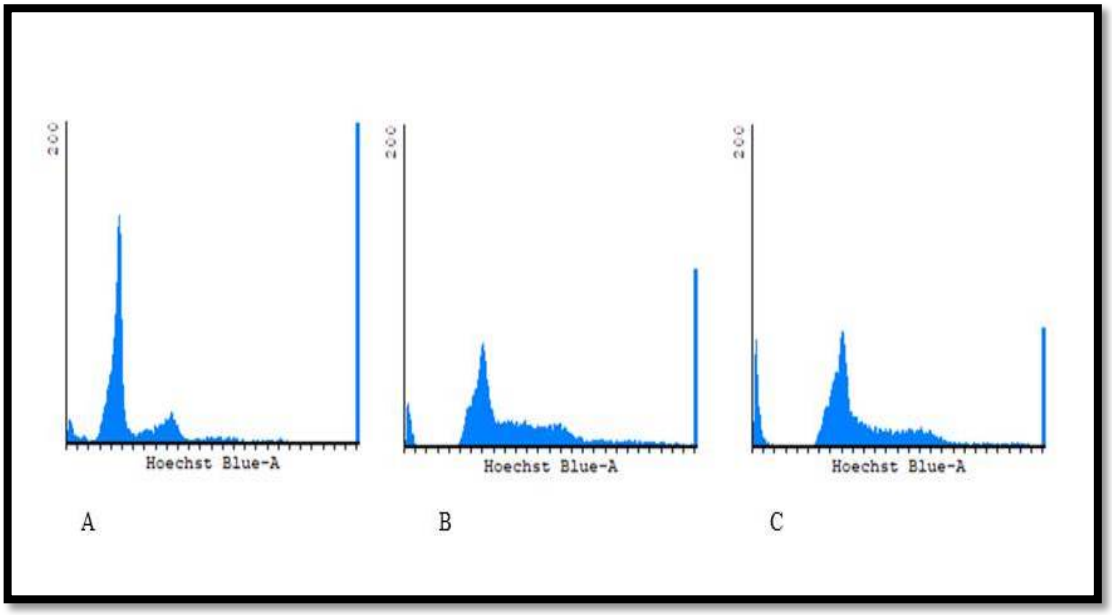

Figure 6.3: Effect of SDEA on cell cycle progression.: (A) Represents vehicle control, (B) represents SDEA treated at the concentration of 5 µg/ml and (C) represents SDEA treated at the concentration of 10 µg/ml.

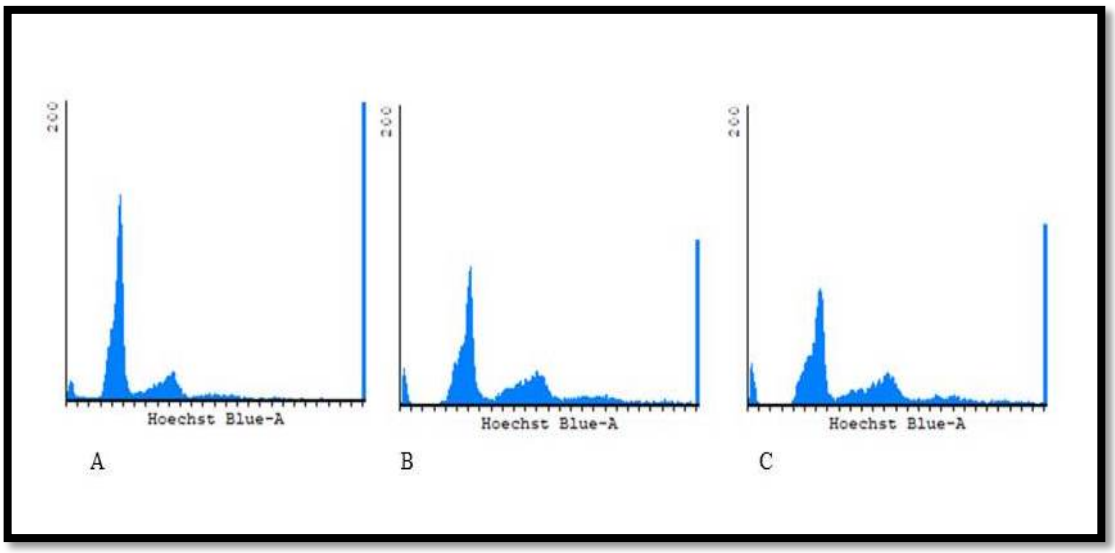

Figure 6.4: Effect of VCEA on cell cycle progression. (A) Represents vehicle control, (B) represents VCEA treated at the concentration of 5 µg/ml and (C) represents VCEA treated at the concentration of 10 µg/ml.

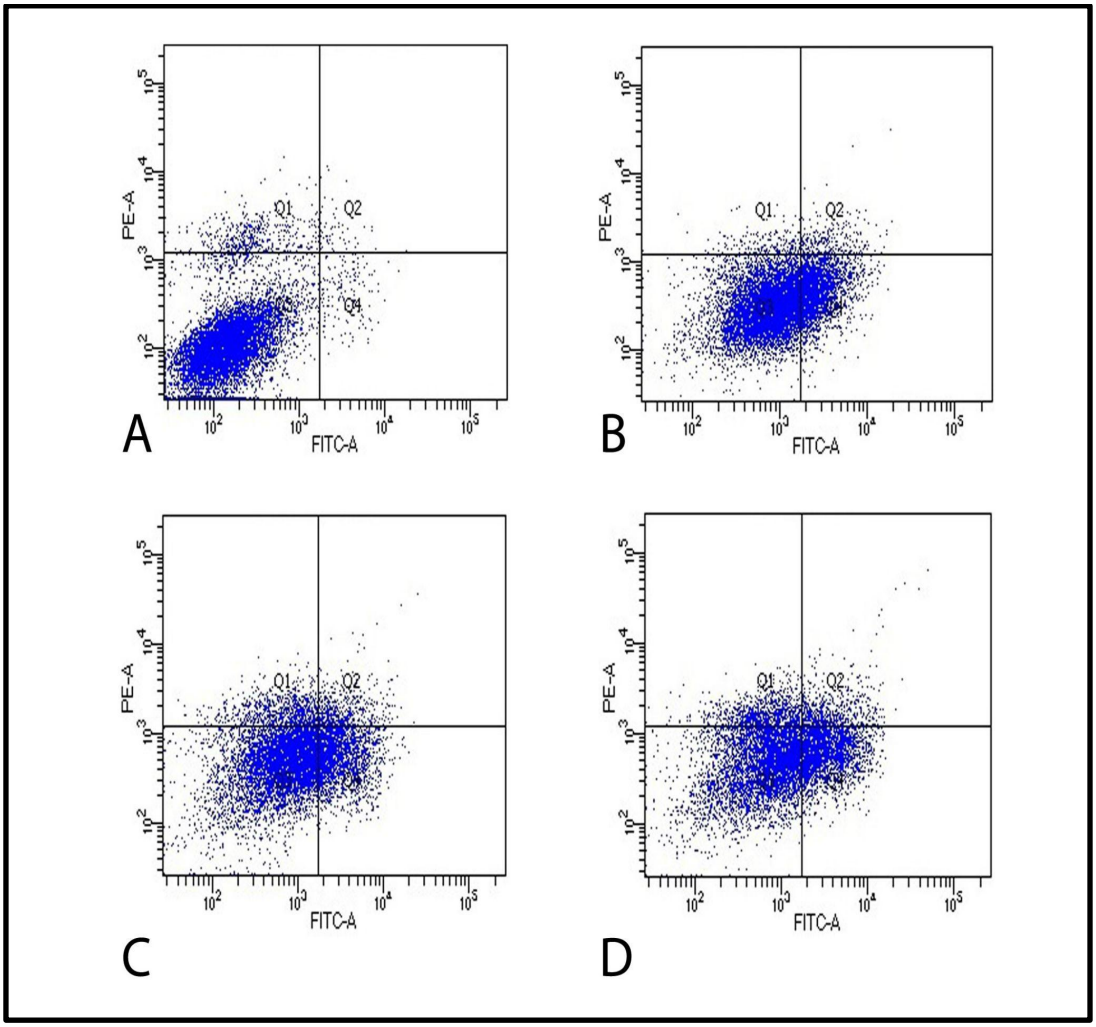

Figure 6.5: Annexin V/ PI associated fluorescence exhibited by YAC-1 Cells after 24 hour treatment with SDEA extract. The percentage of cells in each quarter varies in favour of apoptosis with SDEA treatment. (A) Represents vehicle control, (B) represents 5µg/ml of SDEA and (C) represents 10µg/ml of SDEA treatment and (D) represents 15µg/ml of SDEA. 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 −/+).

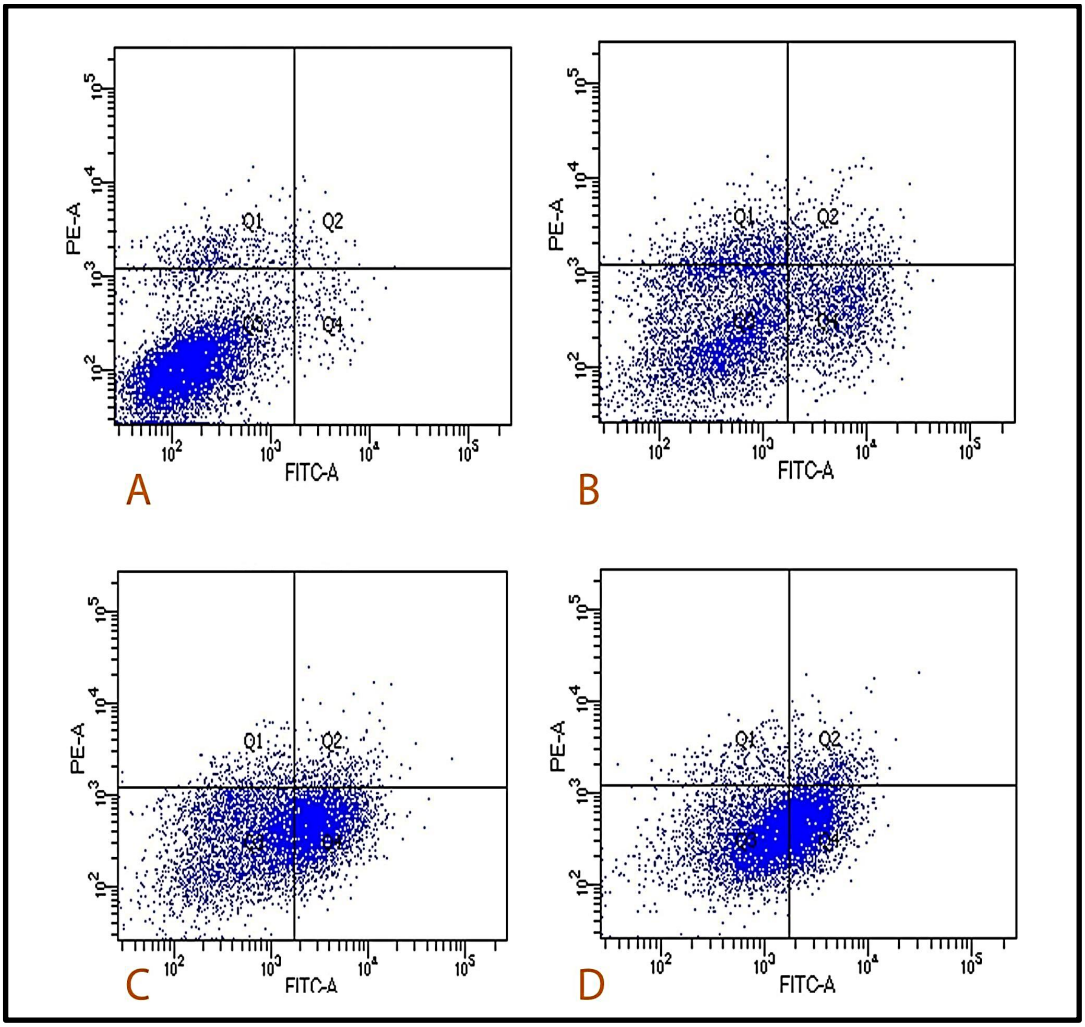

Figure 6.6: Annexin V/ PI associated fluorescence exhibited by YAC-1 Cells after 24 hour treatment withVCEA extract. The percentage of cells in each quarter varies in favour of apoptosis with VCEA treatment. (A) Represents vehicle control, (B) represents 5 µg/ml of VCEA and (C) represents 10 µg/ml of VCEA and 15 µg/ml of VCEA treatment. 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 −/+).

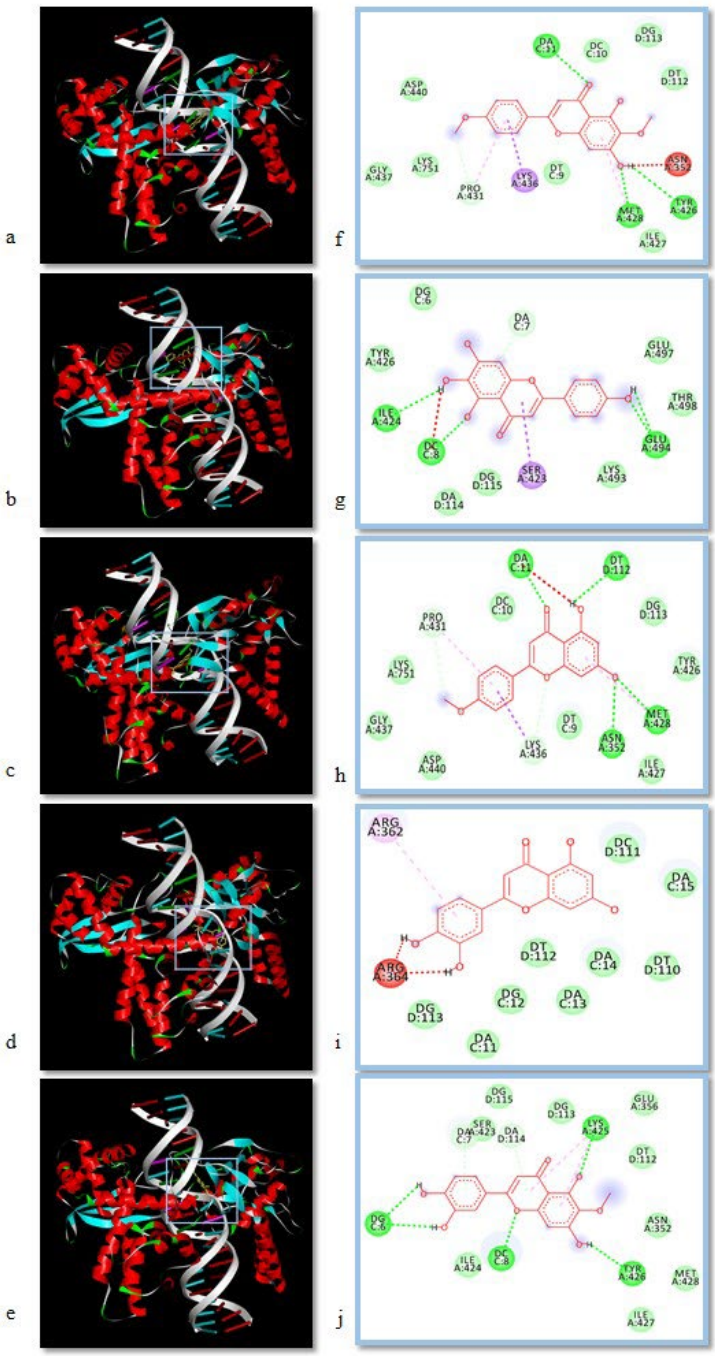

Figure 6.7: Molecular docking interactions of phytocompounds from *S. dulcis* ethyl acetate extract with the enzyme Topoisomerase I **bound to DNA.** The images a, b, c, d and e shows 3D images of binding patterns of the ligands Pectolinarigenin, Scutellarein, Acacetin, Luteolin and Nepetin with Topoisomerase 1 in DNA bound form respectively. The images f, g, h, i and j shows the binding interactions (2D) of Pectolinarigenin, Scutellarein, Acacetin, Luteolin and Nepetin respectively with Topoisomerase 1 in DNA bound form.

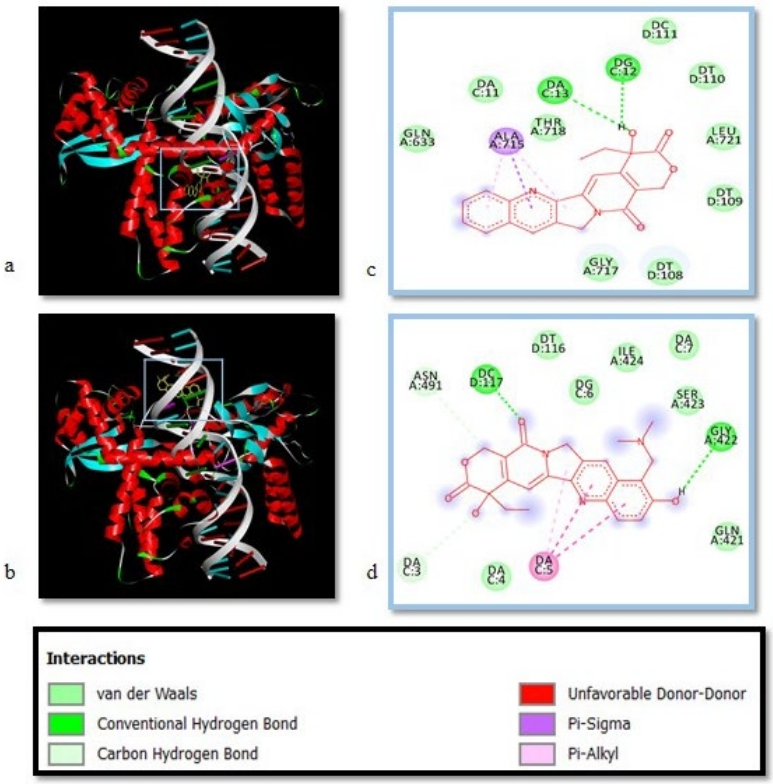

Figure 6.8: Molecular docking interactions reference drugs with the enzyme Topoisomerase I **bound to DNA.** The images a and b shows the binding patterns of the reference drugs Camptothecin and Topotecan with Topoisomerase 1 in DNA bound form respectively. The images c and d shows the binding interactions (2D) of the reference drugs Camptothecin and Topotecan respectively with Topoisomerase 1 in DNA bound form.

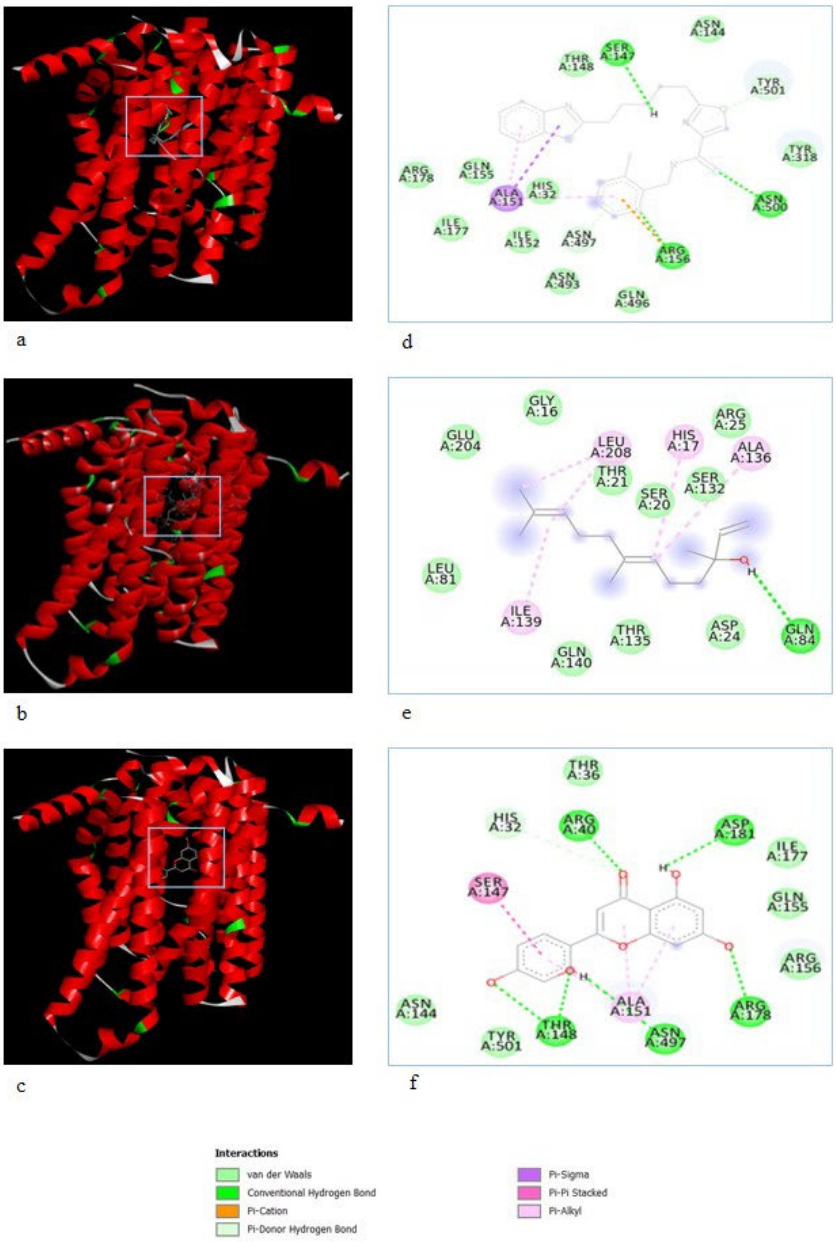

Figure 6.9: Molecular docking interactions of phytocompounds from *V. cinerea* ethyl acetate extract and reference drug with Ferroportin I. The images a, b and c represents the binding patterns of Vamifeport (a reference drug), Nerolidol and Luteolin with Ferroportin I respectively. The images d, e and f shows the binding interactions (2D) of Vamifeport, Nerolidol and Luteolin respectively.

CHAPTER 7

Evaluation of toxicity & antitumor potentials of *S. dulcis* & *V. cinerea* using mice model

7.1. Introduction

The use of indigenous or locally available medicinal plants in the treatment and control of various ailments plays a significant role in the drug discovery process and health management in developing countries (Kalimuthu et al., 2010). Phytochemicals are either beneficial or toxic to mankind. Solvent extracts from plants containing mixtures of phytocompounds may provide better therapeutic effectiveness due to the simultaneous effects of these compounds on multiple pharmacological targets and improves clinical efficacy more than the single compound-based drugs. Many herbalists postulate that isolated ingredients from plants have weaker clinical effects than whole plant extracts, though this statement requires more scientific support in each case (Liang & Fang, 2006). General acceptance, efficacy, relative safety, cost effectiveness are key points which drive research in the field of herbal drugs and formulations. Since the clinical studies are expensive and time consuming, the pre-clinical studies in a suitable animal model following the *in vitro* experiments are very much crucial in the drug discovery process.

The dose at which the drug showing maximum therapeutic efficacy might not be its safe dose always. Some drugs exhibit toxicities at higher doses or on prolonged exposure (Sharif et al., 2015). The investigations on the toxic effects of phytopharmaceuticals are equally important as testing their pharmacological efficacy. Pre-clinical evaluations on safety and pharmacological potentials are

necessary to bring the plant-based compounds into mainstream medicine or evidence based medicine. Acute toxicity studies in suitable animal models help to assess the potential toxicities of drugs in humans and to estimate appropriate safe dosage for multiple-dose toxicity studies and the time related drug-induced clinical observations (Colerangle, 2013). In a subacute toxicity test the toxic effects of drugs on repeated administration for a period of 28 days at the subacute dosage was evaluated in detail. These studies will help to choose dosage levels which can be used for subchronic and chronic toxicity studies as well as in evaluation of pharmacological potentials and also supports initial clinical trials (Colerangle, 2017).

As the cancer incidence and mortality is increasing rapidly around the world and its treatment is still remaining challenging, there exists the demand for constant search for safer natural compounds to treat cancer. It has been seen in previous chapters that ethyl acetate extracts from the plants *S. dulcis* (SDEA) and *V. cinerea* (VCEA) were the most cytotoxic extract to the Dalton's Lymphoma Ascites (DLA) cells. Attempts were also made to identify the active components and explored the possible mechanistic basis of their actions. Lymphomas are the solid tumours of the immune system, arising from the cells of the lymphatic system (Shankland et al., 2012). Dalton's lymphoma is a form of transplantable tumours. It is a T cell lymphoma. It originates naturally in the DBA (H_2^d) mice thymus. It is a highly invasive and deleterious tumour and suppresses the immune system of the tumour-bearing mice (Klein, 1951; Parajuli et al., 1997). Dalton's lymphoma is the useful and interesting model in pre-clinical cancer research for evaluating the chemotherapeutic potentials of new or known drugs. By considering genetic stability and heterogeneity of the transplanted cell line and its immunogenicity

within the host animal, and the appropriate biologic endpoint Dalton's lymphoma solid tumour bearing murine model is ideal animal model for testing the antitumour potentials of natural plant products with reproducible biologic endpoint like local growth (Raj Kumar, 2017).

The current chapter details the toxicological profiling of SDEA and VCEA extracts including the acute and subacute toxicity evaluation in Swiss albino mice. Besides, this chapter also deals with the tumour reduction potentials of these extracts in the DLA solid tumour model in Swiss albino mice using the safe doses obtained from the toxicity study.

7.2. Methodology

7.2.1. Collection of Plant Material and Extraction

The Methods section 3.2.1 and 3.2.2 described the collection and extraction of plant materials in detail. For *in vivo* animal experiments different doses of SDEA and VCEA extracts were prepared in sunflower oil. Also, the sunflower oil alone treated mice were considered as the vehicle control group.

7.2.2. Animals

Female and male Swiss albino mice (25-30 g) were obtained from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur. All animal experiments were conducted by following the guidelines of Institutional Animal Ethics Committee (IAEC) and CPCSEA, Govt. of India.

7.2.3. Toxicity Study

7.2.3.1. Oral acute toxicity

The oral acute toxicity status of SDEA and VCEA extracts was evaluated by strictly following the guidelines of OECD 423. Detailed methodology has been given in Methods section 3.2.8.1.

7.2.3.2. Oral Sub-acute toxicity

The oral sub-acute toxicity estimation of SDEA and VCEA extracts were done by following the guidelines of OECD 407. Detailed methodology has been given in Methods section 3.2.8.2.

7.2.4. Evaluation of tumour reduction potential of SDEA and VCEA

Details of grouping of mice and the procedure used for evaluation of tumour reduction potentials of extracts in DLA cells induced tumour in Swiss albino mice were as described in the method section 3.2.8. Tumour volume of all mice under treatment were recorded during the study period at a 3 day interval. Histopathological analysis (method section 3.2.8.1.) as well as the apoptosis evaluations were conducted using the tumour mass excised from the mice at the end of the study period. Details of the TUNEL assay procedure was described in methods section 3.2.9.

7.2.5. Statistical analysis

The differences in mean between extracts treated and vehicle control were determined using the student's t test. Graph pad Prism software version 5 was used to construct graphical representation of the data. All data were expressed as mean ± standard error of mean. Comparisons were made between vehicle control groups

with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$.

7.3. Results

7.3.1. Acute toxicity studies using SDEA and VCEA extract

No mortality was observed in the animals treated with single oral administration of 2000 mg/kg body weight of SDEA. Similarly no animal deaths were observed in treatment with 2000 mg/kg body weight of VCEA. All mice were found to be normal without any significant changes in body weight, food and water consumption (Figure 7.1; Table 7.1). No abnormalities were found in the necropsy. The mice exhibited no gross behavioural or morphological changes till the end of the observation period (Figure 7.2; Table 7.2)

7.3.2. Sub-acute toxicity studies using SDEA and VCEA extract

The SDEA extract at sub-acute dosage of 400 mg /kg b. wt. ($1/5^{th}$ of acute toxicity dose) was found to be lethal to Swiss albino mice. Therefore the dosages such as 200 mg /kg b. wt. and 100 mg /kg b. wt. were used as high dose and low dose respectively in the sub-acute toxicity study. In the case of VCEA the dosages such as 400 mg /kg b. wt. and 200 mg /kg b. wt. resulted in the death of treated mice before the end of study period. Hence the experiment was repeated with the dosages such as 100 mg /kg b. wt. and 50 mg /kg b. wt. as high dose and low dose respectively in the sub-acute toxicity study with success.

7.3.2.1. Body weight and relative organ weight measurement

No significant changes in body weight and relative organ weight were observed in both SDEA and VCEA treated groups during the 28 day consecutive extract administration at sub-acute dosages (Figure 7.3; Table 7.3).

7.3.2.2. Estimation of Food and water consumption

Figure 7.4 shows the changes in the food intake of male and female mice during 28 days treatment period. SDEA 200 mg/kg b. wt. treated male mice showed significant (p<0.05) difference in the food intake during the last week of study when compared with vehicle control. No other SDEA extract treated groups of male and female showed significant changes in the food intake compared to vehicle control. Also, no significant difference in the food intake of VCEA extracts treated male and female mice were observed compared with vehicle control. Similarly, figure 7.5 shows changes in the water consumption of male and female mice during 28 days treatment period. In male mice, during the last week of study a significant increase in the water intake (p <0.05) of SDEA (200 mg/kg b. wt.) treated mice was observed. Whereas in other SDEA treated groups of male and females no such significant variations were found. Water consumption levels of both male and female VCEA treated groups showed no significant variations compared to the vehicle control.

7.3.2.3. Haematological evaluations

Haematological parameters analysed from the blood of SDEA treated male and female mice showed insignificant changes (p>0.05) compared to those of vehicle controls though slight variations in these parameters were observed. On the contrary, in the case of VCEA extract, among the haematological parameters analysed Hb, RBC, PCV and WBC levels were found to be decreased significantly (p ≤0.05) in the VCEA high-dose (100 mg/kg b.wt) treated animals of both sexes when compared with vehicle controls. All other parameters were not changed significantly in either treated group (Table 7.4 and 7.5).

7.3.2.4. Biochemical evaluations

Different serum biochemical parameters of male and female mice of the treated groups are represented in Tables 7.6 and 7.7 respectively. In SDEA high dose treated male mice, the liver enzymes like serum glutamate-oxaloacetate transaminase (SGOT) and Alkaline phosphatase (ALP) were found to be decreased significantly compared to the vehicle controls. No significant variations were found in the other biochemical parameters analysed in both high and low dose SDEA treated mice from both sexes. In VCEA high dose treated male mice, ALP and total cholesterol levels were significantly decreased (p <0.01) at both dosages and triglycerides and creatinine levels reduced only at high dose treatment. A significant reduction was noticed in the total cholesterol and triglyceride levels of female mice in both dosages. All other parameters showed no considerable changes in VCEA treated male and female mice.

7.3.2.5. Histopathological and necropsy evaluations

Haematoxylin and eosin stained sections of the vital organs dissected from the mice sacrificed at the end of 28 days sub-acute toxicity study showed the appearance of normal tissue architecture (Fig. 7.6 a & b). Liver sections from both male and female extract treated animals showed normal portal triads and central veins. Kupffer cells were found to be normal. Kidney sections from both sexes showed normal glomeruli and renal tubules with normal interstitial tissue. Ovary of treated mice displayed normal graafian follicles. In sections of testis, seminiferous tubules with normal sertoli cells were observed (Johnson & Varghese, 2023b). Necropsy examinations also showed no marked variations in the general morphology of various organs of treated animals from that of untreated and vehicle control animals (Figure 7.7).

7.3.3. Antitumor study using DLA cells induced solid tumour model

7.3.3.1. Effect of SDEA and VCEA on reduction of tumour volume

A significant reduction of solid tumour volume was found in the SDEA as well as VCEA treated group of mice compared to vehicle control. On the 30th day, the tumour volume of the vehicle control animals was found to be 4.81 ± 0.35 cm^3. Whereas a significant reduction (p value ≤ 0.01) in tumour volume was obtained in SDEA treated mice both in low dose (2.08 ± 0.35 cm^3) and high dose (1.48 ± 0.25 cm^3) compared to vehicle control. Similarly, tumour volume in the VCEA treated mice also reduced significantly (*p* value ≤ 0.01) towards the end of the study. The values were 2.17 ± 0.80 cm^3 and 1.69 ± 0.48 cm^3 at the doses of 25 mg/kg b. wt. and 50 mg/kg b. wt. respectively. Figure 7.8 and 7.9 depicts the variations in tumour volume estimated on treatment with SDEA and VCEA extracts respectively.

7.3.3.2. Histopathological evaluation of the tumour mass

Haematoxylin and eosin (H & E) stained sections of the tumour mass isolated from the untreated DLA tumour bearing mice showed pleomorphic, polyhedral, oval or spindle shaped cells having hyperchromatic nuclei. Mitotic cells and tumour giant cells were also found in this H & E stained sections. Tissue sections from the vehicle control also exhibited similar appearance as that of untreated animals. Tissue sections from the animals treated with the reference chemotherapeutic agent, cyclophosphamide showed extensive areas of necrosis with plenty of degenerating cells. A few degenerating tumour cells were observed in tissue sections from the SDEA low dose (50 mg/kg body weight) treated group. Tissue from high dose (100 mg/kg b.wt.) treatment of SDEA showed areas of necrotic cells (Figure 7.10). The VCEA low dose treated group exhibited extensive

necrosis of tumour cells. Only a few viable tumour cells were observed in this tissue sections. Sections from high dose treatment of VCEA showed almost clear necrosis of tumour cells with a few degenerating tumour cells (Figure 7.11).

7.3.3.3. Effect of VCEA on induction of apoptosis in DLA induced tumour

To confirm the induction of apoptotic cell death in DLA induced tumour mass, TUNEL assay was conducted in histological sections of tumour mass. Since the TUNEL assay mixture containing fluorescein isothiocyanate as fluorescent label was used in this study, green fluorescence will be the indication of topological distribution of cells experiencing early stages of apoptosis. Propidium iodide is a nuclear stain which cannot enter the cell unless its membrane integrity is lost, which occurs in the late stage of apoptosis and also in necrosis. TUNEL positive cells were detected clearly in the SDEA and VCEA treated samples compared to the untreated control sample (Figure 7.12).

7.4. Discussion

Treating illness without causing adverse or toxic effects on other parts of the body is the requirement for a successful and effective plant based therapeutic approaches. Acute toxicity studies are helpful in evaluating the adverse effects of a substance on a single high dose administration and this will also be beneficial in selecting dosages for long-term toxicity evaluations (Erhirhie et al., 2018). It was found that the single oral dosage of SDEA and VCEA at 2000 mg/kg body weight was found to be safe in terms of body weight, food and water intake and general behaviour. Mortality was not observed in both the extract treated animals. Therefore it is inferred that the LD_{50} of SDEA and VCEA will be higher than 2000 mg/kg body weight (Johnson & Varghese, 2023b). A previous study reported that the

aqueous extract from *S. dulcis* leaves are safe up to 8000 mg/kg b. wt. in Swiss albino mice (Abere et al., 2015). A study conducted in *V. cinerea* from Malaysia showed that its methanol extract was found to be safe up to 2000 mg/kg b. wt. upon a single oral dosage (Latha et al., 2010). Similarly, in the present study the single dose administration of 2000 mg/kg b. wt. of ethyl acetate extract of these two plants did not impart any toxic effects in mice.

Since clinical applications of acute toxicity data are limited, sub-acute toxicity studies have been followed to get the safe dosages which can be selected for pharmacological studies in mice. OECD guidelines for testing of chemicals Number 407 recommended that the high dose used for the 28-day sub-acute toxicity studies should be the dose which does not cause mortality and severe suffering but can induce toxic effects in animals and a subsequent lower dose (to attain the dose-response) at which no observed adverse effects are is obtained. Oral administration of SDEA at 200 mg/kg b. wt. caused a variation from the normal food (decreased) and water (increased) consumption level at the end of the study period. However this behaviour did not affect the body weight and relative organ weight of treated mice. In SDEA 100 mg/kg b.wt. treated mice, no significant alteration in food and consumption pattern was observed. Daily oral administration of sub lethal doses such as 50 mg/kg b. wt. and 100 mg/kg b. wt. were used for 28 days to investigate the toxic effects of VCEA. Body weight, relative organ weight, food and water consumption level was more or less similar in all the mice regardless of treatment.

It was noted from this study that none of the haematological parameters analysed were altered significantly due to the SDEA treatment at both the dosages, whereas haemoglobin, RBC and Packed cell volume (PCV) were found to be significantly decreased in the VCEA high-dose treated group of animals (both males

and females) when compared with the vehicle control indicating an anaemic condition (Johnson & Varghese, 2023b). However, the values of MCV, MCH and MCHC remained unchanged indicating that the VCEA administration did not affect the oxygenation to tissues (Arika & Nyamai, 2016). In an earlier study evaluating the sub-acute toxicity of methotrexate (an anticancer drug) the reduction of PCV level was correlated with the cytotoxic potentials of the drug on the hematopoietic system (Patel et al., 2014). In this study, the total WBC counts was also altered significantly upon high-dose treatment indicating the selective action of the extract on haematopoiesis. Haematological parameters seemed to be unaltered in animals that received VCEA at the dose of 50 mg/kg body weight (Johnson & Varghese, 2023b).

Liver plays a remarkable role in the drug metabolism. Damage to the liver will be reflected in the blood biochemical parameters. Increased levels of the enzymes SGOT, SGPT and ALP in the serum are excellent markers of liver injury (Giannini et al., 2005). Notably, in the current study with both sexes, SDEA and VCEA extracts did not cause any significant increase in serum levels of these enzymes, slight decrease was observed with the high dose group of animals. Lipid profiles of the animals were not varied significantly in both male and females treated with SDEA. But, in VCEA treated male and female mice cholesterol and triglyceride levels were found to be decreased. The reduction in cholesterol and triglycerides levels can be considered as the potential cardioprotective properties of *V. cinerea*. Previously, a species from the same genus called *Vernonia calvoana* was reported to exert lipid-lowering and cardioprotective properties in Wistar rats (Egbung et al., 2017). Urea and creatinine levels in serum is a useful indicator of renal functioning. It was noted that no increase occurred in the level of these

biomarkers in the SDEA and VCEA-treated mice of both sexes indicating the renal functioning was not affected by the extracts administration. However a slight decrease in serum creatinine level was recorded in VCEA treated males, which is a sign of muscle wastage (Perrone et al., 1992). But the reported body weight gain in these animals does not support muscle wastage (Johnson & Varghese, 2023b).

Microscopic examination of all the H & E stained sections of organs from the treated mice showed normal tissue architecture. Histopathological findings as well as the necropsy examinations confirm the absence of toxic effects on administration of SDEA and VCEA at the dosages used. Hence it is inferred from this sub-acute toxicity profiling data that SDEA (100 mg/kg b. wt. or below) and VCEA (50 mg/kg b. wt. or below) administration does not impart any toxic effects in mice. Since high doses used in the experiments showed variations in some of the parameters studied, these values were omitted for further studies in mice evaluating their pharmacological potentials.

Tumour reduction efficacy of SDEA and VCEA extracts was acceptably revealed in the DLA induced solid tumour model. Oral administration of SDEA extract (at the doses of 50 mg/kg b. wt. and 100 mg/kg b. wt.) and VCEA extract (at the doses of 25 mg/kg b. wt. and 50 mg/kg b. wt.) caused significant reduction ($p < 0.01$) in the DLA induced solid tumour mass. Besides, these doses are well tolerated by the animals during the entire experimental period (30 days) while maintaining the antitumor efficacy. Cyclophosphamide, (our reference drug) is a chemotherapeutic drug used in the treatment of various kinds of cancer, especially lymphoma, exerts its anti-neoplastic effects by alkylation of DNA. Several adverse effects like bladder and gonadal toxicity, secondary malignancies and myelosuppression are often reported on its use in chemotherapy (Ogino & Tadi, 2023). The present study

unveils the potent tumour reduction capacity of ethyl acetate extract of the plants under this study without causing serious side effects in animals.

In vitro studies have undoubtedly proven the apoptosis and anti-cell proliferative properties of the ethyl acetate extracts of SDEA and VCEA towards lymphoma cells. This was further confirmed in mice models in this study. TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labelling) assay is a suitable and well established fluorescence assay for the in situ detection of apoptosis in tissue sections by labelling with the fluorochromes (King, 2007). Fragmentation of DNA into discrete lengths is a key characteristic of cells experiencing apoptosis. In this assay, the green fluorescence obtained is parallel to the extent of apoptosis occurring in cells. The sections were also counter stained with PI to observe the late apoptosis or necrosis. Samples from both SDEA and VCEA high dose treated mice expressed remarkable green fluorescence compared to that of vehicle control indicating the prevalance of cells experiencing early to middle stages of apoptosis. The popular clinically used chemotherapeutic drugs exploit the intact apoptotic signalling pathways to induce cancer cell death by targeting different signalling molecules. Since evasion from apoptosis is a hallmark of cancer, apoptotic proteins are important therapeutic targets and the apoptosis reactivating strategies specifically in cancer cells by natural compounds is a trustful area in discovering drugs for cancer treatment (Pistritto et al., 2016). The potentials of *S. dulcis* and *V. cinerea* ethyl acetate extracts in reducing the tumour burden by triggering apoptosis in cancer cells is clearly evident in this study and are attributed to the biologically active phytocompunds present in these extracts.

Table 7.1 - Acute toxicity analysis - Effect of SDEA and VCEA on food and water consumption of mice				
Treatments	Food consumption (g/group of mice/day)		Water consumption (ml/group of mice/day)	
	Week 1	Week 2	Week 1	Week 2
Vehicle control	10.26 ± 0.73	11.20 ± 0.57	5.33 ± 0.33	6.00 ± 0.58
VCEA (2g/kg b.wt.)	10.03 ± 0.70	10.26 ± 0.43	5.55 ± 0.60	7.66 ± 0.66
SDEA (2g/kg b.wt.)	10.00 ± 0.40	10.05 ± 0.12	7.60 ± 0.48	7.75 ± 0.20
Data are presented as the mean ± SEM. Comparisons were made between vehicle control with the treated groups separately				

Table 7.2 - Acute toxicity analysis - Effect of SDEA and VCEA on general behaviour of mice			
Observations	Vehicle control	SDEA (2g/kg b.wt.)	VCEA (2g/kg b.wt.)
Food intake	Normal	Normal	Normal
Water intake	Normal	Normal	Normal
Body weight	Normal	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
Death	Alive	Alive	Alive

Table 7.3: The effect of SDEA and VCEA administration on relative organ weight (g %) of Swiss albino mice in the sub-acute toxicity study

Parameters	Untreated control	Vehicle control (Sunflower oil)	SDEA 100 mg/kg b.wt.	SDEA 200 mg/kg b.wt.	VCEA 50 mg/kg b.wt.	VCEA 100 mg/kg b.wt.
Males						
Liver	4.86±0.40	4.33±0.25	4.83±0.32	4.67±0.66	4.60±0.24	4.68±0.17
Kidney	1.43±0.09	1.47±0.09	1.63±0.09	1.53±0.09	1.47±0.11	1.45±0.10
Spleen	0.40±0.05	0.35±0.00	0.42±0.04	0.35±0.08	0.34±0.07	0.31±0.03
Brain	0.99±0.10	1.27±0.08	1.25±0.02	1.14±0.11	1.15±0.09	1.24±0.19
Lungs	0.53±0.03	0.69±0.09	0.94±0.17	0.59±0.05	0.51±0.08	0.54±0.03
Stomach	0.72±0.04	0.71±0.03	0.83±0.06	0.90±0.21	0.79±0.16	0.78±0.07
Intestine	4.15±0.45	4.00±0.21	3.76±0.70	4.54±0.91	4.08±0.45	3.79±0.34
Heart	0.44±0.00	0.44±0.02	0.49±0.02	0.46±0.02	0.41±0.06	0.41±0.05
Testis	0.58±0.04	0.53±0.04	0.57±0.03	0.57±0.02	0.50±0.11	0.56±0.01
Females						
Liver	4.31±.038	4.50±0.10	4.32±0.24	4.68±0.46	4.04±0.26	4.31±0.12
Kidney	1.07±0.09	1.11±0.38	1.17±0.02	1.16±0.71	1.23±0.08	1.22±0.04
Spleen	0.32±0.06	0.36±0.08	0.41±0.07	0.47±0.11	0.49±0.11	0.38±0.01
Brain	1.27±0.02	1.36±0.05	1.54±0.09	1.43±0.09	1.24±0.11	1.16±0.08
Lungs	0.58±0.07	0.50±0.01	0.59±0.05	0.60±0.11	0.64±0.05	0.62±0.06
Stomach	0.74±0.08	0.78±0.08	0.90±0.05	0.76±0.11	0.70±0.02	0.73±0.01
Intestine	3.70±0.36	4.60±0.29	4.21±0.28	4.07±0.33	4.27±0.62	5.33±0.30
Heart	0.42±0.06	0.36±0.02	0.43±0.02	0.38±0.44	0.41±0.05	0.41±0.01
Ovary	0.09±0.03	0.07±0.01	0.08±0.01	0.11±0.03	0.09±0.03	0.10±0.02

Data are presented as the mean ± SEM. Comparisons were made between vehicle control with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$

Table 7.4: Evaluation of haematological parameters in male mice on administration of VCEA and SDEA						
Parameters	Untreated control	Vehicle control (Sunflower oil)	SDEA 100 mg/kg b.wt.	SDEA 200 mg/kg b.wt.	VCEA 50 mg/kg b.wt.	VCEA 100 mg/kg b.wt.
Hb(g/dl)	14.67±0.35	15.17±0.27	15.70±0.12	15.23±0.56	14.07±0.48	14.00±0.06**
RBC(10^6/cu mm)	8.07±0.29	7.73±0.27	8.20±0.25	7.90±0.51	7.13±0.15	6.97±0.03*
MCV(10^6/cu mm)	59.00±1.53	62.00±0.58	63.67±0.88	60.33±1.45	59.67±1.20	62.67±0.88
MCH(10^6/cu mm)	18.33±0.33	19.33±0.33	19±0.58	19.00±1.00	19.00±0.00	19.67±0.33
MCHC(10^6/cu mm)	31.33±0.33	32±0.58	30.33±0.88	30.33±0.88	33.00±0.00	32.00±0.58
PCV(10^6/cu mm)	47.67±0.67	48.33±1.76	51.67±0.88	47.00±3.61	44.00±3.06	43.33±0.88*
WBC(10^3/ cu mm)	7.56±0.52	7.83±0.60	7.50±0.45	7.56±0.45	6.16±0.66	5.63±0.84*
Neutrophils (%)	17.0±4.16	11.33±0.33	12.67±0.88	13.33±0.88	13.33±0.88	12.67±0.88
Lymphocytes (%)	77.67±3.76	83.33±1.76	80.33±0.88	80.67±0.88	81.33±1.86	79.33±0.88
Eosinophils (%)	4.67±0.33	3.33±0.33	4.33±0.33	3.33±0.88	4.67±0.67	4.67±0.67
Platelet(10^5/cu mm)	11.0±0.80	10.23±0.20	11.63±1.51	10.67±0.67	9.07±1.01	10.80±0.78
Data are presented as the mean ± SEM. Comparisons were made between vehicle control with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$ (**).						

Table 7.5: Evaluation of haematological parameters in female mice on administration of VCEA and SDEA						
Parameters	Untreated control	Vehicle control (Sunflower oil)	SDEA 100 mg/kg b.wt.	SDEA 200 mg/kg b.wt.	VCEA 50 mg/kg b.wt.	VCEA 100 mg/kg b.wt.
Hb(g/dl)	14.33±0.04	13.90±0.21	13.30±0.32	13.37±0.39	14±0.29	12.83±0.12**
RBC(10^6/cu mm)	8.13±0.16	8.50±0.06	8.13±0.33	7.97±0.26	8.53±0.15	8.03±0.03**
MCV(10^6/cu mm)	56.67±0.41	56.33±0.33	57.67±0.88	56.67±0.33	55.33±0.33	55.33±0.88
MCH(10^6/cu mm)	17.67±0.41	16.33±0.33	16.33±0.88	18.33±0.88	15.67±0.33	15.33±0.33
MCHC(10^6/cu mm)	31.00±0.00	28.67±0.33	29±0.58	29.33±0.33	29.67±0.33	29.00±0.58
PCV(10^6/cu mm)	46.33±0.41	47.33±0.33	46±0.58	45.33±0.88	47.00±0.58	44.00±0.58**
WBC(10^3/ cu mm)	8.70±0.20	8.83±0.16	8.5±1.52	9.03±2.0	8.53±0.57	7.86±0.36*
Neutrophils (%)	14.33±0.41	15.00±1.15	16±1.00	17.67±0.88	15.33±0.33	15.67±0.88
Lymphocytes (%)	80.33±1.12	80.33±0.88	82±0.58	79.00±0.58	80.33±0.33	78.00±1.15
Eosinophils (%)	5.00±0.82	4.33±0.33	4±0.58	4.33±0.33	4.00±0.58	5.33±0.67
Platelet(10^5/cu mm)	10.47±0.73	8.96±1.01	9.17±0.03	10.43±0.78	9.17±0.33	9.33±0.09
Data are presented as the mean±SEM. Comparisons were made between vehicle control with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$.						

Table 7.6: Biochemical parameters from male Swiss albino mice in the sub-acute toxicity study with the administration of SDEA and VCEA						
Parameters	Untreated control	Vehicle control (Sunflower oil)	SDEA 100 mg/kg b.wt.	SDEA 200 mg/kg b.wt.	VCEA 50 mg/kg b.wt.	VCEA 100 mg/kg b.wt.
Liver function						
SGOT (IU/L)	246.67±17.64	304.33±7.54	274.33±12.86	191.00±20.98**	268.33±27.88	263.67±10.17
SGPT (IU/L)	53.67±2.40	68.00±3.79	59.67±1.45	63.00±5.29	57.33±3.76	62±4.00
ALP (U/L)	105.33±1.45	190±0.58	184.00±3.06	135.33±19.81*	184.1±0.58	137.67±8.67**
TB (mg/dL)	0.27±0.03	0.33±0.03	0.33±0.03	0.33±0.03	0.40±0.00	0.33±0.03
TP (g/dL)	6.10±0.10	6.47±0.18	6.17±0.07	6.50±0.10	6.03±0.29	6.20±0.00
Albumin (g/dL)	3.20±0.06	3.30±0.06	3.13±0.09	3.20±0.12	3.23±0.15	3.33±0.03
Globulin (g/dL)	2.97±0.03	3.13±0.13	3.13±0.03	3.20±0.12	2.93±0.26	2.87±0.03
Lipid profile						
Cholesterol (mg/dL)	146.33±20.17	150.67±3.48	158.33±3.28	161.00±6.66	135±0.58**	132.67±1.45**
Triglycerides (mg/dL)	180±15.82	173.33±9.39	159.33±4.70	156.67±5.70	141.67±20.76	113.67±25.67*
HDL (mg/dL)	40.33±0.88	38.33±0.33	40.67±1.45	39.67±0.88	39.35±0.33	39.67±0.88
LDL (mg/dL)	87±17.39	66.33±3.53	79.33±5.49	72.67±1.20	69.67±1.45	68±4.58
VLDL (mg/dL)	19±2.08	30.33±1.45	25.67±2.03	27.00±1.53	25.67±2.33	24±3.79
Kidney function						
Urea (mg/dL)	46.33±4.04	43±0.82	43.67±0.33	45.33±2.03	39.67±2.03	40±1.53
Creatinine (mg/dL)	0.55±0.00	0.54±0.02	0.52±0.00	0.53±0.01	0.48±1.63*	0.47±0.03*
Serum Electrolytes						
Na^+ (mmol/L)	147.33±0.33	143.67±0.33	144.67±0.88	148.67±2.85	144±0.53	145.33±1.20
K^+ (mmol/dL)	9.27±0.32	8.87±0.35	8.70±0.26	9.03±0.55	8.57±0.41	8.10±0.30
Cl^- (mmol/dL)	105.67±0.33	101±0.00	101.33±0.67	102.00±1.00	100.33±0.88	100.33±0.33
HCO_3^- (mmol/dL)	28.33±0.33	23.67±0.33	24.33±0.33	25.33±0.88	24.67±0.88	25.33±1.20
Data are presented as the mean ± SEM. Comparisons were made between vehicle control with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$.						

Table 7.7 Biochemical parameters from female Swiss albino mice in the sub-acute toxicity study with the administration of SDEA and VCEA						
Parameters	Untreated control	Vehicle control (Sunflower oil)	SDEA 100 mg/kg b.wt.	SDEA 200 mg/kg b.wt.	VCEA 50 mg/kg b.wt.	VCEA 100 mg/kg b.wt.
Liver function						
SGOT (IU/L)	288.67±6.12	218±6.00	274.33±39.07	252.67±18.84	230.33±9.91	240.33±55.83
SGPT (IU/L)	82.33±3.27	62.33±0.67	67.00±2.52	63.67±4.18	76.33±9.33	70.33±5.17
ALP (U/L)	185.33±3.67	105.33±12.67	120.00±10.00	122.00±1.15	141.67.±12.33	127.0±0.58
TB (mg/dL)	0.23±0.4	0.30±0.00	0.40±0.06	0.27±0.03	0.33±0.03	0.27±0.03
TP (g/dL)	6.63±0.04	6.83±0.17	6.37±0.31	6.87±0.03	6.67±0.12	6.87±0.12
Albumin (g/dL)	3.37±0.04	3.13±0.07	3.30±0.06	3.20±0.00	3.27±0.07	3.23±0.03
Globulin (g/dL)	3.33±0.08	3.70±0.10	3.50±0.20	3.60±0.06	3.50±0.20	3.60±0.10
Lipid profile						
Cholesterol (mg/dL)	109±2.90	151.00±4.88	153.67±4.48	161.50±2.86	135.00±0.81*	132.5±2.04**
Triglycerides (mg/dL)	114.67±0.88	175.00±13.07	163.33±4.41	156.00±4.90	147.50±28.17*	113.67±25.67*
HDL (mg/dL)	42.00±0.58	38.50±0.41	41.00±1.53	40.50±2.04	39.50±0.41	39.5±1.22
LDL (mg/dL)	45.00±2.31	69.50±2.04	65.67±2.60	56.50±4.95	69.50±8.16	69.5±6.13
VLDL (mg/dL)	22.00±1.00	30.50±2.04	25.33±3.53	28.00±1.63	26.00±3.27	23.5±5.31
Kidney function						
Urea (mg/dL)	46.33±7.34	46.00±3.27	40.67±2.33	43.00±1.15	42.67±3.27	42.50±2.04
Creatinine (mg/dL)	0.55±0.00	0.52±0.02	0.53±0.01	0.53±0.01	0.53±0.02	0.52±0.00
Serum Electrolytes						
Na^+ (mmol/L)	141.33±1.88	148.67±0.88	146.67±0.88	150.00±3.61	147±0.58	147.67±1.20
K^+ (mmol/dL)	9.40±0.12	8.80±0.31	8.33±0.60	9.60±0.31	9.47±0.09	8.83±0.18
Cl^- (mmol/dL)	109±0.58	105±1.15	103.00±0.58	106.00±0.58	107±1.00	106±1.15
HCO_3^-(mmol/dL)	27±0.58	28±0.58	26.33±0.67	29.00±0.58	27.33±0.33	26.67±0.33
Data are presented as the mean ± SEM. Comparisons were made between vehicle control with the treated groups separately. The symbol (*) represents statistical significance at $p \leq 0.05$, (**) represents statistical significance at $p \leq 0.01$.						

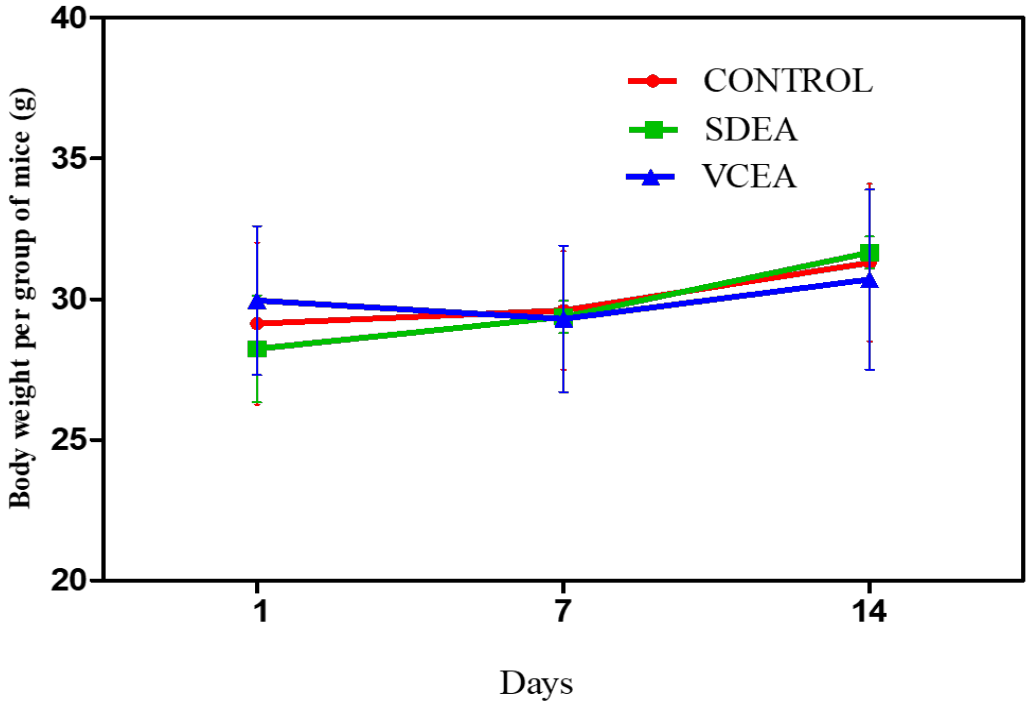

Figure 7.1: Graph showing the effect of SDEA and VCEA extracts on body weight of Swiss albino mice after single acute dose (2000 mg/kg b.wt.) administration. Control - Vehicle solvent (sunflower oil); SDEA - *S. dulcis* Ethyl acetate extract; VCEA - *V. cinerea* Ethyl acetate extract.

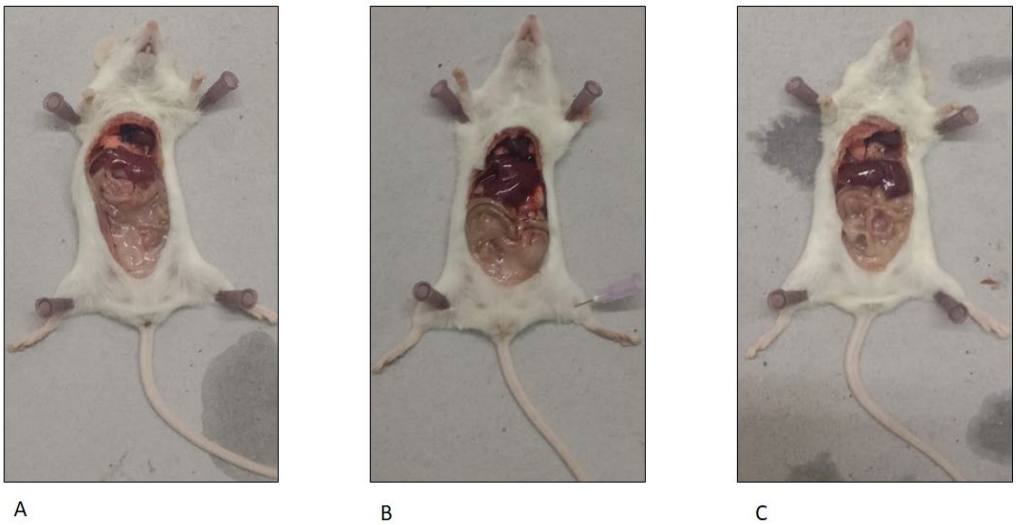

Figure 7.2: Effect of SDEA and VCEA on morphology of various organs of Swiss albino mice observed on 14th day post single acute dose (2000 mg/kg b.wt.) administration. A - Vehicle control; B - SDEA extract; C- VCEA extract.

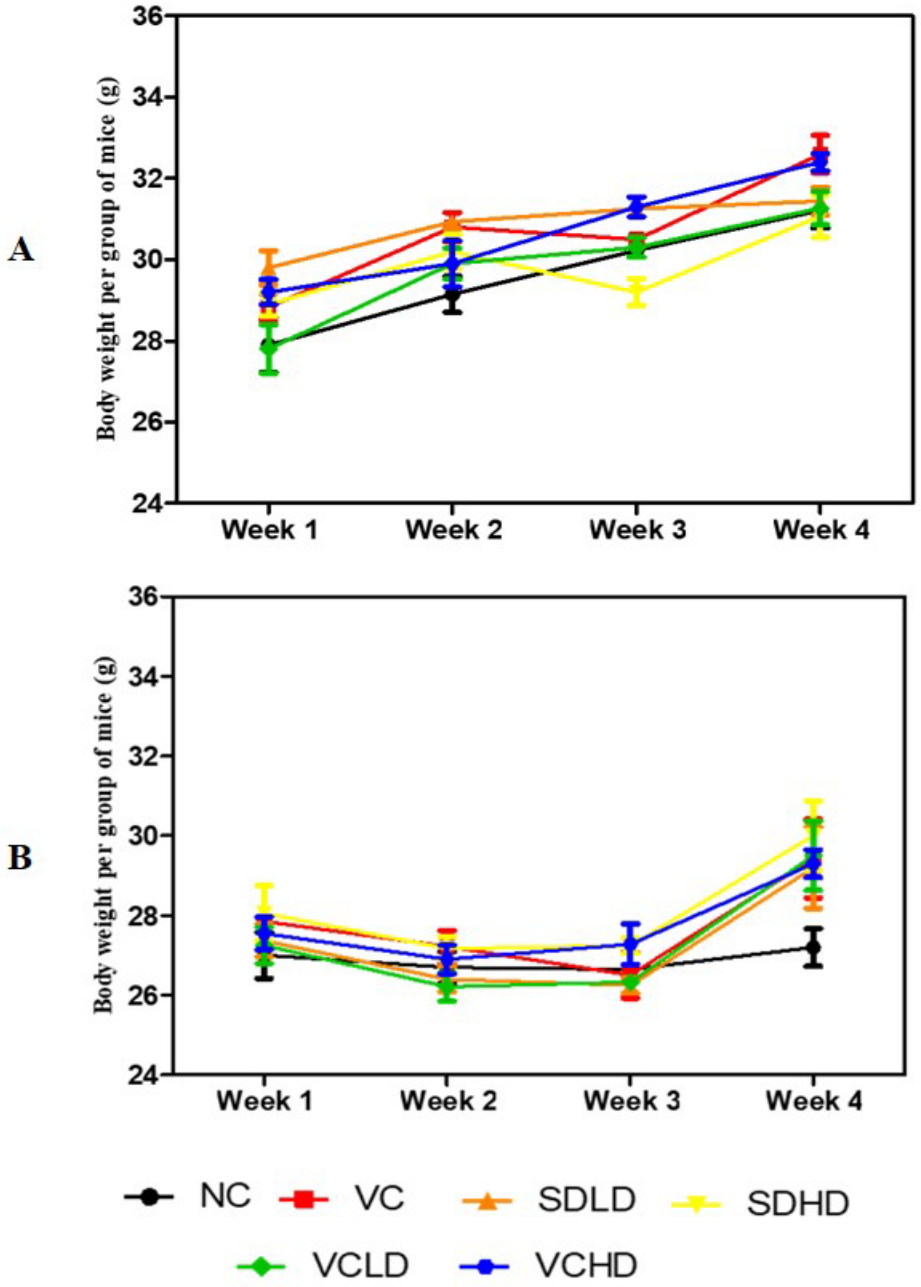

Figure 7.3: Graph showing changes in body weight of Swiss albino mice during 28 days consecutive extract administration. The average body weight per group of mice was recorded at three days interval during the treatment period and averages were calculated for each week. Data are represented as mean ± SEM. A – Male mice; B – Female mice. NC represents negative control (untreated), VC - vehicle control, SDLD - *S. dulcis* low dose (100 mg/kg b.wt.), SDHD – *S. dulcis* high dose (200 mg/kg b.wt.), VCLD – *V. cinerea* low dose (50 mg/kg b.wt.) and VCHD – *V. cinerea* (100 mg/kg b.wt.). Comparisons were made between vehicle control and treated group

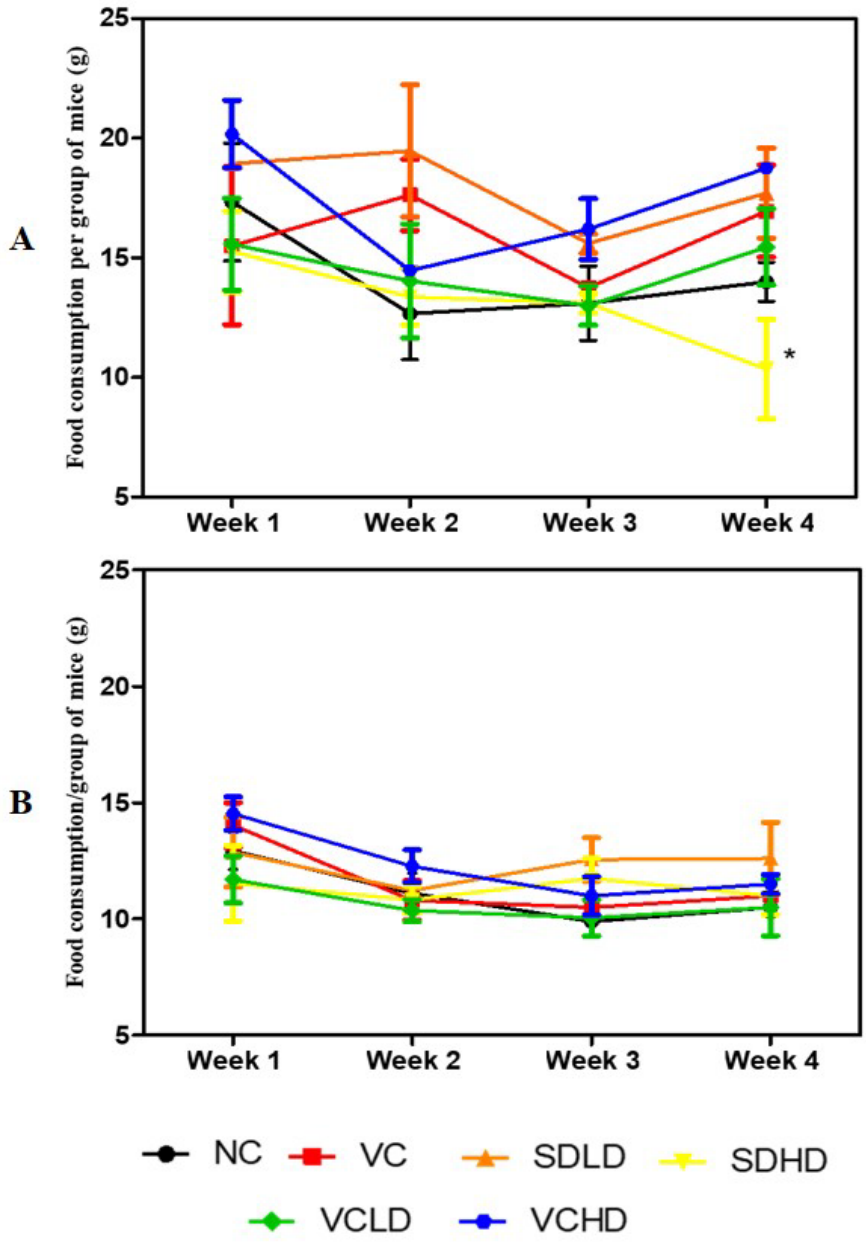

Figure 7.4: Graph showing food consumption levels of Swiss albino mice during 28 days consecutive extract administration. The average food intake per group of mice was recorded at three days interval during the treatment period and averages were calculated for each week. Data are represented as mean ± SEM. A – Male mice; B – Female mice. NC represents negative control (untreated), VC - vehicle control, SDLD - *S. dulcis* low dose (100 mg/kg b.wt.), SDHD – *S. dulcis* high dose (200 mg/kg b.wt.), VCLD – *V. cinerea* low dose (50 mg/kg b.wt.) and VCHD – *V. cinerea* (100 mg/kg b.wt.). Comparisons were made between vehicle control and treated groups. The symbol (*) represents statistical significance at $p \leq 0.01$.

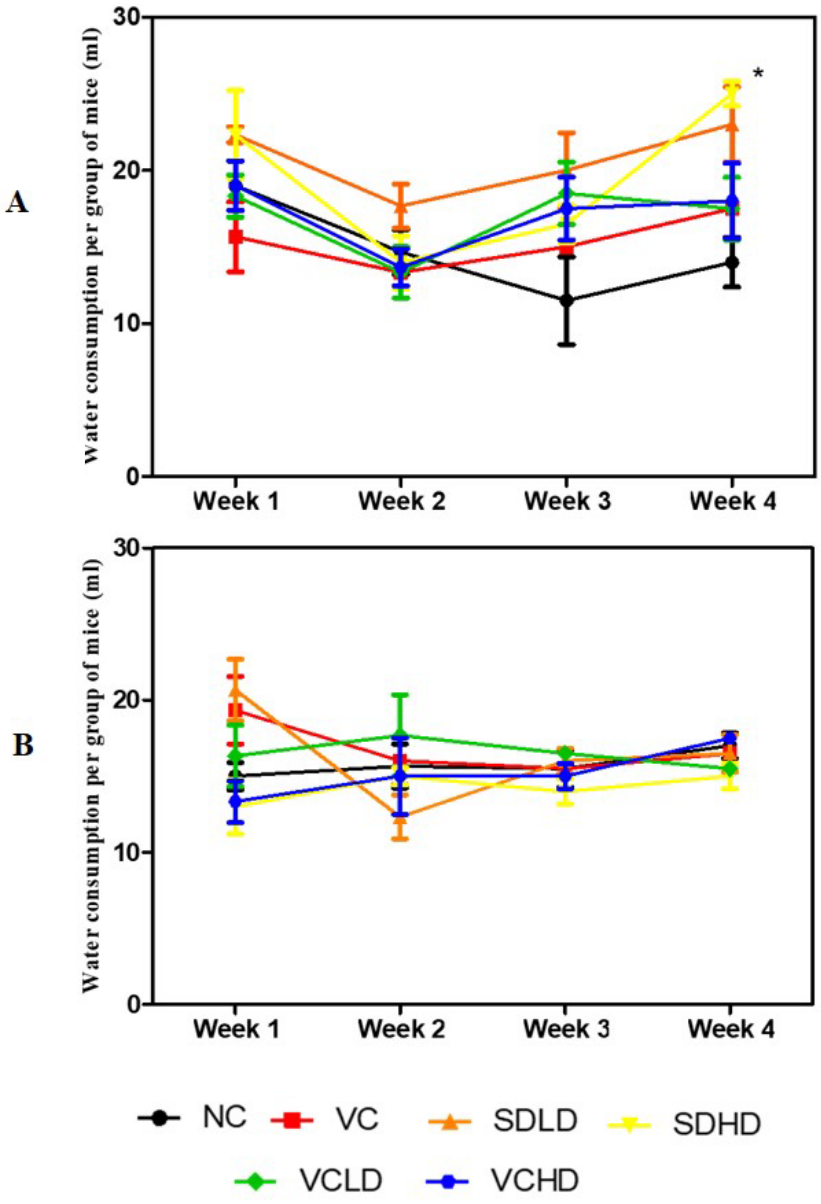

Figure 7.5: Graph showing water consumption levels of Swiss albino mice during 28 days consecutive extract administration. The average food intake per group of mice was recorded at three days interval during the treatment period and averages were calculated for each week. Data are represented as mean ± SEM. A – Male mice; B – Female mice. NC represents negative control (untreated), VC - vehicle control, SDLD - *S. dulcis* low dose (100 mg/kg b.wt.), SDHD – *S. dulcis* high dose (200 mg/kg b.wt.), VCLD – *V. cinerea* low dose (50 mg/kg b.wt.) and VCHD – *V. cinerea* (100 mg/kg b.wt.). Comparisons were made between vehicle control and treated groups. The symbol (*) represents statistical significance at $p \leq 0.01$.

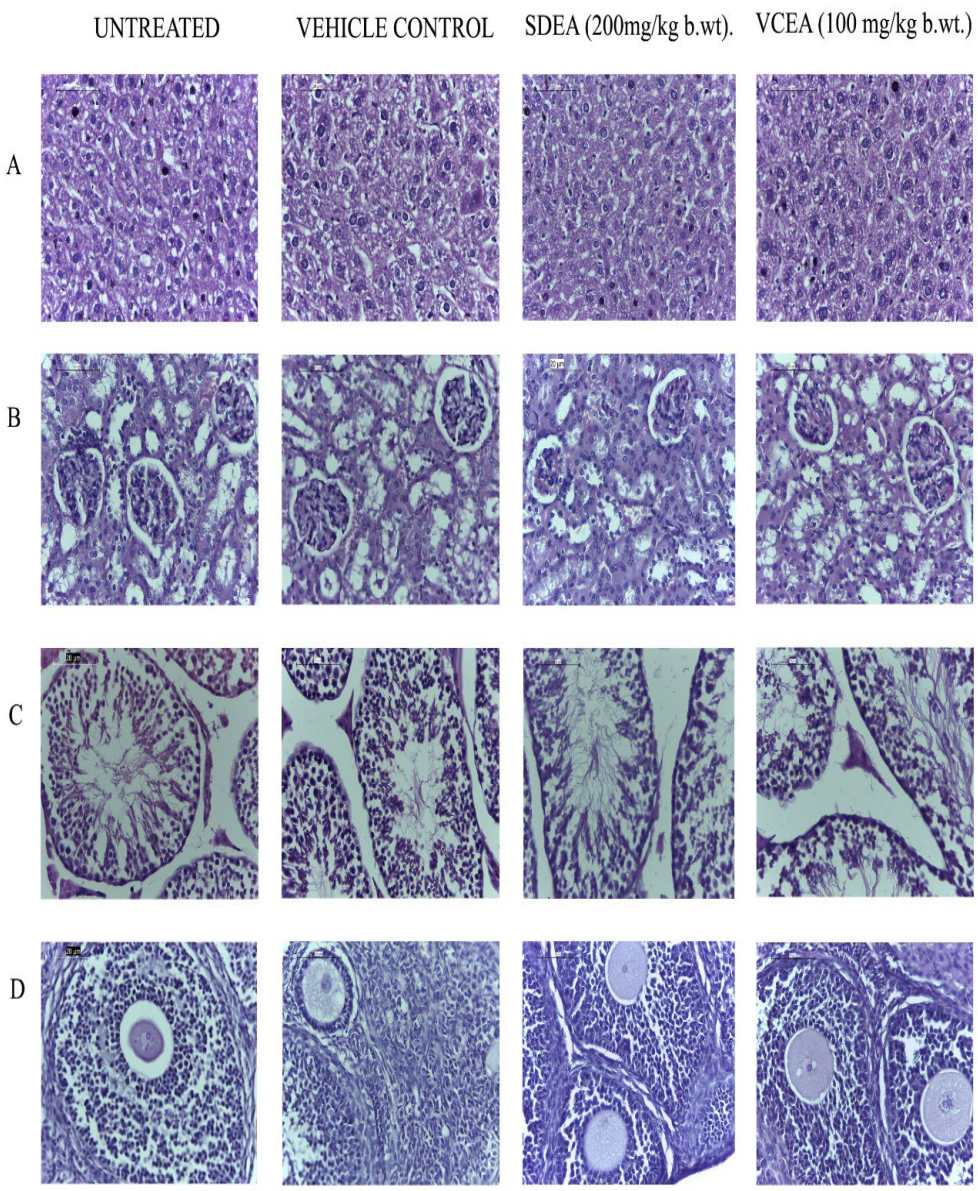

Figure 7.6a. Histopathological examination of vital organs of Swiss albino mice using haematoxylin and eosin stain (H&E) post 28 days treatment with SDEA and VCEA. A represents liver; B – kidney; C – testis; D – ovary.

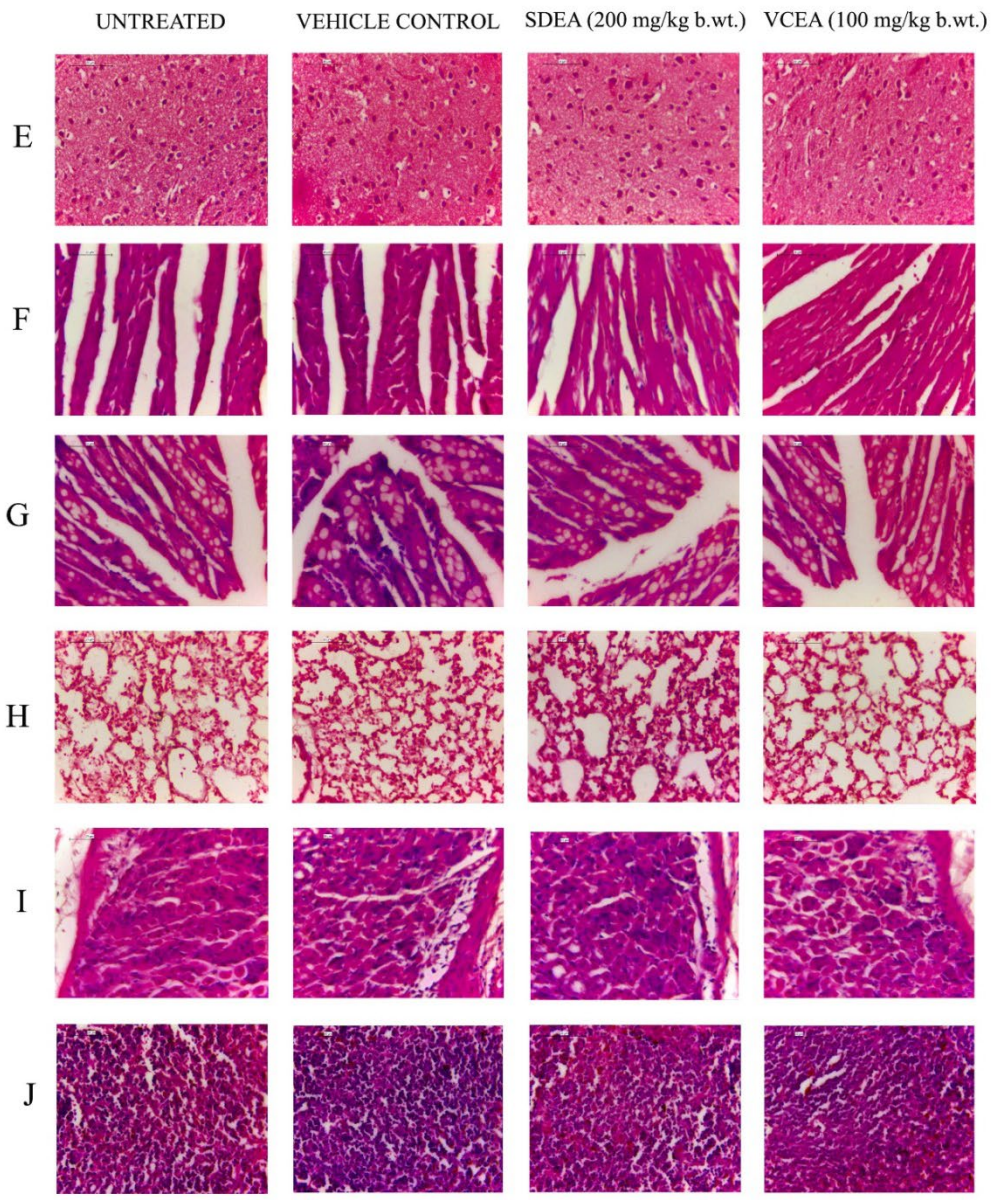

Figure 7.6b: Histopathological examination of vital organs of Swiss albino using haematoxylin and eosin stain (H&E) post 28 days treatment with SDEA and VCEA. E represents brain; F – heart; G – intestine; H – lungs; I – stomach and J - spleen

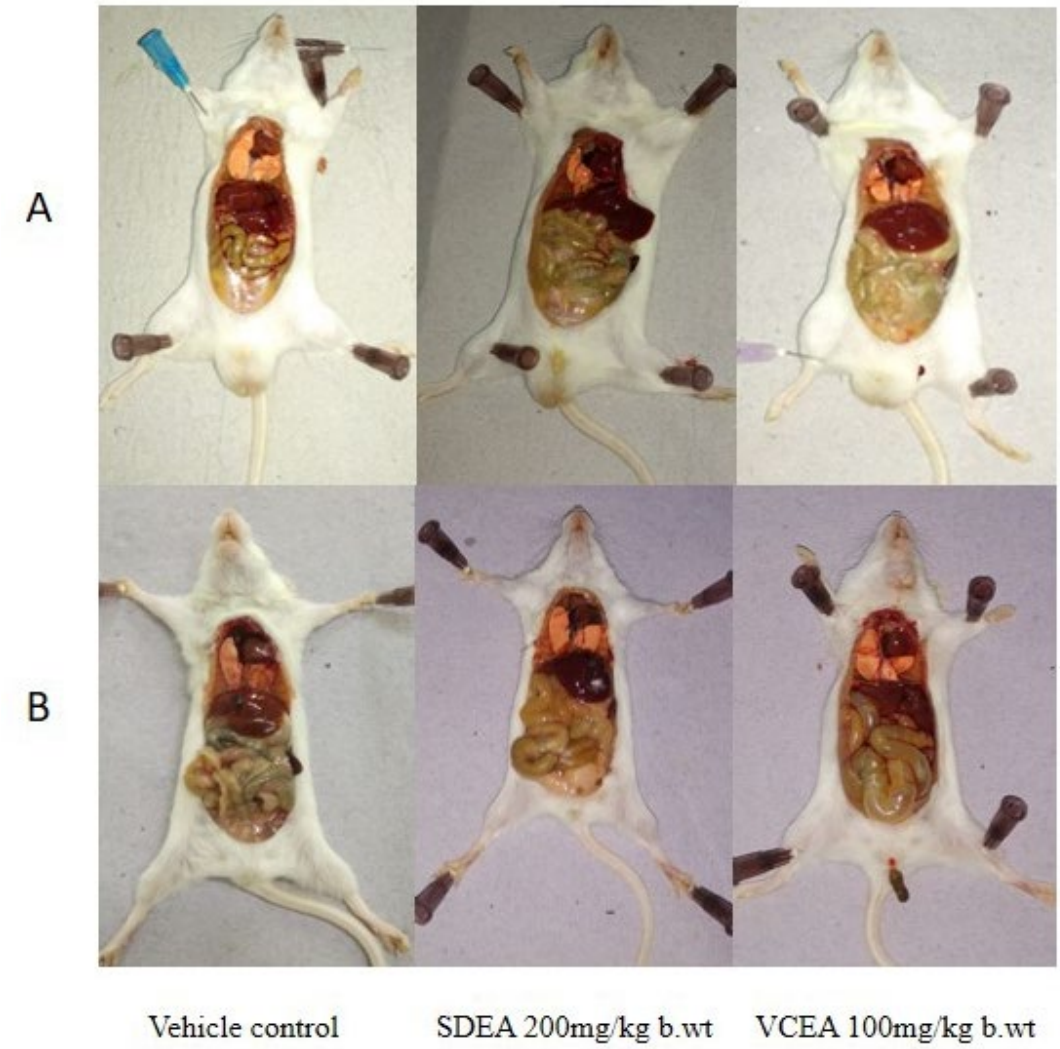

Figure 7.7: Effect of SDEA and VCEA on morphology of various organs of Swiss albino mice observed at the end of sub-acute toxicity study following consecutive plant extract administration for 28 days. A – male; B – female.

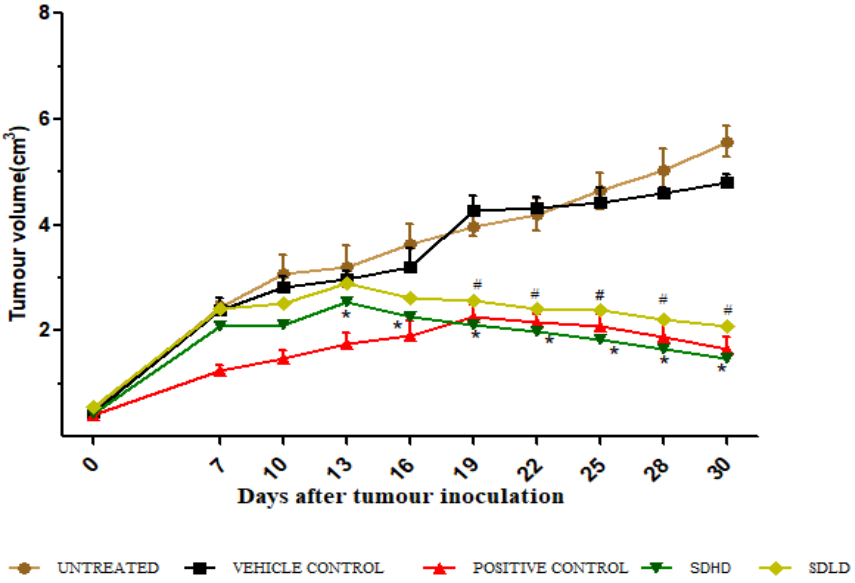

Figure 7.8: Effect of SDEA on DLA induced solid tumour model. Comparisons were made between vehicle control with SDHD (SDEA 100 mg/kg b. wt.) and SDLD (SDEA 50 mg/kg b. wt) separately at each time point. The symbol (*) represents statistical significance of SDHD at $p \leq 0.01$, (#) represents statistical significance of SDLD at $p \leq 0.01$

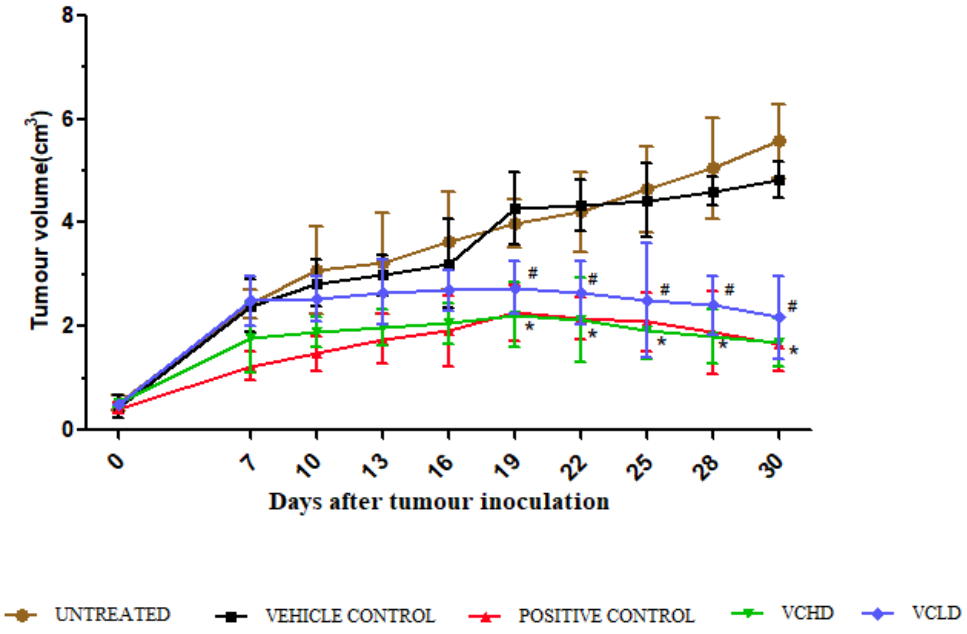

Figure 7.9: Effect of VCEA on DLA induced solid tumour model. Comparisons were made between vehicle control with VCHD (VCEA 100mg/kg b. wt.) and VCLD (VCEA 50mg/kg b. wt) separately at each time point. The symbol (*) represents statistical significance of VCHD at $p \leq 0.01$, (#) represents statistical significance of VCLD at $p \leq 0.01$.

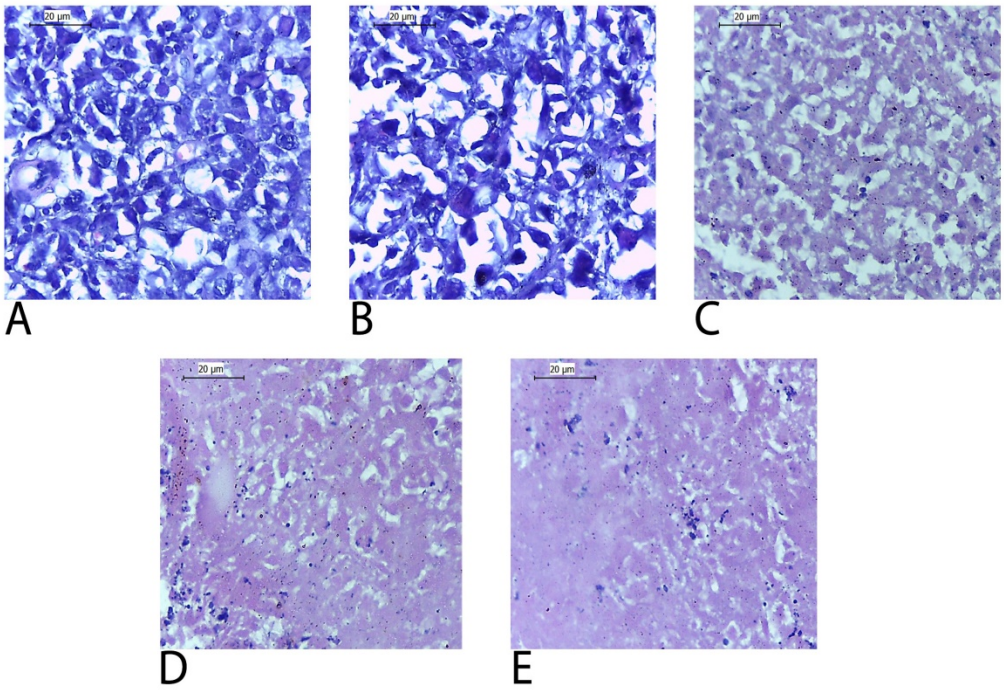

Figure 7.10: Haematoxylin and eosin stained sections of tumour mass excised from DLA induced solid tumour bearing mice treated with SDEA extract. (A) Represents control without any treatment, (B) treated with vehicle only, (C) treated with Cyclophosphamide, (D) treated with 50 mg/kg b. wt. SDEA and (E) treated with 100 mg/kg b. wt. SDEA. (Magnification ×40).

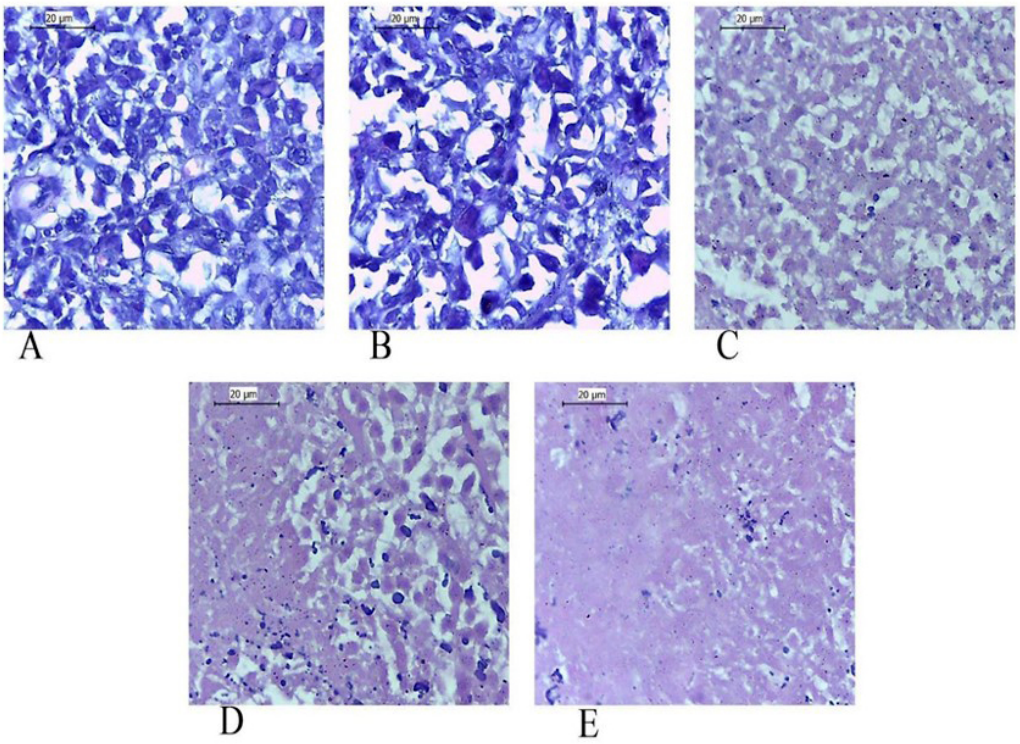

Figure 7.11: Haematoxylin and eosin stained sections of tumour mass excised from DLA induced solid tumour bearing mice treated with VCEA extract. (A) Represents control without any treatment, (B) treated with vehicle only, (C) treated with Cyclophosphamide, (D) treated with 25 mg/kg b. wt. VCEA and (E) treated with 50 mg/kg b. wt. VCEA. (Magnification ×40).

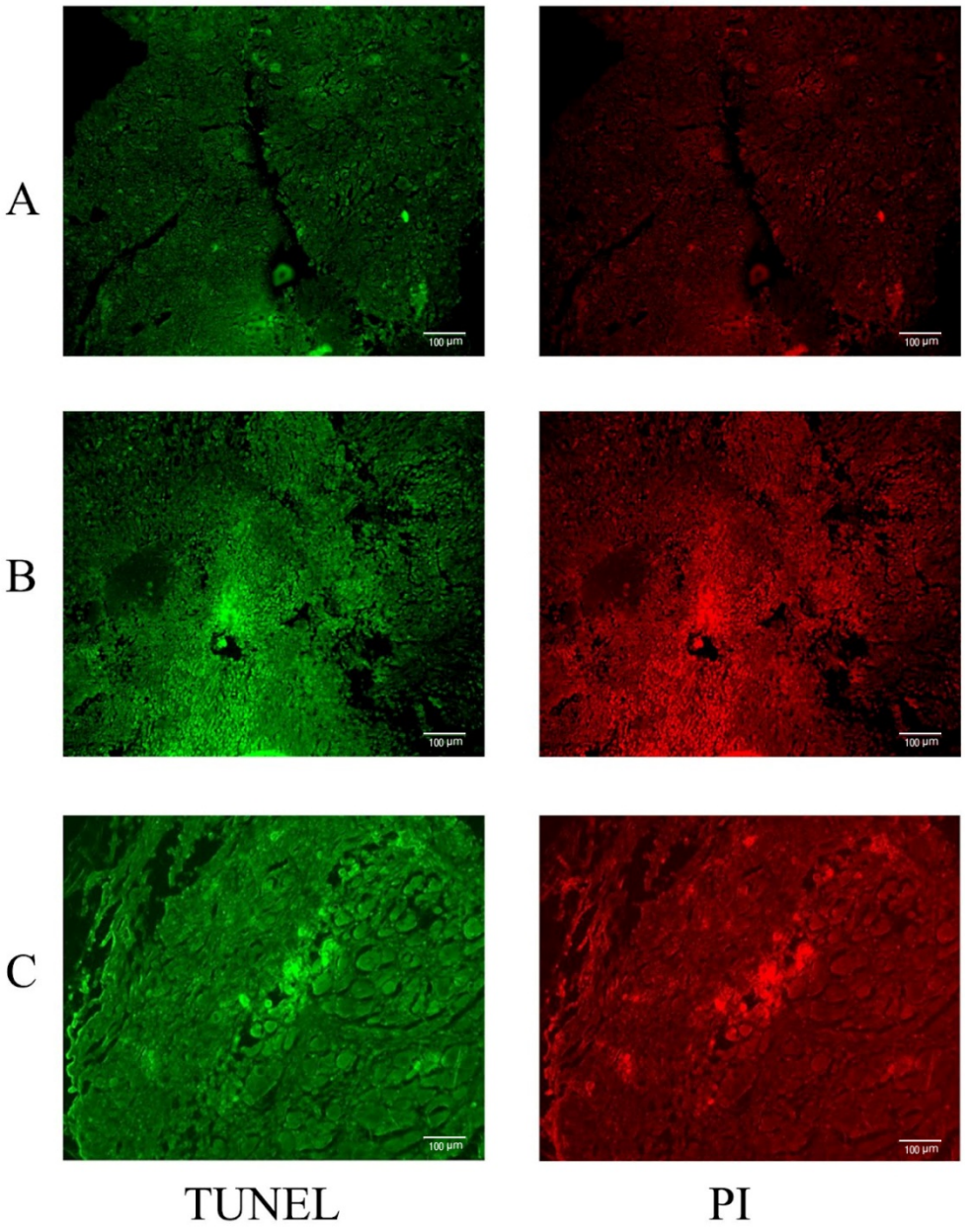

Figure 7.12: Representative pictures of TUNEL staining for apoptotic cells in DLA induced tumour tissue excised from mice treated with plant extracts. (A) represents untreated control and (B) SDEA treated at the dose of 100 mg/kg b. wt. and (C) VCEA treated at the dose of 50 mg/kg b. wt. Green fluorescence indicates early to middle stages of apoptosis with dUTP-FITC positive cells. Red fluorescence indicates late stages of apoptosis and necrosis. (Magnification ×100).

SUMMARY AND CONCLUSION

Transformation of a normal cell from mortal to immortal state is fundamentally driven by the accumulation of mutations and associated genetic or chromosomal instabilities. Deregulation of cell cycle and evasion of apoptosis are the key features of cells in this regard. There are several chemotherapeutic drugs used in clinical practices which targets these specific hallmarks of cancer cells. The limitations of currently used cancer drugs include (1) inability to discriminate normal proliferating cells from cancer cells leading to severe side effects (2) need for high dose administration of drugs in order to reach the target site and (3) multidrug resistance as a result of prolonged use of a single drug or combination of drugs with similar mode of action. Plant secondary metabolites are effective, less toxic and pharmacologically active compounds since they are co-evolved with their target sites and can elicit alternate modes of cancer cell death. Considering the promising role of phytochemicals in cancer treatment, two ethnomedically important plants found in India namely, *Scoparia dulcis* and *Vernonia cinerea* were selected in this study to evaluate their tumour reduction potentials and to delineate the underlying mechanisms using *in vitro*, *in vivo* and *in silico* approaches.

Genoprotective potentials of the plant extracts were assessed in both prokaryotic and eukaryotic cells. It was evident from Ames assay that ethyl acetate extracts of both plants are capable of exerting antimutagenic effects against the potential mutagen Sodium azide in *Salmonella typhimurium* TA 100 strains. It is inferred that it could possibly be mediated by the direct inactivation or blocking of

mutagens by extract components before their interaction with DNA. Additionally all the extracts of *S. dulcis* significantly reduced the induction of micronuclei by the Cyclophosphamide treatment in both simultaneous and post treatment protocols. However, ethyl acetate extract showed a remarkable reduction compared to others. In the case of *V. cinerea,* only the ethyl acetate extract showed a significant reduction in the number of micronuclei and that too only in simultaneous mode of treatment than others. Here also the desmutagenic action can be inferred as the predominant mechanism occurred because of the strong inhibition of micronuclei formation observed when the extract was co-treated with the cyclophosphamide rather than in the post cyclophosphamide treatment. The possibility of desmutagenic actions of these extracts by scavenging the free radicals can be excluded since they showed least activity in all the free radical scavenging assays conducted. Overall, it can be concluded from this study that the extract phytoconstituents act together in blocking the interaction of genotoxic agent with the DNA by chemical inactivation or modulation of xenobiotic metabolising enzymes. The observed reductions in the CBPI and increase in cytostasis of ethyl acetate extracts (of both plants) treatment reflects its ability to interfere with the proliferation of cells with damaged genes which in turn gives sufficient time for apoptotic or repair machinery to act further.

Cytotoxicity analysis of various organic and aqueous extracts of *S. dulcis* and *V. cinerea* revealed that ethyl acetate extracts of both plants possessed good cytotoxic potentials against lymphoma cell lines (IC_{50} value obtained for *S. dulcis* and *V. cinerea* ethyl extracts were 15.41 µg/ml and 61.24 µg/ml respectively). Hence these extracts were subjected to further anticancer studies *in vitro*. *S. dulcis* ethyl acetate extract inhibited the proliferation of lymphoma cells in a concentration and time dependent manner. It also exerted cell cycle arrest at S phase that led to

apoptotic cell death. The potential of this extract components to interfere with the cell cycle at S phase indicated their role in replication inhibition. Topoisomerase I is a known target of some clinical anticancer drugs such as Camptothecin which interfere with DNA replication in cancer cells. Therefore *in silico* studies were conducted to evaluate the binding affinities of the extract compounds identified from *S. dulcis* ethyl acetate extract. It was evident from this study that Pectolinarigenin, Scutellarein, Acacetin, Luteolin and Nepetin strongly bind to topoisomerase I - DNA complex, indicating their potential to act as topoisomerase poisons. *V. cinerea* ethyl acetate extract also inhibited cell proliferation of lymphoma cells in concentration and time dependent manner. This extract induced G2/M phase arrest leading to apoptotic cell death. Most of the agents inducing G2/M phase arrest often induce accumulation of reactive oxygen species inside cells. This prompted us to test whether the cell death induced by *V. cinerea* ethyl acetate extract within three hours of incubation in lymphoma cells is by ferroptosis (an iron and lipid peroxide mediated cell death). Since iron metabolism, lipid metabolism and antioxidant metabolisms are more active in cancer cells compared to normal cells, ferroptosis inducers can easily sensitize cancer cells specifically. Ferroportin-1 is a membrane protein involved in iron effluxing from cells to maintain iron homeostasis. Hepcidin is a protein facilitating closing of this channel protein when the iron level inside the cells are sufficient. In this study, the binding affinity of *V. cinerea* ethyl acetate extract compounds against the hepcidin binding site of ferroportin-1 was assessed by *in silico* approaches and found that Nerolidol and Luteolin strongly bind with this protein interacting site.

In vivo acute and subacute toxicity analysis of ethyl acetate extracts of these plants enabled us to select the safe dosages for further antitumour studies in mice.

Oral administration of these extracts in Swiss albino mice, bearing DLA cells induced solid tumour, resulted in significant tumour volume reduction. Histopathological analysis of tumour tissues confirmed the apoptotic phenotype indicating the proapoptotic potentials of these extract components. Since the tumour reduction potentials of *S. dulcis* and *V. cinerea* ethyl acetate extracts are attributed to their biologically active phytocompounds, further studies using these in different lymphoma preclinical models are required to promote their chemotherapeutic potentials against lymphoma.

RECOMMENDATIONS

The present study was mainly focused on the genoprotective and chemotherapeutic potentials of *S. dulcis* & *V. cinerea* crude extracts and their mechanism of action preferably attributing to their phytoconstituents. Several pharmacologically relevant phytochemicals have been identified from the ethyl acetate extract of these plants. Several other compounds including Pectolinarigenin, Luteolin, Nepetin, Eupalitin, Tectorigenin, 5-Demethylnobiletin, D germacrene and betabisabolene from *S. dulcis* as well as Nerolidol, Genistein, Tectorigenin, Eupalitin, Nevadensin and Acevaltrate from *V. cinerea* are identified for the first time in these plants. For conducting further pre-clinical studies, bioassay guided fractionation studies and isolation of bioactive compounds using advanced chromatographic techniques are required. To test their drug likeliness, ADME studies using *in silico* methods and toxicological studies and bioavailability studies using *in vivo* models are recommended.

Ability of the compounds from ethyl acetate extract of *S. dulcis* & *V. cinerea* in protecting DNA from genotoxic actions of xenobiotics was evident in the current study indicating the potential role of these compounds in chemoprevention. Desmutgenic actions, specifically, the blocking of genotoxic agents from induction of DNA damage was speculated as the genoprotective mechanism in this study. However further studies are recommended to confirm these mechanisms using the individual extract components. DNA nicking assay and Comet assays are useful *in vitro* assays in this regard. In addition to this, *in vivo* micronucleus assay is recommended as confirmation to their genoprotective potentials.

The phytochemicals extracted using ethyl acetate was found to be capable of inducing antiproliferating effects in lymphoma cells by triggering arrest at particular

phase of cell cycle that led to apoptotic cell death of these cells. This effects of the phytocompounds present in the ethyl acetate extract of *S. dulcis* & *V. cinerea* was also involved in the reduction of solid tumour volume in experimental mice indicating their potential in alleviating tumour burden. The tumour reduction potential of these extracts can also be done using different human lymphoma cell lines such as Jurkat and Raji cell lines to further explore their broad anti-lymphoma activities.

The potentials of SDEA constituents in inhibiting DNA topoisomerase -1 was determined by *in silico* approaches in the present study. This can be further proven by *in vitro* assays based on relaxation of supercoiled DNA using compounds having high binding affinity to topoisomerase -1. The remarkable binding affinity of Nerolidol and Luteolin (constituents of VCEA) to Ferroportin -1, a protein involved in iron metabolism was also revealed in this study. Further *in vitro* studies are required to confirm these interactions and to determine the ferroptosis inducing potentials by these interactions.

The *in vivo* acute and subacute toxicity studies conducted in swiss albino mice enabled to recommend a safe dose of the drug for antitumour studies of 30 days duration in same strain of mice. In order to confirm the non-toxic nature of the ethyl acetate extracts, studies can be undertaken in different animal models such as Balb/c mice and Wistar rats. A chronic toxicity analysis is also recommended for selecting dosages for various pharmacological studies using animals with longer duration. A significant decrease in the total cholesterol and triglycerides levels after 28 days of oral administration using *V. cinerea* ethyl acetate extract was noted in this study indicating its hypolipidemic potentials. Therefore experiments to confirm this potential can also be further explored in future.

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PUBLICATIONS

Research publications

1. **Johnson, J.,** & Varghese, L. (2024). Genoprotective potentials of two traditional medicinal plants *Scoparia dulcis* L. and *Vernonia cinerea* (L.) Less. *Journal of Applied Biology and Biotechnology. 12* (2); 167-172. DOI: 10.7324/JABB.2024.142447
2. **Johnson, J.,** & Varghese, L. (2023). Tumor Reduction Potentials of *Vernonia cinerea* Sesquiterpenes by Induction of Ferroptosis. *Journal of Herbs, Spices & Medicinal Plants*. *29* (4); 438-451. DOI: 10.1080/10496475.2023.2217491
3. **Johnson, J.,** & Varghese, L. (2023). Evaluation of acute and subacute toxicity of *Vernonia cinerea* (L.) Less using mice model. *Indian Journal of Natural Products & Resources*, *14* (2); 270-277. DOI: 10.56042/ijnpr.v14i2.4211
4. **Johnson, J.,** & Varghese, L. (2024). Phytoconstituents of *Scoparia dulcis* inhibit lymphoma progression by triggering cell cycle arrest and apoptosis. *RPS Pharmacy and Pharmacology Reports* – submitted.

Research paper presentations in National & International Seminar/Webinar

1. Presented a paper entitled "Evaluation of Antigenotoxic effects of a traditional medicinal plant" in the seminar "Recent Trends in Disease Prevention & Health Management" conducted by CSIR-NIIST on 14-15 December 2022.
2. Presented a poster entitled "Evaluation of Tumor reduction potential of *Vernonia cinerea* (L.) Less" in the 41st International Annual Conference of Indian Association for Cancer Research (IACR) conducted at Amity Institute of Molecular Medicine and Stem Cell Research, Amity University, Noida on 2nd to 5th March 2022.
3. Presented a paper entitled "Antimutagenic effects of a medicinal plant *Scoparia dulcis* L." in the International webinar and Symposium on "Trends in Modern Biology" organized by Department of Zoology, University of Calicut during 25th to 27th August 2021.
4. Presented a paper entitled "Antiangiogenic and antibacterial activities of an ayurvedic medicinal plant" in the Seminar on " Recent Trends & Challenges in Developing Green Chemical Methods for Industrial & Medicinal Applications" organized by Department of Chemistry, Christ College (Autonomous), Irinjalakuda during 19-20 February 2020.