Biospectrum analysis of allied taxa of *Nothapodytes* found in Kerala with special reference to anticancer properties

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CERTIFICATE

This is to certify that the thesis entitled ***Biospectrum analysis of allied taxa of Nothapodytes found in Kerala with special reference to anticancer properties*** is a bona-fide account of the research work carried out by Mrs. Divya Menon K under my guidance and supervision at Amala Cancer Research Centre, Amala Nagar, Thrissur and no part thereof has been presented for the award of any other degree, diploma or other similar titles and a plagiarism check is made.

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DECLARATION

I hereby declare that the thesis entitled **Biospectrum analysis of allied taxa of Nothapodytes found in Kerala with special reference to anticancer properties** is based on the original research carried out by me at Amala Cancer Research Centre, Kerala under the guidance of Dr. T. D. Babu, Associate Professor, Department of Biochemistry, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India and no part thereof has been presented for the award of any other degree, diploma or other similar titles and no plagiarism is made in the thesis.

Place: Amala Nagar
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DIVYA MENON. K.

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Last but not least, just one line:-

Ohm Namo Bhagavathae Vaasudevaaya…….

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ABBREVIATIONS

AIF	:	Apoptosis Inducing Factor
AMF	:	Active methanolic fraction of *Apodytes dimdiata*
CAM	:	Complementary and Alternative Medicine
COX	:	Cyclooxygenase
CPCSEA	:	Control and Supervision of Experiments on Animals :
CPT	:	Camptothecin
DISC	:	Death Inducing Signalling Complex
DLA	:	Daltons Lymphoma Ascites
DMBA	:	7,12-Dimethylbenz[a]anthracene
DMSO	:	Dimethyl Sulphoxide
EAC	:	Ehrlichs Ascites Carcinoma
ECM	:	Extra Cellular matrix
EEC	:	European Economic Community
EGF	:	Epithelial Growth Factor
FADD	:	Fas-Associated Death Domain
FAP	:	Familial Adenomatous Polyposis
FDA	:	Food and Drug Administration
FDG-PET	:	Fluorodeoxy glucose Positron Emission Tomography
FGF	:	Fibroblast Growth Factor
FTIR	:	Fourier Transform Infra Red
GCMS	:	Gas Chromatography Mass Spectrometry
HBV	:	Hepatitis B Virus
HCC	:	Hepatocellular Carcinoma
HCV	:	Hepatitis C Virus
HER	:	Human Epidermal Growth Factor
HPLC	:	High Perfomance Liquid Chromatography
HPTLC	:	High Performance Thin Layer chromatography
HPV	:	Human Papillomavirus
HTLV	:	Human T-Cell Leukemia Virus
IARC	:	International Agency for Research on Cancer
IP3	:	Phosphatidyl inositol-3, 4, 5

JAK	:	Janus Kinase
KVASU	:	Kerala Veterinary and Animal Sciences University
LDCT	:	Low-Dose Computed Tomography
LDL	:	Low Density Lipoprotein
LOX	:	Lipoxygenase
MDR	:	Multiple Drug Resistance
MTT	:	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide
NaF	:	Sodium Fluoride
NaN3	:	Sodium azide
NIST	:	National Institute Standard and Technology
NMR	:	Nuclear Magnetic Resonance
NO	:	Nitric Oxide
NPDA	:	4-nitro-o-phenylenediamine
NSAID	:	Non-steroidal anti-inflammatory
PAH	:	Polycyclic Aromatic Hydrocarbons
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
PDGF	:	Platelet Derioved Growth Factor
PI	:	Propidium Iodide
PKC	:	Receptor protein kinase C
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute medium
RT-qPCR	:	Quantitative Reverse Transcription PCR
SMACs	:	Small Mitochondria derived Activator of Caspases
STATs	:	Signal transducers and activators of transcription
TAMs	:	Tumour-associated macrophages
TBARS	:	Thiobarbituric Acid Reacting Substance
TCA	:	Trichloro acetic acid
TCM	:	Traditional Chinese Medicine
TLC	:	Thin Layer Chromatography
TRADD	:	TNFR-associated Death Domain
VC	:	Vehicle Control
VEGF	:	Vascular Endothelial Growth Factor
WHO	:	World Health Organisation

Introduction

INTRODUCTION

Cancer is a general term that we use for more than two hundred different types of cancer diseases. Even though there is a worldwide pursuit for the past 50 years on all the pros and cones of cancer including the involvement of various growth regulating molecules and its signaling pathways in carcinogenesis, as a matter of fact, the disease still remains a hard nut to be cracked. Although there are 100 different types of cancers, all of them share one important characteristic in common that they divide abnormally by disrupting normal growth restrictions. As normal cells transform to cancer cells, they break all the regulations of growth, and divide in an uncontrollable fashion, invading other tissues and eventually turning out to be fatal to the organism. The definition for cancer still exists as "the uncontrolled proliferation of cells" that results in tumor formation and metastasis. The causes involved in this abnormal transformation of cells are numerous. Recent studies clearly state that the accumulation of mutations by external or internal factors and further disruption of molecular signaling pathways are said to play a major role. So far, we can conclude that the cancer results not from a single event or factor, rather, a series of events are usually required for a normal cell to become malignant. As the obscurities on cancer persist, it is reported by the scientists of Johns Hopkins University School of Medicine in a leading magazine that two thirds of cancer occurs due to bad luck (Dallas, 2015).

In one of the studies conducted by WHO, it is estimated that nearly 5, 00,000 people die due to cancer in United States every year and at this rate, the disease is anticipated to become the nation's leading killer in the coming years (Cancer Facts and Figures, 2014). Approximately, 80 per 1, 00,000 populations is affected by cancer in India (population being 1.27 billion) (Ali *et al.*, 2011). Around, 8, 00,000 new cases of cancer and 5, 50,000 deaths occur each year in India. The most common cancers reported in India are lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) (Ali *et al.*, 2011).

Fortunately, the research that we have been continuing for the past few decades have helped us to get a better understanding on the disease and find some preventive and curative agents. Understanding on some exogenous and endogenous carcinogenic

chemicals and its mechanisms in carcinogenesis has been a landmark and helped to prevent the disease by reducing its consumption or exposure. Additionally, the early detection practices by diagnostics including Low-Dose Computed Tomography (LDCT) screening test, identifying BRAC1 and BRAC2 genes in breast cancer are some of the greatest achievements. Cancer immune prevention is another emerging field where the research focuses on improving the body's ability to heal and protect itself from cancer-causing agents, including infectious causes such as the human papilloma virus. Likewise, continued research globally in various angles will help us to move forward.

On human impact, cancer not only results in unpredictable death, but also emotional trauma and financial burden affects the society adversely in diverse ways. Disease management, surgery, radiotherapy and chemotherapy are the important treatment strategy for cancer patients. Chemotherapy has always been the mainstay among the treatment modalities. But, the side effects and drug resistance are the two important limitations. An economically bearable treatment for cancer has also become demanding. Rather than synthetic sources, plant sources have been considered safe because of lesser side effects and cost effectiveness. Researchers may take years to find an answer, or sometimes it might end up in another question. Nevertheless, further studies seem to be inevitable in any situation. Identifying innovative genes and pathways, diagnosing new causative factors and exploring new drug resources are some of the important approaches that are followed in cancer research.

The process of transition from a healthy cell to a cancerous one is a process that usually takes many years and may be influenced by dietary, hereditary and behavioral factors (lifestyle). Hence, chemopreventive agents can help in preventing the transformation or at least aid in slowing down the process. Plant medicines are considered always safer and better for human health than synthetic drugs, since human beings have co-evolved with plants. Moreover, the plants synthesize a variety of chemicals or compounds called 'secondary metabolites' as a part of defensive mechanisms. Some of the important secondary metabolite with biological activities are alkaloids, flavonoids, polyphenolics, terpenes etc. Considering the effectiveness and fewer side effects to human body, the search of suitable metabolites for cancer prevention as well as treatment using plants is been continued globally.

India possesses a wide variety of medicinal plants and it is one of the countries in the world blessed with incredible biodiversity. Western Ghats of India has one of the richest biodiversity of flora and fauna and is considered as a hot spot. Out of the numerous plant wealth of Western Ghats, about 50 species are found to be of high demand in folklore and other medicinal practices. *Rhincanthus nasuta*, *Momordica dioica*, *Cinnamomum zeylanicum*, *Ophiorhizza mungos* are some of the plant species that are frequently used in the treatment of cancer (Suja, 2005). Many plants in these areas are yet to be explored and studied extensively. In recent years, plants previously with unknown pharmacological activities have been extensively investigated as a source of medicinal agents. The family Icacinaceae comprising of 400 species received attention when camptothecin, an important anti-cancer alkaloid was detected in high quantities from the plant, *Nothapodytes nimmoniana* of this family. Moreover, the members of the family Icacinaceae contains much iridoid type of compounds (Harinantenainaa *et al.*, 2006) which possess anti-oxidant, anti-inflammatory, immunomodulatory and anti-tumor properties. More than 13 members of the family Icacinaceae are known for the presence of camptothecin. Considering the importance of this family, presently, an attempt was made to analyse certain unexplored members of the family Icacinaceae found in Western Ghats of Kerala. Eventhough *Nothapodytes nimmoniana* has been well investigated for its pharmacological properties, its allied taxa like *Apodytes dimidiata*, *Sarcostigma kleinii*, *Miquelia dentata* and *Gomphandra tetrandra* are yet to be explored for revealing its pharmacological activities, emphasizing on cancer. Thus, an attempt is being made through this study to find out a candidate who could probably be useful in the advancements of cancer research.

Chapter 1

Review of Literature

1.1 Introduction

Tumorigenesis is a multistep process that begins with cellular transformation which progresses to hyper proliferation and culminates to invasive with angiogenic properties and finally establishes a metastatic form (Hahn and Weinberg, 2002). The carcinogenic process is triggered by various endogenous tumour promoters or exogenous carcinogens (Yuspa, 2000). According to the survey conducted by International Agency for Research on Cancer (IARC) (expand), a 50% increase in cancer rates is expected within another 20 years.

Cancer is clonal in origin. A single abnormal cell with altered DNA undergoes a multistep mutational alterations and leads to the production of a mass of abnormal cells called malignant tumour. These primary clones of cells undergo further malignant transformation repetitively; invade adjacent tissues by breaking tissue barriers and basal membrane and spreading to the other parts of the body; the process of metastasis. Hence, the alterations of DNA by mutations are considered the prime event in the process of carcinogenesis. Usually, DNA mutations, that occurs at a frequency of one in every 20 million per gene per cell division. In the total lifetime of an individual the average number of cells produced is 10^{16} (10 million cells). Thus, it is likely that human populations anywhere across the world would also show similar frequencies. But, it is seen that the statistics is not evenly distributed. Some countries or population in certain areas are affected more with cancer than the others. Obviously we have to assume that certain genetic or environmental factors may be influencing the population. Certain populations might be carrying greater cancer susceptible genes or certain areas in which the population live would be contributing larger to the cancer incidence. Benign is considered non-cancerous and malignant is cancerous. Benign tumours rarely are life threatening and do not spread to other parts of the body and can often be removed. It continues to represent largest cause of mortality in the world and claims over 6 million. Cancer kills annually about 3500 per million populations around the world. A large number of chemo preventive agents are used to cure various cancers, but they produce side effects that prevent their extensive usage. According to World Health Organisation (WHO), 2013 it is estimated that there will be approximately 19.3 million new cancer cases per year by 2025, due to ageing and life style. It is reported that in economically developed countries, 78% of all newly diagnosed cancer cases occur at age 55 and older and 58% in case of developing countries.

The importance of medicinal plants in providing healthcare against various ailments including infectious diseases is well documented. Many medicinal herbs have been reported to possess antimicrobial, counter irritant, sedative, anti-inflammatory, and expectorant properties. Many pharmacological investigations are being carried whole over the world to identify new drugs or to find new structures for the development of novel therapeutic agents for the treatment of human diseases such as cancer and infectious diseases (Newman *et al.*, 2003). Although more than 1500 anticancer drugs are in the stages of active development, and 500 of the drugs under clinical trials, there is an urgent need to develop much effective and less toxic drugs (Pandey and Maduri, 2010).

1.2 Causes of cancer

Both genes and environmental factors are the crucial for cancer development. The statistics shows that approximately, 90% of the total cancers are caused by environmental factors. But, the aetiology of cancer is widely distributed and several exogenous and endogenous factors play a key role in carcinogenic processes (Camargo *et al.*, 1999; Gutiérrez and Salsamendi, 2001).

1.2.1 Exogenous factors

Exogenous factors include physical agents like radiation, chemical and biological agents, nutritional habits and life style (Pitot and Dragan, 1991; 1993; Lutz, 2002).

1.2.1.1 Viruses and cancer

Even though humans are infected with many viruses, only a few are known to cause cancer. DNA viruses and Retroviruses (a type of RNA virus) is known to cause human cancer (Klein, 2002).The oncogenic RNA viruses are retroviruses and able to integrate their genome with host genome which trigger oncogenesis (Klein, 2002). Human T lymphotropic Virus (HTLV-1) and Hepatitis C virus (HCV) are the examples for RNA viruses (Gallo, 2005).The papillomavirus causes genital carcinomas, hepatitis B virus causes liver carcinoma and chromosome instability (Kremsdorf, 2006), Epstein-Barr virus causes Burkitt's lymphoma and nasopharyngeal carcinoma (Ruddon, 2007), Hepatitis C Virus

(HCV) causes B cell lymphoma (Zuckerman and Zuckerman, 2002) and human T-cell leukemia virus causes T-cell lymphoma. The cancer causing viruses possess oncogenes which provide them the ability to promote cancer. These oncogenes are similar to proto-oncogenes in animals. Retroviruses acquire this proto-oncogene from the infected animal cells. c-SIS proto-oncogene is a normal gene and promotes cell growth and v-SIS, is the oncogene product. When a virus that has v-SIS infects the cells, then overproduction of growth factors occur, leading to high levels of cell growth resulting in the formation of tumour cells. Viruses can also cause cancer by inserting their DNA into the host cell chromosome. This integration mutates the proto-oncogene into an oncogene. Moreover, insertion of the virus DNA near a gene in the chromosome that regulates cell growth division results in the overexpression of the gene.

1.2.1.2 Environmental factors

Several environmental factors are involved in acquiring cancer and are considered carcinogenic when there is a correlation between exposure to an agent and the occurrence of a specific type of cancer. X-rays, UV light, tobacco products, viruses, pollutants and chemicals are the main exogenic carcinogenic factors. Non-fibrous materials are involved in sarcomas, adenocarcinomas and squamous cell carcinomas and malignant lymphomas etc (Simonato *et al.*, 1989). X-rays and other sources of radiation are also reported to be potent mutagens. Constant exposure to radiation induces free radical formation which alters the genomic stability and enhances the rate of mutations and induces chromosomal aberrations in the descendants of their radiated cell even after many generations of replication (Little, 2000). Tobacco smoke causes cancer in lung, oesophagus, bladder and pancreas and contributes to as many as half of all cancer deaths. The molecular responses in the human skin are primarily initiated by UV via photochemical generation of ROS mainly by the formation of superoxide anion, hydrogen peroxide, hydroxyl, and singlet oxygen. UV exposure to skin can form photoproduct radicals that inactivate the functions of DNA. Many chemicals are reported to be cancerous (benzene, arsenic etc) and based on the mode of action, the chemicals are classified into direct acting (do not require metabolic activation) and indirect acting carcinogens (requires metabolic activation). Some examples of direct acting carcinogens are ethyleneimine, ethylene oxide, methyl methane sulfonate,

and cyclophosphamide (Phillips *et al.*, 1997). Indirect acting chemicals produces highly reactive electrophiles from the chemicals by phase I enzymes (cytochrome P450enzymes) (Miller, 1978). Out of the total carcinogens, 95% of them fall into three major categories as proposed by Perantoni (1998) as follows

(i) Alkylating agents: Chemicals that form DNA adducts by transferring alkyl groups, often methyl or ethyl groups to nucleotides. Eg. aflatoxins, nitrosamines etc.

(ii) Alkylating agents: Chemicals that form adducts by transferring the aromatic or multi-ringed compounds to a nucleotide. Eg. Polycyclic aromatic hydrocarbons (PAH).

(iii) Aryl hydroxylamine: Chemicals that transfer aromatic amines to nucleotides and forms DNA adducts. Eg.2-napthylamines, acetamino fluorine, aniline dyes (Perantoni, 1998).

One of the best ways to prevent cancer is to understand the carcinogenicity of chemical factors and try to avoiding them. The effect of these external agents is not independent of cancer genes and it is the constant exposure to these factors that triggers the susceptible cells. Genetic variations amongst the individuals also affect the susceptibility to the carcinogenic effects of environmental agents.

1.2.1.3 Life style and food habits

The first evidence brought out linking cancer and life style was reported by an English physician, John Hill who in 1761 noted that nasal cancer is linked to the use of tobacco snuff (Hejmadi, 2010). Over a half million deaths occurring worldwide are due to the preferences and changes that occurred in the life style like obesity, lack of exercise, eating junk foods (high salt/nitrate content leads to stomach cancer), use of tobacco and alcohol (leads to mouth and throat cancer). More than 50% of all cancers are strongly associated with habits like alcoholism, smoking, tobacco and chewing (Carroll *et al.*, 1975). The body's antioxidants get depleted by the oxidants present in cigarette smoke, mainly nitrogen oxides (Lykkesfeldt *et al.*, 2000). Drinking alcohol increases the risk of bladder and liver cancer. Smoking along with alcohol consumption generates a higher risk of breast, liver, stomach and urinary bladder cancers. The high intake of red meat and low fibre diet has also been considered to be the cause of the high incidence of gastric cancer in the USA (Umadevi,

2004). A carcinogenic chemical, acrylamide is formed when carbohydrate is cooked at high temperatures and this is highly seen in potato chips and French fries (Tareke *et al.*, 2002). Regular intake of food low in fibre and rich in oil and fat increase the risk of oesophageal and stomach cancer. Initiation and progression of cancer are well aided by the exposure to carcinogens. These carcinogens can be present in the food, water, air and sunlight that we are exposed to. Only 10% of cancer is caused by genetic factors. Rest all are environmental factors, including our lifestyle (http://pdfsr.com/pdf/cell-biology-and-cancer).

1.2.2 Endogenous factors

Endogenous factors that contribute to carcinogenesis include genetic factors, chronic inflammation, hormonal factors, aging and free radicals (Cohen *et al.*, 1993, Barrett and Anderson 1993, Koivusalo *et al.*, 1994, Minamoto *et al.*, 2000, Gutierrez and Salsamendi, 2001).

1.2.2.1 Age

Even though cancer is prevalent in persons of all age groups, the statistics shows that the rate is high amongst the aged populations. Two third of the total cancer deaths and 60% of new cancers are reported in individuals above the age of 65. It is seen that the risk of caners like breast, prostate and lung increases with age. Various reasons pointed out to substantiate this fact is that as age increases, greater exposure to carcinogens might have occurred, a person's immune system gets weakened, genetic mutation gets accumulated at a higher rate and body's repair mechanisms slow down. All these factors may lead to the development of cancer.

1.2.2.2 Genetic factors

Some type of cancer occasionally run in families, either caused by genetic or environmental factors or both. Some cancers like breast, prostate and ovary have been largely attributed to the presence of specific mutant genes. Cancer, that is genetically influenced, can be divided into 3 main categories; inherited, familial and sporadic. In inherited type, there is occurrence of cancer throughout the generations. This occurs when a mutated gene gets

directly passed on from a parent to offspring. Mutations in multiple susceptible genes lead to familial type and occur more frequently than the inherited type. But, their occurrence is unpredictable since it involves multiple genes. When an individual randomly develops cancer, apart from the familial pattern, it is categorised as the sporadic type. Three of the most studied cancer involving susceptible genes is breast; prostate and colorectal. They involve genes which largely contribute to the familial aggregate forms of cancer. Hereditary cancers are primarily caused by an inherited genetic defect. It is estimated that about 0.3% of the population are carriers of a genetic mutation (Roukos, 2009). There are certain susceptible genes reported in case of inherited mutations like, mutations in the genes BRCA1 and BRCA2 increases the risk of breast cancer and ovarian cancer more than 75% and hereditary non-polyposis colorectal cancer which is present in about 3% of people with colorectal cancer (Cunningham *et al.*, 2010). p53 is another tumor suppressor gene which gets commonly mutated and in fact, more than 50% of all cancers involve a missing or damaged p53 gene. These are mostly acquired mutations and germline mutations are rare. Certain hormonal factors are also thought to be involved in the development of cancer and play a crucial role in development of about 20% of all cancers, for eg. Breast, prostate, ovary and endometrium (Henderson *et al.*, 1991; Henderson and Feigelson, 2000). Even though oestrogen is essential for the normal functioning of a woman's reproductive system and for normal breast development, it's over production is thought to be increase the risk for breast cancer.

1.2.2.3 Free radicals

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in their atomic or molecular orbitals (Halliwell and Gutteridge, 1999). Free radicals are generated when an atom or a molecule either gains or losses an electron. Free radicals are formed naturally in the body and play an important role in many normal cellular processes (http://www.cancer.gov/about-cancer/causes-prevention/risk/diet/antioxidants-fact-sheet). When the free radicals exceed the normal concentrations, they become hazardous to the body and damage DNA, proteins and cell membranes. The damage to cells caused by free radicals, especially the damage to DNA play a major role in the development of cancer and other health conditions (Diplock *et al.*, 1998, Valko *et al.*,

2007). It is studied that ionizing radiation and other environmental toxins abnormally increases the level of free radicals in the body. The ionizing radiation may cause the atom to lose an electron, forming free radicals. Some common environmental toxins such as cigarette smoke, metals etc, stimulate the production of free radicals. Free radicals that contain the oxygen are the most common type of free radicals produced in living tissue and they are commonly known as "reactive oxygen species," or "ROS". These free radicals are removed by the antioxidants, by neutralising them. The body itself has some defence mechanisms to tackle free radicals by generating endogenous antioxidants. However, we have dietary antioxidant which we obtain through food. Fruits, vegetables, and grains are rich sources of dietary antioxidants (http://www.cancer.gov/about-cancer/causes-prevention/risk/diet/antioxidants-fact-sheet). Free radicals are omnipresent in our body by normal metabolism processes and inflammatory responses and are produced as a part of defence mechanism to fight invading microorganisms (Hussain *et al.*, 2003).

Types of free radicals

Even though free radicals containing oxygen atom are said to be Reactive Oxygen Species (ROS), most free radicals are ROS such as superoxide ion, hydroxyl radical, hydrogen peroxide and singlet oxygen (http://www.phytochemicals.info/free-radicals.php).

1.2.2.3A Superoxide ion

This is an oxygen molecule with an extra electron (leaving the molecule with only one unpaired electron) and the chemical formula is O^{-}_{2} (http://www.exrx.net/Nutrition/Antioxidants/Introduction.html). The mitochondrial respiratory chain is the major source of superoxide anion radical (Sawyer, 1981) and also by several other enzymes, example xanthine oxidase (Muller *et al.*, 2007). They are deployed to evade the attacking foreign agents. In the oxygen-dependent killing mechanism employed by phagocyte, superoxide is produced enormously by the enzyme NADPH oxidase. These free radicals damage mitochondria, DNA and other biomolecules. Superoxide dismutase is the antioxidant enzyme produced inside the body to neutralize superoxide ions. Superoxide anion gains importance as the product of the one-electron reduction of dioxygen O_2.

1.2.2.3B Hydroxyl radical (**$OH^{\bullet}$**)

This is the neutral form of the hydroxide ion (OH^-) formed by the reduction of an oxygen molecule in the electron transport chain (http://www.phytochemicals.info/free-radicals.php) and by the decomposition of hydroperoxides (ROOH). Hydroxyl radicals unlike superoxide cannot be eliminated by an enzymatic reaction. The hydroxyl radical has a very short half-life (appx 10^{-9} sec) and is highly reactive (Helmut, 1993) and damages important biological molecules such as carbohydrates, DNA, lipids and proteins (http://www.phytochemicals.info/free-radicals.php; Reiter *et al.*, 1997). Hydroxyl radicals are sometimes produced as a by-product of immune defence action endogenous antioxidants such as melatonin and glutathione and dietary antioxidants like vitamin E is involved in the scavenging peroxyl radicals (Reiter *et al.*, 1995).

1.2.2.3C Singlet oxygen

Singlet oxygen is commonly referred to the particular singlet electronic excited state or the exited states of molecular oxygen (O_2), those with an electronic structure that is singlet. It can be formed during radical reactions and also cause further reactions, but not considered as a free radical. Singlet oxygen causes oxidation of LDL cholesterol. Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation (http://www.exrx.net/Nutrition/Antioxidants/Introduction.html). Singlet oxygen can interact with other molecules leading to the formation of new free radicals. Polyphenol are good scavengers of reactive oxygen species antioxidants and prevent many deleterious oxidative effects (Karp, 2005)

1.2.2.3D Hydrogen peroxide

Hydrogen peroxide is formed as a by-product of oxygen metabolism and it is involved in the production of many reactive oxygen species. It is not considered as a free radical and are neutralized by peroxidises. Hydrogen peroxide is distinctive in the fact that it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted as water (http://www.exrx.net/Nutrition/Antioxidants/Introduction.html). When H_2O_2 is converted to water, glutathione peroxidase converts glutathione to oxidized glutathione. If H_2O_2 is not converted into water, $1O_2$ is formed. The free-radical theory of aging explains how hydrogen peroxide can decompose into a hydroxyl radical and how superoxide

radical reacts with water to form hydrogen peroxide (Weindruch and Richard, 1996). The hydroxyl radicals produced by these cellular metabolism damages vital cellular components, especially mitochondria (Giorgio *et al.*, 2007). Hydrogen peroxide has been closely linked to the formation of cancer (Lopez-Lazaro, 2007).

1.2.2.3E **Reactive nitrogen species** (RNS)

They are derived from the radicals, nitric oxide ($^{\bullet}NO$) and superoxide (O_2^-) which are produced via by nitric oxide synthase 2 (NOS_2) and NADPH oxidase, respectively. They are formed when nitric oxide reacts with superoxide to form peroxynitrite ($ONOO^-$) (Squadrito and Pryor, 1998; Droge, 2002). Various amino acids react with RNS that are produced by peroxynitrite and alters cytoskeletal organization, enzymatic activity and weakens the cell signal transduction (Pathcher *et al.*, 2007).

Usually, the body maintains a strong antioxidant defence mechanism to defend the attack of free radicals. But during increased oxygen flux or stress, free radical production may destruct the balance redox status, thus resulting in lipid peroxidation. Thus, free radicals have said to play a major role in the cause of cancer and other degenerative diseases.

1.3 Hallmarks of cancer

Certain mutations in a cell causes the disruption in the cell cycle pattern, thus uncontrolled proliferation occurs, which results in tumour development. Though, we can simply explain the disease in this manner is not as simple as it seems. There are innumerable complex mechanisms in the cellular and molecular levels by which a normal cell gets transformed to a cancerous one. A cell does not perform by itself, but they rely upon certain external signals by which the whole communication is synchronised. To summarise these events, we have to look into the main changes that take place in a cell that results in cancer. Below listed some points mentioning the hallmarks of cancer.

1.3.1 Immortality

Normal cells follow a finite pattern of replication. There is an intrinsic cellular mechanism which controls the normal cells to divide a restricted number of times and prevent further replications (Hanahan and Weinberg, 2011). All the organisms do have a definite shape and

size because of this controlled division. One of the main characteristics of cancer is its ability to divide indefinitely. This possibly occurs due to certain disruptions in the internal cellular mechanisms that control cell division. In usual cases, it is seen that normal cells divide and finally the growth rates slows down and form senescent cells (cells which are alive but do not divide). The control mechanism behind the senescence is thought to be the telomeres. Telomeres protect chromosome by preventing end to end fusion. After every round of DNA replication, a short piece of the telomere is lost and the chromosomes get shorter and shorter. Finally, genetic changes occur and the cell will die. Cancer cells overcome this by activating telomerase, an enzyme that maintains telomere length (Artandi and DePinho, 2010). This over expression of telomerase protects the ends of chromosomes and allows continued cell proliferation. Recent research have indicated that telomerases are spotted with certain additional functions that aid in tumor growth such as rendering resistance to apoptosis, association with chromatin, DNA damage repair and RNA-dependent RNA polymerase function. Studies indicate that enabling immortality to cancer cells is also aided by the loss of tumour suppressor genes like p53.

1.3.2 Angiogenesis

Angiogenesis is a complex process which involves the activation, proliferation, and directed migration of endothelial cells to form. This is considered very essential for the sustained tumour growth and metastasis. Several pro-angiogenic growth factors control the signalling mechanism involved on tumour angiogenesis (Hicklin and Ellis, 2005). Vascular endothelial growth factor (VEGF) was first concerned in angiogenesis when it directed normal blood vessels to become hyperpermeable (Senger *et al.*, 1983). The angiogenic switch is turned on when pro-angiogenic factors like VEGF are over expressed (Bergers and Benjamin, 2003). Angiogenesis facilitates tumour expansion and local invasion by supplying oxygen and nutrients and by the production of growth factors that benefit tumour cells. Even though many anti-angiogenic drugs are in the clinical trials, the angiogenic switch activation differs among tumour types.

1.3.3 Sustaining proliferative signalling

The normal cells maintain cell homeostasis by strictly controlling growth proliferative signals that are activated only when necessary. Whereas in cancer cells, such regulation is overpassed and they proliferate without a controlled signalling input (Hanahan and

Weinberg, 2011). It is seen that in cancer cells, there is increased growth factor and receptor production. Along with that, they create a microenvironment for obtaining more growth factors at the risk of the normal cells. Alteration in receptors and activation of signalling proteins also aids in the sustained signalling (Bardeesy and Sharpless, 2006). Cancer cells also interrupt negative feedback loops that controls signalling pathway. For example, it is not the overactive RAS signalling that results in oncogenic activity of RAS, but it is due to the disruption of normal negative feedback mechanisms operated by the oncogenic GTPase which causes the same.

1.3.3.1 Oncogenes

Proto-oncogenes constitute a class of genes which normally stimulate cell division or inhibit cell death. The mutated forms of these form the oncogenes**.** In non-transformed cells, oncogenes are usually seen in an inactive form (proto-oncogenes) and are activated by gene mutations resulting in a gain of function. In normal cells, proto-oncogenes code signalling proteins which act via signal transduction pathway. Whereas, the oncogenes activate the signalling cascade continuously, and results in over expression of growth factors. Proto-oncogenes identified in cellular genome includes, c-erbB, c-myc, c-myb, C-H-ras (Bishop, 1987). Most of these genes encode growth factors like: sis (PDGF B chain) and hst (FGF-like factor). Oncogenes associated with tyrosine kinase activity include src family of oncogenes, eg, erbB (EGF receptor) (Hunter, 1991). Membrane-associated oncogene products are guanine nucleotide binding proteins such as the Ras family. The cytoplasmic oncoproteins with serine/threonine protein kinase activity includes, products of Raf and cot genes. Oncogenes that code for nuclear transcription factors are myc, fos, jun and erb A (Hunter, 1991).

1.3.3.2 Signalling pathways

1.3.3.2A Human epidermal growth factor receptor (HER) signalling

The loss of regulation over HER-mediated signalling pathways results in the proliferation and metastasis (Menard *et al.*, 2000). The 4 structurally related receptors of HER includes, HER1 (EGFR), HER2, HER3, and HER4 (Sliwkowski, 2004). In HER-mediated signalling, the receptors are activated by two mechanisms, dimerization or receptor pairing

(Sorkin and Goh, 2008). Dimerization leads to the activation tyrosine kinase domain and phosphorylation of tyrosine residues. The recruitment of these molecules at these docking sites leads to the activation of various signalling cascades like, MAPK or PI3K/Akt l pathway (Campiglio *et al.*, 1999). Angiogenesis, increased cell proliferation and reduced apoptosis are the end results of the over expression of these receptors (Hynes and Stern, 1992).

1.3.3.2B MAPK signalling

This signalling pathway significantly controls gene expression and cellular growth (Pearson *et al.*, 2001). The MAPK signalling pathway initiated by receptor tyrosine kinases consists of MAPK that is activated via phosphorylation of MAPKK (MAPK kinase kinase or MAP2K), which in turn is phosphorylated by a MAP3K (Kolch *et al.*, 2005; Peyssonnaux and Eychene 2001). The signalling molecules involved are Ras, Raf, MEK, and ERK (Sebolt-Leopold, Herrera, 2004). MEK communicates signals from Ras and Raf by activating ERK.ERK stimulates transcription factors in the nucleus, thereby controlling cellular functions and can lead to cancer if disregulated (Ussar and Voss, 2004). Resistance to apoptosis and chemotherapy, and uncontrolled cell proliferation occurs if the signalling pathway is disrupted (Mc Cubrey *et al.*, 2007).

1.3.3.2C PI_3 Kinase/AKT pathway

The phosphorylated lipid, phosphatidyl inositol-3, 4, 5 (IP3) and phosphatidyl inositol are produced during signalling events and play a major role in the activation of signalling components (Cantley and Neel, 1999). Irregular activation of the PI3K pathway has been associated with cancers like breast cancer and lung cancer. Akt is activated when attached to cell surface and regulates cell proliferation and growth (Vivanco and Sawyers, 2002). Dephosphorylation of IP3 by inhibiting Akt phosphorylation by a tumour suppressor, PTEN (phosphatase and tensin homolog) is observed in a number of human cancers (Weaver and Ward, 2001).

1.3.3.2D JAK/STAT pathway

JAK-STAT pathway is initiated when the activation of STAT transcription factor family proteins occur by the binding of cytokine molecules like interferons (IFN) and interleukins

to their receptors. The receptors get oligomerized and activate the Janus Kinase (JAK) family of tyrosine kinases. The cytoplasmic domain of the receptor gets phosphorylated by activated JAKs, recruiting signalling proteins, such as STATs (signal transducers and activators of transcription). STATs are phosphorylated by JAKs and regulate gene transcription. Recent studies have shown that JAK mutations are associated with hematopoietic malignancies, especially myeloproliferative neoplasms (Butcher *et al.*, 2008).

1.3.4 Inflammation

Tumour-associated inflammation creates a microenvironment that aid in tumour growth. The tumour cells kind of imitate the inflammatory conditions that are usually seen in normal cells. These inflammatory conditions supply the essential growth factors, extracellular matrix (ECM)–modifying enzymes that promote angiogenesis and promotes metastasis. The genetic mutations evolved by the action of chemicals facilitated by the early inflammatory molecules of the disease helps in the establishment of tumour growth (Grivennikov and Karin, 2010).

1.3.5 Evading growth suppressors

The growth suppressors produce anti-growth signals and in normal cells and the multiplication process is strictly regulated by pro- and anti-proliferation signals. Induction of the G_0 phase and a post-mitotic state can inhibit proliferation in normal cells (Caldon *et al.*, 2010). It is seen that most of the cancer cells evade these growth suppressors for continuous multiplication.

1.3.5.1 Tumour suppressor genes

They are a class of regulatory genes that encode proteins that restrain cell division and mutations in these genes lead to a loss of function, resulting in increased cell proliferation. Studies show that cancer is also resulted by the inhibition or deletion of some specific regions of chromosomes which codes for tumour suppressor genes (Ruddon, 2007). The retinoblastoma protein (Rb) and p53 are the main tumour suppressors most commonly dysregulated in cancer cells. They are also expressed in normal tissue, and control the cell cycle (Ringshausen *et al.*, 2006).

1.3.5.1A Rb

The Rb gene, the defective gene in retinoblastoma causes paediatric tumours in retina (Cavenee *et al.*, 1983). These genes actively inhibit cell cycle at G_1. Cancer cells with mutated Rb exhibits uncontrolled cell proliferation. Hyperphosphorylation, binding to oncoproteins or mutations can impair the role of Rb to inhibit cell cycle progression (Defeo-Jones *et al.*, 1991). In most of the retinoblastomas and 80% of small-cell lung carcinomas, the rb gene are associated with the deletion or mutation (Sherr and Mc Cormick, 2002).

1.3.5.1B p53

They are the most important tumour suppressor genes and act as the central regulator of apoptosis. A loss of function in p53 allows the cell division to proceed even under DNA damaged conditions. The most common mutation leading to cancer p53 mutation at least 50% of all human cancers have been found to contain p53 abnormality (Hollstein *et al.*, 1991). p53 induces G_1 arrest or apoptotic cell death under stressed or damaged conditions (Kastan, 1997). Li-Fraumeni syndrome results from a germ line (egg or sperm) mutation in p53 and mutated p53 gene are also seen in breast cancer (Hartmann *et al.*, 1997).

Examples of other tumour suppressor genes include Adenomatous polyposis coli (APC) gene causing familial adenomatous polyposis of the colon (FPC) accounts for about 10% of colorectal cancers (Levine, 1993). Mutations BRCA1 and BRCA2 causes breast cancer (King *et al.*, 2003).

1.3.6 Invasion and metastasis

Tissue invasion and metastasis involves changes in the way cells attach to other cells and to the extracellular matrix (Hanahan and Weinberg, 2011). Tumour metastasis consists of a number of steps and the stages are described as:-

The first step in the cancer development process is hyperplasia, where there are too many cells resulted from uncontrolled cell division. These cells appear normally though some changes have occurred in them. These cells continue to grow and accumulate

abnormalities, resulting in a condition called dysplasia. In the anaplastic stages, the cells completely lose their original function but remain in their original location. The last step is metastasis in which the tumour cells invade other tissues including the bloodstream, and spread to other locations (Hart and Saini 1992; Takeichi, 1993). Non-invasive tumours are classified as benign whereas the invasive ones are malignant (a) Carcinomas result from altered epithelial cells (b) Sarcoma occurs by the changes in muscle, bone or connective tissue (c) Leukemia results from destruction to WBC (d) Changes in the lymphatic system causes lymphoma and finally (e) Myelomas are associated with antibodies.

It is the input and signals from the surrounding tissue that helps the tumour cells for the molecular cross-talk. The epidermal growth factor (EGF) required for the progression of cancer is supplied by tumour-associated macrophages (TAMs). In the process of metastasis, a group of cells invade the Extra Cellular matrix (ECM) of the host tissue, enter the circulation, and induce a new blood supply (Liotta et al., 1991). Type IV collagenases, plasmin (activated by plasminogen activator), metalloproteases and thiol proteases etc are involved in the process.

1.3.7 Apoptosis/Evading cell death

Cancer cells tend to avoid apoptosis, whereas the normal cells initiate apoptosis as a result of DNA damage and cellular stress (Jin and El-Deiry, 2005). Cancer cells are not only characterised by its proliferative ability but also marked by its reduced cell death. This is where apoptosis demands attention. Apoptosis occurs normally in cells and tissues. Apoptosis involves characteristic morphological and biochemical changes and can be induced by defects in cell cycle; stress and other DNA damaged conditions (Kerr *et al.*, 1972). Disruption of cell membrane, chromosomal degradation and nucleus fragmentation are hallmarks of apoptosis. Apoptosis is a key strategy for eliminating cancerous cells (Shankar *et al.*, 2008) and most of the chemotherapeutic drugs like tamoxifen (Han *et al.*, 2009), cisplatin and doxorubicin (Lupertz *et al.*, 2010) cause cell cycle arrest and induce apoptosis in eliminating the neoplastic cells. The nuclear DNA of apoptotic cells is cleaved by endogenous endonucleases in multiples of 180 base pairs and they show a typical laddering pattern of oligonucleosomal fragments (Telford *et al.*, 1991). Finally, the remnants are engulfed by nearby cells in a tissue and vanish, typically within 24 hr (Wyllie *et al.*, 1980). The two classic pathways of apoptotic signalling in mammalian cells are:-

1.3.7.1 Death receptor-mediated apoptosis: The extrinsic pathway

The pathway is initiated by external factors such as toxins, cytokines, NO etc. The extrinsic pathway is activated by death receptors on the plasma membranes by molecules like tumour necrosis factor receptor (TNFR) and Fas/CD 95. This results in the formation of death inducing signalling complex (DISC), leading to the initiation of caspase cascades through caspase 8.The TNF-induced model and the Fas-Fas ligand-mediated model, both involve TNFR family coupled to extrinsic signals. The DISC–FADD/TRADD complex with the help of death effector domains (DED, D4/D5), binds to the initiator caspases 8 and 10, resulting in the autocatalytic cleavage of procaspase-8 and activation of downstream executioner caspases (Hengartner, 2000; Krammer, 2000; Thorburn, 2004). The level of TNF receptor associated factor 2 (TRAF2), a protein which inhibits apoptosis family (IAP) of proteins is seen elevated in numerous tumours. This is capable of activating the cell survival pathway leading to apoptosis resistant tumours (Karna and Yang, 2009).

1.3.7.2 Mitochondria-mediated intrinsic pathway

This pathway is initiated by intrinsic stresses such as DNA damage, oncogene activation and deprivation of survival factors and are characterised by permeabilisation of mitochondria and release of cytochrome c into the cytoplasm. This is followed by the formation of a protein complex, apoptososme and initiates capsase cascade through caspase 9. Release of other pro-apoptotic proteins such as endonuclease G, SMACs (small mitochondria derived activator of caspases) and apoptosis-inducing factor are also seen. In the cytosol, cytochrome c combines with ATP, c terminus of Apaf-1 and oligomerises Apaf-1 accompanied by the recruitment of procaspase 9 to form an apoptosome, which activates caspase 9 and subsequently the effector caspase 3 leading to cell death. The mitochondrial permeability transition pore (PTP) and Bax play vital roles in this process (Crompton, 1999; Reed and Kroemer, 2000). The Bcl-2 family of proteins are thought to play an essential role in regulating the intrinsic pathway as many cancers resist the apoptotic pathway through dysregulation of BCL-2 family members. This is achieved either by down-regulating pro-apoptotic proteins, or by increasing the BCL-2 expression (Letai, 2008). The resistance to apoptosis is also gained by down regulating downstream effector molecules.

1.3.7.3 Caspases- The executioners of apoptosis

The caspases are cysteine–aspartic acid-proteases present as inactive pro-enzymes (procaspases) and activated by proteolytic cleavage. They are categorised as initiator and effector caspases. The initiator caspases cleaves the pre-forms of effector caspases and these are in turn activated by binding to specific adapter proteins. CASP- 2, 8, 9 and 10 are examples of initiator caspases. The effector caspases like CASP 3, 6 and 7, which are activated by initiator caspases cleave other protein substrates within the cell to trigger apoptosis (Boatright and Salvesen, 2003; Motadi *et al.*, 2007). The caspases targets many proteins like, Inhibitor of DNA se (ICAD), nuclear lamins, FAK (folk adhesion kinase), cytoskeletal proteins (actin, myosin, tubulin) etc. Regulation of Bcl-2 family of proteins, activation of caspases, and changes in the levels of the inhibitor of apoptosis family (IAP) of proteins play important role in the execution and regulation of the apoptotic cell death mediated by both pathways (Reed, 1998; Tamm *et al.*, 1998; Reed, 2000).

1.3.7.4 Apoptosis regulators

Apoptosis in mammals are primarily regulated by Bcl-2 family proteins. Depending upon the sub-cellular localization, they can inhibit (anti-apoptotic members) or promote apoptosis (pro-apoptotic members).

Bcl-2-Subfamilies are:-

1. Bcl-2 subfamily (anti-apoptotic): Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1
2. Bax subfamily (pro-apoptotic): Bax, Bak and Bok
3. BH3 subfamily (pro-appoptotic): Bad, Bid, Bik, BimL etc

The fate of cell, whether death or life is determined by the balance of activity of pro-apoptotic and anti-apoptotic Bcl-2 family members, which in turn regulate each other.

1.3.7.5 Apoptosis markers

There are various apoptosis markers identified based on their ability to induce cell death, DNA fragmentation etc. Some of the examples are:-

(a) AIF (Apoptosis inducing factor)- They are released from mitochondria and mediated DNA fragmentation
(b) P53- They initiates apoptosis by activating pro-apoptotic Bcl2 family members and repressing anti-apoptotic proteins.
(c) Caspase 3- Targets many cellular proteins to bring about cell death.
(d) Annexin V- It is a phospholipid- binding protein with a high affinity for phsphatidylserine (PS), detected by flow cytometric analysis.

1.3.8 Evading immune destruction and reprogramming energy metabolism

In normal cells, an active immune system prevails and eliminates cancer cells before they form a tumour mass (Hanahan and Weinberg, 2011). However, cancer cells preliminary elimination enters to the equilibrium phase, in which the immune system have control over cancer cell growth but cannot completely, get rid of the foreign body. They later enter into escape phase. In this phase the malignant cells continue to divide and grow (Prendergast, 2008).

Reprogramming energy metabolism is one the emerging hallmark in cancer cells. The cancer cells slightly alter the metabolic parameters for their continuous growth. For eg, cancer cells make changes in glucose metabolism and up regulates glucose transporters. Additionally, reprogramming energy metabolism is widely applied in clinical settings today through the use of [^{18}F] fluorodeoxy glucose positron emission tomography (FDG-PET) that helps to capture images of tumours with increased glucose uptake (Chen and Chen, 2011).

1.4 Cancer and treatment

Since there are numerous types of cancers, different treatment are preferred for each person based on the type, locality and extend of spread. Since a complete eradication has not been possible, what cancer treatment does is to either kill the cancer cells or reduce the pain or extend to extend life span. The major treatment includes surgery, radiation, chemotherapy and immunotherapy (Cancer Facts and Figures, 2013).

1.4.1 Surgery

Surgery involves the removal of the solid tumour mass and is preferred during early or benign stages. In fact, surgery was one of the predominant cancer treatments during the

treatment first half of the 20th century (Michael *et al.*, 2006) but now they are done along with other advanced treatments. The various types of Surgery according to American Cancer Society are:-

Preventive (prophylactic) surgery: The body tissue that is likely to become cancerous is removed. For example, pre-cancerous polyps may be removed from the colon during a colonoscopy.

Diagnostic surgery: It involves diagnosis cancer by dissecting out a piece of tissue (called a sample) and testing it, often as biopsy.

Curative surgery: This is done before or after the operation along with other treatments like chemotherapy or radiation therapy for the local control of the lesion in primary cancer.

1.4.2 Radiation therapy

Radiotherapy uses high doses of radiation to kill cancer cells and stop them from spreading. At low doses, radiation is given as an x-ray to see and take pictures inside our body. Cancer treatment works in much the same way, but radiations are given at higher doses either by using an external or an internal beam. Radiation can be used to shrink cancer or slow the growth of cancer. Since radiation targets damaging DNA and prevents its replication, the rapidly dividing cancer cell is killed. But the normal cell which does not divide is also killed in the process. When a cure is not possible, radiation may be used to shrink cancer tumours in order to reduce pressure.

1.4.3 Chemotherapy

Studies indicate that the agents used for chemotherapy have helped in reducing death or have increased the life span of cancer patients (Suggit and Bibby, 2005). Chemotherapy drugs target rapidly growing cells by interfering in the S phase of the cell cycle, to prevent the DNA replication. DNA has become a critical target in chemotherapy by inhibiting the DNA replication (Zahid, 2002).

Chemotherapeutic agents can be classified based on which stage of cell cycle do they exert their action. Cell cycle specific drugs like alkylating agents and anti-tumour antibiotics

follow a linear dose-response curve, ie greater the dose of drug, greater the cytotoxicity. However, cell-cycle-phase–specific drugs like antimetabolites kill cells during a specific phase and are unable to work in the resting phase and further increase in drug concentration cannot kill cells (Page, 2007).

1.4.3.1 Alkylating agents

Alkylating agents directly damage DNA to prevent the cancer cell from reproducing. These classes of drug are not phase-specific; rather they work in all phases of the cell cycle. Lung, breast, and ovary cancer, Hodgkin disease, multiple myeloma, and sarcoma can be treated by alkylating agents. Damage to bone marrow and in rare cases, acute leukemiais effects of alkylating agents. Examples of alkylating agents are- alkyl sulfonates: Triazines: dacarbazine (DTIC) and temozolomide (Temodar), ethylenimines: thiotepa and altretamine, nitrogen mustards: such as mechlorethamine (nitrogen mustard), chlorambucil, cyclophosphamide (Cytoxan) and melphalan; nitrosoureas: which include streptozocin, carmustine (BCNU), and lomustine.

1.4.3.2 Antimetabolites

These agents' damage cells during the S phase by interfering with DNA and RNA synthesis and are commonly used to treat leukaemia, breast cancer, ovary and the intestinal tract. Examples of antimetabolites are floxuridine, hydroxyurea, thioguanine, 5-fluorouracil (5-FU), 6-mercaptopurine (6-MP). Methotrexate (MTX), is the most widely used antifolate which act against urothelial cancer, breast cancer, head and neck cancer, colorectal cancer, etc (Chu and Allegra, 1996).

1.4.3.3 Anti-tumour and anthracycline antibiotics

Anti-tumour antiobiotics are natural in origin that interacts with DNA by means of inducing DNA strand breakage or by forming intercalations and also by inhibiting topoisomerase II. For example dactinomycin gets intercalated between guanine-cytosine base pairs in the DNA helix, inhibiting the activity of RNA and DNA polymerases. Antitumor drugs are mainly comprised of anthracycline antibiotics and they work by intercalating into DNA and inhibit DNA and RNA synthesis, produce oxygen radicals and

also alter membrane signal transduction. Doxorubicin, idarubicin and dauxorubicin are the important anticancer agents (Arcamone *et al.*, 1997).

1.4.3.4 Topoisomerase inhibitors

The DNA topoisomerases are nuclear enzymes that play a significant role in relaxation and untangling of DNA regions in order to allow proper replication without any torsional stress. The plant-derived camptothecins (irinotecan, topotecan) act as inhibitors of topoisomerase I; the plant-derived epopodophyllotoxins (etoposide and teniposide) and the microbial derived anthracyclines (e.g. doxorubicin, epirubicin) act as inhibitors of topoisomerase II. Camptothecins exerts its action by stabilizing the covalent topoisomerase I-DNA complex, thus preventing relegation (Hsiang *et al.*, 1985). Even though camptothecin derivates have undergone clinical trials, due to their unpredictable toxicity several synthetic camptothecin analogues like lurtotecan, karenitecin and gimatecan) are under various stages of trials (Hartmann and Lipp, 2006; Teicher, 2008).

1.4.3.5 Mitotic inhibitors

They are widely used in cancer treatment, as they are able to stop the continuous cell division of cancer cells by inhibiting mitosis. This inhibition is achieved by the disruption of microtubules that play an essential role in cell division by pulling the cells apart. During cell division, get assembled by polymerisation of tubules and later gets disassembled. The mitotic inhibitors interfere in this process, ie during the mitosis (M) phase of the cell cycle, and prevent the proper separation of daughter chromosomes. Some of the mitotic inhibitors frequently used in cancer treatment include paclitaxel, vinblastine, and vinorelbine (Cancer Facts and Figures, 2007). Some of the specific agents are discussed below-

1.4.3.5A Taxanes

Taxanes are derived from the plant genus, Taxus (yews). They are terpene class of compounds originally obtained from the Pacific yew tree, but now synthesised artificially also. They exert their action by stabilizing the formation of microtubules, thereby disrupting its functioning and finally inhibiting the cell division. For eg. Paclitaxel is a taxane class of drug used for treating breast cancer, ovarian cancer and some types of

sarcoma (Saville *et al.*, 1995). Docetaxel is another example of taxanes used to treat breast and ovarian cancer (Lyseng-Williamson and Fenton, 2005).

1.4.3.5B Vinca alkaloids

Vinca alkaloids are isolated from the periwinkle plant *Catharanthus roseus* or *Vinca rosea*. Vincristine, vinblastine and vindesine are the first vinca alkaloids with anti-tumour activity to be identified. Vinca alkaloids disrupt the mitotic spindle assembly by binding specifically to β-tubulin, inhibiting its polymeriseation with α-tubulin to form microtubules. This leads to mitotic cell cycle arrest and inhibits the division of cells. The alkaloid amine, vinblastine is used to treat breast cancer, leukaemia, non-small cell lung cancer and Hodgkin's lymphoma. Various other classes of vinca alkaloids like vincristine is used to treat lymphoma and, lung cancer, vindesine for the treatment of lung cancer, melanoma and lymphoma (Ngan *et al.*, 2001).

Other mitotic inhibitors include colchicines (alkaloid obtained from *Colchicum autumnale*), podophyllotoxin from May apple and Griseofulvin (derived from Penicillium species).

1.5 Side effects of chemotherapy

One of the main drawbacks of chemotherapy is that along with targeting the cancer cells, normal cells also undergoes cell death. While focussing on the fast dividing cancer cells, certain fast dividing normal cells like, blood cells in the bone marrow, cells in digestive tract (mouth, oesophagus), hair follicle cells and cells in the reproductive system (sexual organs) are also spared unknowingly, sometimes effecting vital organs also (Brenner and Stevens, 2010). The other side effects include- vomiting, headache, loss of immunity, stomach problems, etc.

1.6 Multiple Drug Resistance/ MDR

One of the major obstacles to effective chemotherapy is the resistance shown by tumor cells to multiple chemotherapeutic drugs (Gottesman and Pastan, 1993). Multidrug resistance (MDR) can be defined as the resistance offered by cancer cells to the cytotoxic or cytostatic action of various drugs used in chemotherapy (Gottesman, 1993). It is seen

that in the case of non-transport based cellular MDR mechanisms, certain enzymes negatively influences the activity of desired drug without obstructing its concentration. For e.g. Glutathione-Stransferase (GST), catalyzes the removal of chemotherapeutic drug by conjugating them with polar molecules (Batist *et al.*, 1986). According to the classical cellular mechanism of MDR, the drugs are expelled by an efflux from the cancer cell by using energy dependant membrane transport proteins. For eg: ABC transporters such as P-glycoprotein shows such efflux and are often seen overexpressed in cancer cells (Gottesman, 2002).

1.7 Chemoprevention

Cancer chemoprevention is defined as the use of natural, synthetic, agents to reverse, suppress, or prevent cancer development to an invasive form). According to Tsao et al., 2004, a successful chemopreventive mechanism should focus on the thrust areas like

- Framing a tumor-specific risk model for determining the high-risk factors
- Increase the use of pre-clinical models to develop novel chemopreventive agents
- To identify surrogate endpoints using molecular alterations
- Identify new targets for drug action

Since carcinogenesis is a multi-step process marked by initiation, promotion and progression stages, the most effective intervention would be possible at the promotion phase. This would inhibit the conversion of premalignant cells to malignant (Trosko, 2005). The chemoprevention concept relies on the use of using natural or synthetic compounds in delaying or preventing this transformation (Brenner and Gescher, 2005). Chemopreventive agents are classified into blocking agents and suppressing agents based on their mode of action (Wattenberg, 1985). Blocking agents prevent carcinogens by blocking the interaction between chemical carcinogens or endogenous free radicals and DNA, or prevent metabolic activation of pro-carcinogens (Yu and Kong, 2007). Suppressing agents inhibit the malignant transformation of initiated cells, in either the promotion or the progression stages (Manson, 2003). They act by inhibiting the signal transduction pathways to downregulate the tumour promoters (Karin, 2002), which would otherwise lead to cell proliferation.

Various phytochemicals like gingerol, sylimarin, curcumin, lycopenee, vitamin C etc are known chemopreventive agents. These dietary agents are believed to suppress the inflammatory processes that lead to the initiation of carcinogenesis by inhibiting angiogenesis and metastasis (Aggarwal and Shishodia, 2006). Agents like carotenoids, flavonoids, flavonolignans, omega-3 fatty acids and otherpolyphenolic compounds inhibit, delay or reverse cancer (Palozza and Krinsky, 1992).

Examples of chemoprevention

- The use of Tamoxifen, an estrogen blocker to reduces the risk of developing breast cancer,
- Lowering prostate cancer risk by Finasteride (Propecia, Proscar)
- Aspirin and other non-steroidal anti-inflammatory drugs are also used to lower the risk of various types of cancer

1.8 Scope of the present study

1.8.1 Plant as sources of anti-carcinogenic agents

Since time immemorial, different parts of plants are used for the treatment and prevention of many ailments (Chah *et al*., 2006).The use of folk medicines (implemented by the use of herbs) is practised throughout the world for pharmacological treatment of diseases as part of their tradition (Schulz *et al*., 2001). Examples of important healing practices that used herbs include Traditional Chinese medicine (TCM), Japanese traditional medicine and Ayurveda, Indian traditional medicine. Plants owe an extensive history of use in the treatment of cancer. In earlier times, cancer could not be properly defined and many of the references of cancer like hard swellings, calluses etc., are written based on the external characteristics of the disease. Plants, marine and other microorganisms have contributed to almost 60% of the total anti-cancer agents used today. The search for anti-cancer agents started off during 1950 with the discovery of Vinca group of alkaloids. Plant derived compounds like vinblastin, vincrsitine, taxol, camptothecin derivatives like topotecan and irinotecan have played a major role in the development of many the clinical useful anti-cancer agents (Cragg and Newman, 2005).

Phytochemicals are naturally occurring, biologically active chemical compounds in plants. Theses phytochemicals are bestowed with lot many medicinal properties. Most phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer. As early as in 1940's, podophyllin (alcoholic extract of dried root of Phodophyllum species) was used to cure the so called venereal warts and *P. peltatum* to cure cancer. Semi-synthetic derivatives of Epidophyllotoxin (an isomer of podophyllotoxin) like etoposide and teniposide is used for the treatment of cancer (Cragg and Newman, 2005). Homoharringtonine is another plant derived agent obtained from Cephalotaxus harringtonia. Moreover, of the 2069 clinical trials recorded by NCI, 248 or around 12% are taxane derived drugs, including placitaxel. Using the foundation of traditional medicinal practises, lot of herbal products have been incorporated into so-called 'alternative', 'complementary', or 'integrative' medical system (Tyler, 2000). Complementary and alternative medicines (CAM) are being used world-wide for cancer and the usage is as high as 80% (Ernst and Cassileth, 1998). And more than 35.9% of cancer patients are users of complementary and alternative medicine (CAM) (Molassiotis *et al.*, 2005). Herbal medicine has been found effective for cancer treatment over the last century. Herbal medicines are also in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects (Tilburta and Kaptchuk, 2008). Medicinal plants have become vital and truthful sources for anticancer agents, and therefore efforts on discovering novel plants with biological activity is in the run (Newman *et al.*, 2003).

1.8.2 Western Ghats- The treasure house of plant biodiversity

Western Ghats is one among the 34 global hotspots of biodiversity (Mittermeier *et al.*, 2005), renowned for its medicinal plants resources. By virtue of its location, the Kerala State occupies biodiversity rich areas of the Nilgiri Hills, Anamalai High Ranges and Agasthyamalai Hills, the three important phytogeographical subunits of southern Western Ghats. The area is remarkable for its convoluted terrain with diverse tropical habitats. The great topographic heterogeneity, from sea level to the highest point, the Anaimudi peak (2695 m) and a strong rainfall gradient from 500 mm in valleys in the east to 7000 mm along west-facing slopes, combine to give rise to a rich diversity of life forms and

vegetation types, including montane wet temperate forest (shola) and grassland, tropical wet evergreen forest, semi-evergreen forest, moist deciduous forest, lateritic plateaus, dry deciduous forest and dry thorn forests. Number of Edaphic types and micro-habitat formations are also seen within these major habitats. All these habitats are the treasure house of medicinally important species used in modern and various Indian Systems of Medicine.

A total of 5094 taxa of flowering plants were reported from the Kerala part of Western Ghats (Sasidharan, 2014). This includes 1246 medicinal plants species used in folk, modern and the classical Systems of Medicines. The medicinal plants are important renewable resources which are comprehensively utilized in distinct areas of health management, both for traditional as well as modern system of medicine. Over the centuries, the use of medicinal plants has become a significant element of daily life, in spite of the developments in modern medical and pharmaceuticals research and functions complimentary to modern medicine. Isolation and characterization of active ingredients for therapeutic use based on ancient knowledge system has been in practice for many decades. Plants can provide biologically active molecules and lead compounds for the development of modified derivatives with enhanced activity and or reduced toxicity. Only minor shares of the plants were effectively examined and so far, majority of the plants especially the endemic species are still under explored for its medicinal properties and pharmaceutically active compounds.

1.8.3 Diversity of the family Icacinaceae (allied taxa of *Nothapodytes*) in Kerala

Nothapodytes nimmoniana (Syn. Mappia foetida) is a small tree inhabited to the high elevation and medium elevation evergreen and semi-evergreen forests, found in almost all the Districts of Kerala, except Alappuzha. The plant is well known because of the presence of an anti-cancerous compound, camptothecin. The plant is belonging to the family Icacinaceae. In Kerala the family is represented with seven species (Sasidharan, 2014) with broad biological spectrum. Herbaceous climber, shrubs, woody climbers and trees are there in this family. *Apodytes dimidiata* Meyer ex Arn., *Gomphandra tetrandra* (Wall.) Sleumer, *Miquelia dentata* Bedd., *Pyrenacantha volubilis* Wight and *Sarcostigma kleinii* Wight &Arn. are the other members in this family. Among these, *Miquelia dentata* is rare and endemic species to southern part Western Ghats. Another species *Gomphandra coriacea* is endemic to Western Ghats-Sri Lankan Hotspot of Biodiversity. *Gomphandra tetrandra* and *Pyrenacantha volubilis* are

distributed to evergreen forests of South-east Asia. Even if some of the plants are with wider distribution pattern their population is very low.

1.8.4 The importance of family Icacinaceae

Icacinaceae family consists of more than 400 species grouped in almost 55 genera. They comprise trees up to 40 m high, shrubs and lianas. In this family, there are also several commercially important timber trees (e.g., Apodytes and Cantleya) and some ornamentals (Citronella, Pennantia). Many genera produce edible fruits and some are cultivated to obtain products such as starch (from the seeds of Casimirella), oil (from the fruits of Poraqueiba) (Howard, 1942). The family is known to produce various phytochemicals such as monoterpenoids, quinone alkaloids, flavonoids, iridoids, saponins and proanthocyanina etc. The family Icacinaceae gained attention by the discovery of an anti-cancer alkaloid, camptothecin from a Chinese tree, *Camptotheca acuminata*. It was discovered in 1966 by M. E. Wall and M. C. Wani in the systematic screening of natural products for anticancer drugs. Camptothecin, a monoterpeneindole alkaloid, is regarded as one of the most promising anticancer drugs of the twenty-first century and causes DNA damage by stabilizing the covalent topoisomerase I-DNA complex, thus preventing relegation (Hsiang *et al*., 1985). Camptothecin was used in clinical trials, during 1970's, but was dropped due to severe bladder toxicity. Later, camptothecin was isolated from a variety of plant species including *Merriliodendron megacarpum*, *Pyrenacantha kleiniana* and *Nothapodytes nimmoniana* (family Icacinaceae), *Ophirrohiza mungos* and *O. pumila* (family Rubiaceae), *Eravatamia heyneana* (family Apocynaceae) and *Mostuea brunonis* (family Loganiaceae) (Govindachari *et al*., 1972). High concentration of CPT (about 0.3% dry weight) was reported from *N. nimmoniana* in a study conducted by Sharma *et al*., 2008. Two CPT analogues of camptothecin, topotecan and irinotecan have been approved and are used in cancer chemotherapy today (Samuelsson, 2004).

1.8.4 A *Nothapodytes nimmonina*

Nothapodytes nimmoniana is a small tree (3-8 m) belonging to the Icacinaceae family. The plant demands attention as it is a rich source of camptothecin (Govindhachari and Vishwanathan, 1972). It is distributed in Indomalasyia and Indochina regions. In India,

they are seen along the Western Ghats, South India, Munnar, Wayanad, Maharashtra and Goa. It is a shrubby small tree, with broad dark green leaves and flowers.

Since the discovery of camptothecin from *N. nimmoniana*, there has been an indiscriminate extraction of the trees from many parts of India, especially from the Western Ghats. For establishing high-yielding clonal populations by *in vitro* production systems many populations of *N. nimmoniana* were screened (Ajay *et al.*, 2010).

Nothapodytes nimmoniana contains camptothecin as its active constituent which is used in the treatment of cancer. The cellular target of camptothecin is DNA topoisomerase I. Irinotecan and topotecan, two water soluble derivatives of camptothecin, have been approved by the Food and Drug Administration (FDA) of the United States of America for treating colorectal and ovarian cancer (Sharma *et al.*, 2008). A sensitive and reliable HPLC method has been developed for quantification of camptothecin in the dry stem powder of *N. nimmoniana* was done by Dighe *et al.*, 2007. It is reported that petroleum ether, chloroform and methanolic extracts of leaves and stem of *Nothapodytes nimmoniana* were tested for its antibacterial activity and the methanolic fraction were found to be more effective against all the tested organisms (Nandhakumar *et al.*, 2002). Anti-inflammatory activity of *N. nimmoniana* has also been studied by carrageenan induced hind paw edema in rats (Sheeja *et al.*, 2006). Callus assessment in different genotypes of N. nimmoniana for camptothecin content has been analysed (Karadi *et al.*, 2008). In a study conducted by Satheeshkumar et al., 2000 *in vitro* multiplication of *N. nimmoniana* through seedling explants cultures was reported. Puri *et al.*, 2007 reported immunomodulatory activity of an extract of the novel fungal endophyte Entrophospora infrequens, isolated from *N. foetida* (Wight) Sleumer. The endophyte *E. infrequens* was found to synthesize camptothecin. The free radical scavenging activity of methanolic extracts of *N. nimmoniana* determined using various antioxidant assays is also reported (Namdeo *et al.*, 2010).

1.8.5 The allied taxa of *Nothapodytes* chosen for the study

Apodytes dimidiata, *Gomphandra tetrandra*, *Miquelia dentata* and *Sarcostigma kleinii* were the allied taxa of *Nothapoytes nimmonina* subjected to the present study. Studies indicate that camptothecin has been isolated from 13 members of the Icacinaceae family by screening the leaf, stem, bark, fruit, root etc of the plant specimens (Ramesha *et al.*, 2013). The highest

amount of camptothecin (% dry weight) was detected in the seeds of *Miquelia dentata* (1-1.4). Trace amount of camptothecin was reported from *A. dimidiata* stem bark (0.0051 ± 0.0014), *Miquelia dentata* twig, leaf, root, fruit etc (0.003- 1.4), *Gomphandra tetrandra* leaf, and stem bark (0.00045- 0.006) and *Sarcostigma kleinii* fruit, leaf and stem bark (0.0003- 0.018).

Apodytes dimdiata E. Mey. Ex Arn., a small bushy tree belonging to the family Icacinaceae is distributed in Southern and Eastern parts of Africa, Western Ghats of India and other Asian countries. The plant is commonly known as umDakane to the rural communities of KwaZulu-Natal (Watt and Breyer-Brandwijk, 1962) and used as molluscicide for schiostosomiasis control. An iridoid glycoside, genipin was isolated from the bark of *A. dimidiata* in relation to its molluscicdal activity (Drewes and Kayoga, 1996). The plant is widely used in Zulu traditional medicines for treating gastrointestinal ailments, helminthes (Gestner, 1938; Bryant, 1966; Hutchings *et al.*, 1996) and the leaves are used as a remedy for ear inflammation (Watt and Breyer-Brandwijk, 1962). In addition, an iridoid glycoside genipin, 10-monoacetate derivative of genipin and an acetylated eudesmane glucoside was isolated from bark and leaf, respectively in association with the molluscicidal activity of *A. dimidiata* (Drews *et al.*, 1996; Harinantenaina *et al.*, 2006). Six new saponins, apodytine A-F with anti-protozoal activity were also isolated from the leaves of *A. dimidiata* (Foubert *et al.*, 2011). Based on toxicity assessment of mammals, the European Economic Community (EEC), South Africa Bureau of Standards (SABS) and the World Health Organisation (WHO) have classified the plant as non-toxic and non-irritating (Brackenbury *et al.*, 1997).

Reports suggest that the antiepileptic activity of *Sarcostigma kleinii* was studied using chemically induced seizures (Sabu *et al.*, 2015). The phenolic content of *Sarcostigma kleinii* was also reported in another study (Arunachalam *et al.*, 2011). Shwetha *et al.*, 2012 has reported that three fungi isolated from the fruit and seeds of *Miquelia dentata* are capable of producing 9-methoxy CPT and 10-hydroxy CPT . There are no reports of any biological or phytochemical studies conducted in *Gomphandra tetrandra.*

The literature evidence suggests that the allied taxa of *Nothapodytes* found in Western Ghats of Kerala have not been subjected to extensive studies in an anti-cancer background apart from the screening for new sources of camptothecin from the family Icacinaceaea. It is viewed that initial studies should comprise a series of investigations on *in vitro* and *in vivo* mechanistic assays which include studying the effect of the agent under investigation on

important processes such as inhibition of proliferation, inflammation or induction of apoptosis, followed by exploring the prevention of tumour development, decrease in overall burden, or prolongation of occurrence (Steward and Brown, 2013). Models relevant to carcinogenic models are also encouraged (Scott *et al.*, 2009). Keeping this in mind, the present study aims to explore the biological properties of the plants, *Sarcostigma kleinii*, *Gomphandra tetrandra*, *Apodytes dimidiata* and *Miquelia dentata* focussing on its cytotoxic, antioxidant, anti-inflammatory and phytochemical aspects.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Plant materials

Leaves of *Apodytes dimidiata* E. Mey. Ex Arn, *Gomphandra tetrandra* Wight, *Miquelia dentata* Bedd., and *Sarcostigma kleinii* were used for the study (Figure 2.1). The plant specimens were collected from Periya forest, Wayanad District of Kerala, India (Altitude: 810 m, Geographical location: Lat. N 11° 51' 03.19", Long. 75° 48' 05.54") during the month of June, 2011 and identified by Dr. V B Sreekumar, Taxonomist, Kerala Forest Research Institute (KFRI) Peechi, Thrissur, Kerala. Voucher specimens were lodged in the Herbarium of KFRI. The systematics and peculiarities of these taxa are described below.

2.1.1.1 *Apodytes dimidiata* Meyer ex Arn.

Local names: Slatemathi, Karineeli

Habitat: Evergreen and Semi-evergreen forests

Altitude range: 500-1500 m asl (above sea level)

Distribution: Palaeotropics (Old world tropics)

In Kerala: Wayanad, Silent Valley, Idukki (Thekkadi)

Flowering and fruiting: April - October

Botanical description: Small tree, to 18 m high, bark to 1.5 cm thick, greyish-black, rough, scaly, with short gaping fissures; blaze red. Leaves simple, alternate, estipulate; petiole 8-25 mm long, slender, glabrous, grooved above; lamina 3.8-12.6 x 1.5-6 cm, ovate, elliptic-ovate, obovate or obovate-oblong, base acute, obtuse or oblique, apex acuminate, margin entire, revolute, glabrous, coriaceous; lateral nerves 4-10 pairs, slender, pinnate, prominent, intercostae reticulate, faint. Flowers bisexual, 5-8 mm across, white, in terminal or sub-terminal corymbose cymes; pedicel short; calyx small, 5 toothed, pubescent, persistent; petals 5, free, oblong, acute, valvate, disc absent; stamens 5, filaments dilated; anthers oblong, bipartite at base, medi-fixed, longer than filaments; ovary superior, oblique, gibbous at base, 1-celled, ovules 2, pendulous, superposed; style slender, stigma oblique. Fruit a drupe 15-18 x 10-12 mm, obliquely appendaged; stone crustaceous; seed one, pendulous.

Parts used: leaf

Properties and uses: An infusion from the root bark is used as an enema for intestinal parasites. The leaves are used in the treatment of ear inflammation (Quattrocchi, 2012).

2.1.1.2 ***Gomphandra tetrandra*** (Wall) Sleumer

Local names: Chottamaram, Kambilichedi

Habitat: Evergreen and Semi-evergreen forests

Altitude range: 400-1300 m asl

Distribution: South and south-east Asia

In Kerala: All districts

Flowering and fruiting: September - December

Botanical description: Shrubs to small trees, bark dark grey, smooth; blaze yellow; young branches green. Leaves simple, alternate, estipulate; petiole 5-15 mm long, slender, grooved above, glabrous; lamina 3-12 x 1.8-5 cm, elliptic, obovate or elliptic-obovate, base acute, apex obtuse, obtusely acute, acute or acuminate, margin entire, coriaceous; lateral nerves 3-7 pairs, pinnate, slender, faint, intercostae obscure. Flowers polygamo-dioecious, greenish-white, in leaf opposed cymes or rarely in axillary cymes; calyx minute, cup-shaped; lobes 4-5; corolla 4 mm long; lobes 4-5, acute, connate into a tube, lobes inflexed at apex; male flowers : stamens 4-5, filaments thick, fleshy often connate, usually with a tuft of club-shaped hairs on the back at the apex; anthers pendulous from the interior apex of the filaments, dehiscing longitudinally; disc thick, annular or 0; pistillode pubescent; female flowers : ovary superior, oblong, 1-celled, ovules 2; style conic; stigma small or discoid. Fruit a drupe, 15 x 8 mm, white, oblong-terete, crowned with the remains of persistent stigmas; endocarp wrinkled; seed pendulous, longitudinally surrounded by raphe.

Parts used: leaf

Properties and uses: Information is not available

2.1.1.3 ***Miquelia dentata*** Bedd

Local names: Palluvalli

Habitat: Evergreen and Semi-evergreen forests

Altitude range: 200 to 1200 m asl

Distribution: Endemic to southern Western Ghats

In Kerala: Almost all districts

Flowering and fruiting: April – September

Botanical description: Glabrous perennial climbers. Leaves to 8-16 x 3-7 cm, ovate, apex acute, base rounded or truncate, dentate, more sharply near the base; petiole 3-4 cm long. Flowers unisexual, in peduncled globose spike; peduncle to 6 cm long; calyx cupular, 4-toothed; petals 4, 3 mm long, oblong; stamens 4 or 5, in male flowers; ovary 1-celled, 4-angled, style absent, stigma 4-angled, ovules 2, pendulous. Drupe to 2 x 1 cm, 3-8 together, ovoid, sessile, yellow; seeds 1.5 x 1 cm, stalked, pitted.

Parts used: Leaves

Properties and uses: Information is not available

2.1.1.4 *Sarcostigma kleinii* Wight & Arn

Local names: Erumathali, Odal, Vattodal, Vellodal

Habitat: Evergreen and Semi-evergreen forests

Altitude range: up to 1500 m asl

Distribution: Indo-Malaysia

In Kerala: Almost all districts

Flowering and fruiting: February – June

Botanical description: Large woody climbers, young shoot glabrous. Leaves 17-25 x 8-15 cm, oblong-lanceolate, apex acuminate, base rounded or obtuse; lateral nerves 6-9 pairs, reticulate; petiole 5-12 cm long. Spikes to 35 cm long, slender, axillary or from tubercles. Flowers 3-6 together, yellow; calyx cupular, 2 mm across, 5-toothed; petals 3-5 mm long, oblong, recurved; stamens 5, anthers versatile, staminodes 5; female flowers mostly from old wood, ovary 1-celled, densely hairy; stigma sessile, discoid; pistillode in male flowers conical. Fruit a drupe to 3 x 1.5 cm, ovoid, orange-yellow on maturity, glabrous.

Parts used: leaf

Properties and uses: Powdered bark mixed with honey is given in rheumatism. Oil from seeds is used against rheumatism, leprosy and piles (Quattrocchi, 2012).

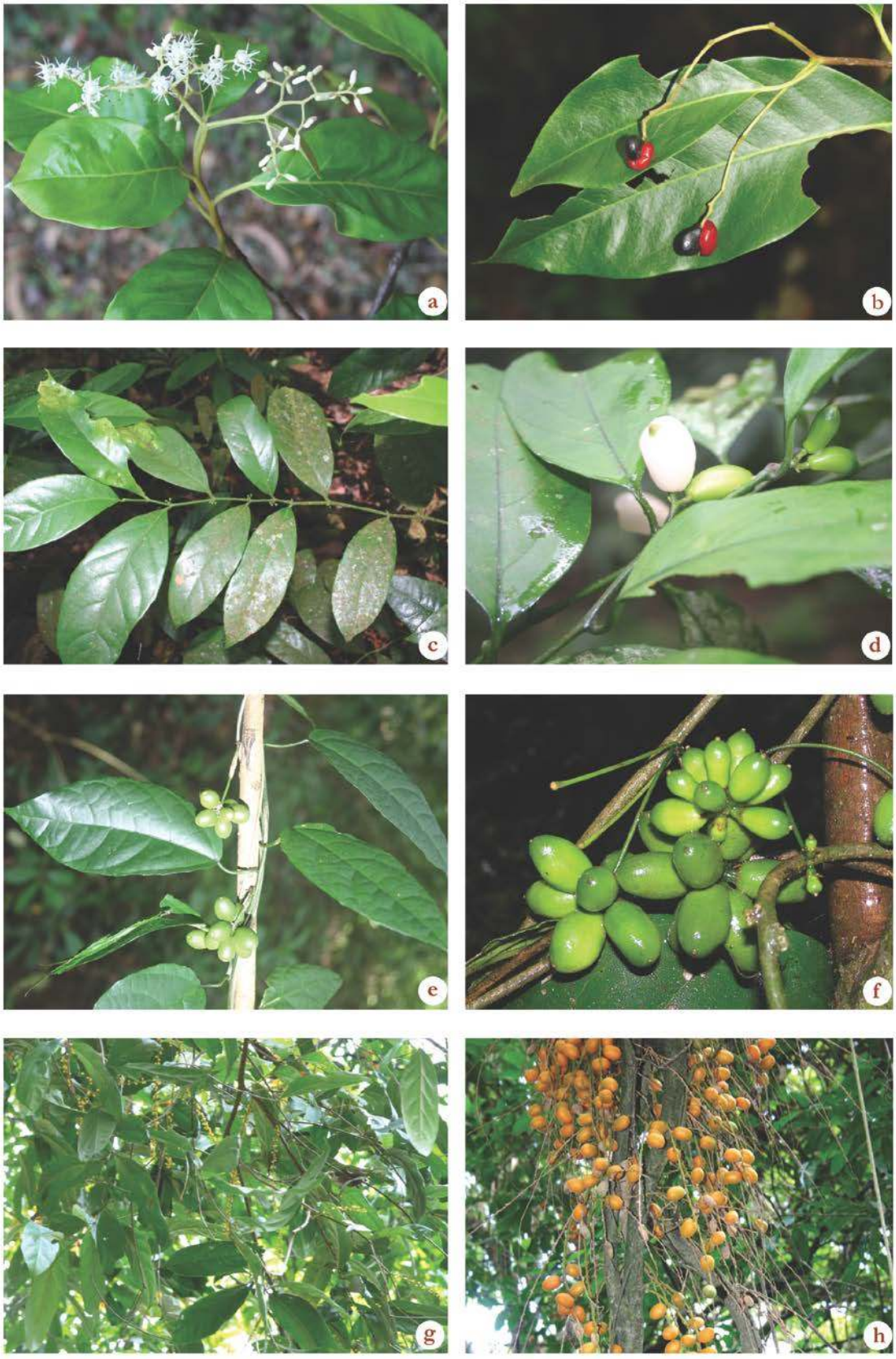

Figure 2.1. The allied taxa of *Nothapodytes* seen in Western Ghats of Kerala
a-b *Apodytes dimidiata*, **c-d** *Gomphandra tetrandra*, **e-f** *Miquelia dentata*, **g-h** *Sarcostigma kleinii*

2.1.2. Chemicals

Acetic acid	:	Merck Specialities, Pvt., Ltd.
Acetonitrile (HPLC)	:	„
Acetone	:	”
Acridine orange	:	„
Agarose	:	Sisco Research Laboratories, India
Agar agar	:	„
Ascorbic acid	:	Merck Specialities, Pvt., Ltd.
2,2-Azobiz-3-ethylbenzthiazoline-6-sulfonic acid (ABTS)	:	Sigma Aldrich, USA
Biotin	:	Sisco Research Laboratories, India
Bovine Serum Albumin (BSA)	:	Pan Biotech, South America
Bromophenol blue	:	Sigma Aldrich, USA
Butanol	:	Merck Specialities, Pvt., Ltd.
Camptothecin (98 % pure)	:	Sigma Aldrich,USA
Carboxymethyl cellulose	:	Sisco Research Laboratories, India
Carrageenan	:	Sigma Aldrich, USA
Chloroform	:	Merck Specialities, Pvt., Ltd.
Cyclophosphamide (CP)	:	Neon Laboratories Ltd, Mumbai, India.
Copper sulphate ($CuSO4.5H_2O$)	:	Merck Specialities, Pvt., Ltd.
Deoxyribose	:	Sisco Research Laboratories, India
Diclofenac	:	Torrent labs Pvt Ltd, Ahmedabad
Dimethyl sulfoxide (DMSO)	:	Merck Specialities, Pvt., Ltd.
3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT)	:	Sigma Aldrich,USA
5-5’dithiobis (2-nitrobenzoic acid)(DTNB)	:	Sisco Research Laboratories, India
Di-potassium hydrogen phosphate (KH_2PO_4)	:	Merck Specialities, Pvt., Ltd.
Ethidium Bromide	:	Genei, Bangalore

Ethylene diamine tetra acetic acid (EDTA)	:	Merck Specialities, Pvt., Ltd.
Ferrous ammonium sulphate (FeSO4)	:	„
Folin's reagent	:	Sisco Research Laboratories, India
Formaldehyde	:	Merck Specialities, Pvt., Ltd.
Genipin powder (99 % pure)	:	Sigma Aldrich,USA
Glutathione reduced (GSH)	:	„
Glucose-6-phosphate	:	„
HEPES buffer	:	„
Hydrochloric acid	:	Merck Specialities, Pvt., Ltd.
Hydrogen peroxide	:	„
L-Glutamine	:	Sigma Aldrich, USA
L-Histidine	:	Sisco Research Laboratories, India
Methanol (HPLC)	:	Merck Specialities, Pvt., Ltd.
Molecular grade water	:	Merck Specialities, Pvt., Ltd.
Nicotinamide adenine dinucleotidePhosphate reduced (NADPH)	:	Sisco Research Laboratories, India
NADP	:	„
Nitrobluetetrazolium (NBT)	:	„
4-nitro-o-phenylendiamine (NPDA)	:	Sigma Aldrich, USA
Nutrient broth	:	Hi-media, Mumbai
Potassium hydroxide (KOH)	:	„
Pottasium chloride (KCl)	:	„
Propidium iodide	:	Sigma Aldrich, USA
Pyridine	:	Merck Specialities, Pvt., Ltd.
Riboflavin	:	Sisco Research Laboratories, India
RNase A	:	Gene laboratories, Pvt Ltd
Rosewell Park MemorialInstitute (RPMI) medium	:	Sigma Aldrich, USA

Silica gel for columnchromatography	:	Merck Specialities, Pvt., Ltd.
Silica gel G	:	„
Sodium acetate	:	”
Sodium azide	:	Sisco Research Laboratories, India
Sodium dodecyl sulfate	:	Merck Specialities, Pvt., Ltd.
Sodium dihydrogen phosphatedehydrate ($Na_2HPO_4.2H_2O$)	:	„
Sodium potassium tartrate	:	„
Sodium bicarboate (Na_2CO_3)	:	„
Sodium hydroxide (NaOH)	:	„
Sulfuric acid	:	„
Thiobarbituric acid (TBA)	:	Hi-Media, Mumbai
Trisbuffer	:	Merck Specialities, Pvt., Ltd.
Tris-HCl	:	”
Trisodium citrate	:	”
Triton X-10	:	„
Trypan blue	:	Spectrum Pvt Ltd, Cochin
Trypsin	:	Sigma Aldrich,USA

2.1.3. Diagnostic reagent kit

Alkaline phosphatase kit	:	Span Diagnostics, Surat, India,
cDNADirectTM	:	GeNei, Bangalore, India
Creatinine kit	:	Span Diagnostics, Surat, India,
Hemoglobin kit	:	Agappe Diagnostics Ltd
Serum glutamate oxaloacetatetransaminase kit	:	Span Diagnostics, Surat, India
Serum glutamate pyruvatetransaminase kit	:	”
Total protein	:	„
Urea kit	:	Agappae Diagnostics
SYBR Premix ExTaq (TliRNaseH Plus)	:	TaKaRa

2.1.4 Instruments

Deep freezer (-70ºC)	:	Eppendof, Germany
Deep freezer (-20ºC)	:	Remi
Electrophoresis unit	:	Bangalore Genei
Fluorescent microscope	:	Leica, German Radicle, Ambala
Gel documentation system	:	Uvitech, UK and Biotech R & D laboratories, Yercaud
High speed cooling centrifuge	:	Remi Laboratory Instruments
Horizontal Laminar flow hood	:	Cleanair, Chennai
Hot air oven	:	Rotex Instruments Pvt Ltd, India
Incubator	:	„
Inverted microscope	:	Leica, German Radicle, Ambala
Microcentrifuge	:	Tarsons Products Private Limited, Kolkata
PCR	:	Eppendorff
pH meter	:	Elico Limited
Phase contrast microscope	:	Leica, German Radicle, Ambala
Soxhlet apparatus	:	Rotex Instruments Pvt Ltd, India
Upright research microscope	:	Meiji, Japan; Labex, Labovision
UV/Visible spectrophotometer	:	PG instruments, UK
RT-PCR	:	AB Biosystem 7300
HPTLC	:	CAMAG system
Flow Cytometer	:	Beckman Coulter, U.S.A
H^1 NMR	:	Bruker 500 MHz
LC-MS	:	LC-1200 infinity-MS-6120 Quadrapole LC/MS, Agilent, USA
HPLC	:	SPD-10A VP (Shimadzu)
GC-MS	:	GC-MS-QP 2010 (Shimadzu)

2.1.5 Cell lines

Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cells, obtained from Adayar Cancer Institute, Chennai, India were maintained in peritoneal cavity of mice at Amala Cancer Research Centre, Thrissur, Kerala. Other cell lines used for study, Vero

(monkey kidney cells), HeLa (human cervical cancer cells) and SKBR3 (human breast cancer cells), Hep-2 (Human larynx cancer cells), Jurkat (human leukemic cells) were obtained from National Centre for Cell Science (NCCS), Pune and maintained in RPMI-1640 medium as recommended by the supplier.

2.1.6 Animals

Swiss albino mice (Male and female 20-30 g) and Wistar rats (180-200 g) were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University (KVASU), Thrissur, Kerala were maintained under standard environmental conditions (22-28ºC, 60-70% relative humidity and a 12 h dark/light cycle) and fed with standard rat feed (Sai Durga Feeds and Foods, Bangalore) and water ad libitum. The entire animal experiments in the study were carried out with the prior approval from Institutional Animal Ethics Committee by strictly following the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by Animal Welfare Division, Government of India (Reg. No. 149/1999/CPCSEA).

2.2 Methods

2.2.1. Preparation of plant extracts

Since leaf is a sustainable source and also to maintain uniformity, leaf part of the plants were chosen for the study. Leaves of plants were dried under shade and powdered using a mixer grinder. Approximately, 30 g of the leaf powder was extracted separately with 250 mL of petroleum benzene, chloroform, acetone and methanol using soxhlet apparatus for 24 h for each extraction. The extracts obtained were concentrated to dryness and the residue collected was dissolved in minimum dimethyl sulphoxide (DMSO) and resuspended in phosphate buffer saline (PBS). Out of these extracts, crude methanolic extracts of plants were found to be most active (cytotoxicity was confirmed by short-term *in vitro* assay and antioxidant property by *in vitro* free radical scavenging assays). Thus, the methanolic extract of all the plants was used for further studies.

2.2.1.1 Preparation of AMF (active methanolic fraction) from *Apodytes dimidata* crude methanolic fraction

The methanolic fraction of A. dimidiata leaf obtained from soxhlet was purified by loading on to a column (600 × 30 mm) packed with 60-120 mesh silica gel and eluted successively by passing various solvents (150 mL each) of different polarities such as petroleum benzene, chloroform, acetone and methanol for further purification. The individual fractions obtained from various solvents were dissolved in DMSO and resuspended in PBS. Of this, the extract obtained in methanol from the column showed the highest cytotoxic and antioxidant activity (cytotoxicity was confirmed by short-term *in vitro* assay and antioxidant property by *in vitro* free radical scavenging assays). Therefore, further studies were carried out using the active methanolic fraction (AMF) obtained from column.

2.2.2 *In vitro* antioxidant activity assays

2.2.2.1 Superoxide free radical scavenging assay

The ability of the plant extracts to scavenge superoxide ion was assessed by the method proposed by McCord and Fridovich, 1969. Plant extracts varying from concentration ranges 50-500 µg/mL was made up to a final volume of 3mL reaction mixture containing 50 µM NBT, 2 µM riboflavin, 6 µM EDTA, 0.0015% NaCN and phosphate buffer (67 mM, pH 7.8). A control was also kept without adding the plant extract. After adding all the ingredients, the optical density (OD) of the sample was measured at 560 nm under dark. Further, they were illuminated with an incandescent lamp for 15-20 min and optical density was measured against the same wavelength. The 50% inhibitory concentration (IC_{50}) of the test material was calculated using the formula

$$\% \text{ inhibition} = \frac{\text{Absorbance of control - Absorbance of test material}}{\text{Absorbance of control}} \times 100$$

2.2.2.2 Hydroxyl radical scavenging assay

Scavenging ability of the plant extracts for hydroxyl radicals liberated *in vitro* assay was determined by the method suggested by Ohkawa et al (1979). The deoxyribose in the reaction mixture (containing 2.8 mM deoxyribose, 0.1 mM $FeCl_3$, 1 mM H_2O_2, 0.1 mM

EDTA, 0.1 mM ascorbic acid, 20 mM KH2PO4-KOH of pH 7.4 in 1 mL volume with plant extract of various concentrations) gets degraded to thiobarbituric acid reacting substance (TBARS) by the effect of hydroxyl radicals generated from Fe2+/ascorbate/H_2O_2 system when incubated for 1 h at 37°C (Kunchandy and Rao, 1990). It is the formation of TBARS that is measured at 535 nm and compared with the control and extract was added in experimental groups to determine the IC_{50} value using the previous formula.

2.2.2.3 Inhibition of lipid peroxidation

The ability of the extract to inhibit the lipid peroxidation in the liver homogenate of rat in an *in vitro* assay system was assessed. The homogenate (25% w/v) was prepared in 20 mM Tris-HCl buffer (pH 7.0). Then, 0.1 mL of homogenate, 0.16 mM $FeSO_4(NH_4)2SO_46H_2O$, 30 mM KCl, 0.06 mM ascorbic acid and various concentrations of plant extracts were added to make upto a final volume of 500 μL. The reaction mixture was incubated for 1 h at 37°C. Further, 100 μL was removed and 0.2 mL 8% SDS, 1.5 mL 0.8% TBA, and 1.5 mL 20% acetic acid (pH 3.5) was made upto a total volume of 4 mL by distilled water. The tubes were then kept in a water bath at 95°C for 1 h. The tubes were then cooled, added 1 mL distilled water and 5 mL butanol: pyridine mixture (15:1, v/v). It was mixed well and was centrifuged at 3,500 rpm for 10 min. Each tube contained an upper organic layer and was removed for measuring the absorbance at 532 nm. Using the optical density of the control and sample, the inhibition in lipid peroxidation was calculated.

2.2.2.4 DPPH radical scavenging ability

The method determined by Williams et al (1995) was followed to examine the antioxidant ability of the extract. Stable radical, 2, 2-diphenyl-1-picryl hydrazyl (DPPH) suspended in methanol serves as the substrate in the reaction mixture (375 µL DPPH and made upto 2 mL by methanol for control, and for the test add plant extract and made up to 2 mL by methanol). DPPH free radical formed gets reduced to a non-radical form DPPH-H by the antioxidants which donate hydrogen. A blank sample containing DPPH and ethanol alone was prepared, and in the treated tubes plant extracts was added to make upto a final

volume of 3 mL. Dark purple coloured solution was seen in the control tube which gradually disappeared as the concentration of plant extract increased. Absorbance was measured at 515 nm after 20 min incubation at room temperature. Percentage inhibition was calculated using the previously used equation.

2.2.2.5 ABTS free radical scavenging assay

The method described by Alzoreky and Nakahara (2001) was used to measure the formation of ABTS (2, 2-azobis-3-ethylbenthiozoline-6-sulfonic acid) radical. The principle working mechanism involved is that the ABTS gets oxidised to its cationic form, $ABTS^+$ by ferryl myoglobin generated by the reaction of H_2O_2 and metmyoglobin. The $ABTS^+$ has slight green/blue colour which gets diminished by the presence of plant extract or other antioxidants. The stock solution comprising of 400 μM myoglobin (MbIII), 500 μM ABTS diammonium salt, 740 μM potassium ferrycyanaide and 450 μM H_2O_2 were prepared in PBS (pH 7.4). Myoglobin was used to prepare metmyglobin by mixing with equal volume of potassium ferrycyanide solution. Various concentrations of plant extracts, ABTS (150 μM), MbIII (2.25 μM) and PBS was added, made up to a total volume of 2 mL. Further, 75 μM H_2O_2 was added to start up the reaction and absorbance was obtained at 734 nm.

2.2.2.6 Ferric reducing antioxidant power (FRAP) assay

The reducing power of the extract was determined by the method of Nenadis et al (2007). FRAP reagent was prepared by mixing 2.5 mL 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), 20 mM 2.5 mL ferric chloride solution, and 25 mL acetate buffer. The reaction mixture was set up by adding 900 μL of FRAP reagent (freshly prepared) and different concentrations of plant extracts, incubated at 37°C for 15 min. The reading was taken at 595 nm and using a standard graph, the equivalent ferric reducing ability of the extract was calculated.

2.2.2.7 Nitric oxide radical inhibition assay

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in BALB/c mice. Macrophages were washed with PBS and resuspended in RPMI-1640 with 10% FCS. The cells were plated in 96-well culture plates and incubated for 2 h at 37°C, in a 5% CO_2

atmosphere. After incubation, non-adherent cells were removed and the adherent macrophages were incubated (2×10^6 cells/well) in complete medium (RPMI-1640, 10% FCS, 100 µg/mL streptomycin and penicillin, 2 mM glutamine). Macrophages were cultured in the presence of various concentrations of AMF and the standard drug, ascorbic acid. After 24 h, plates were centrifuged and the supernatant was used for the estimation of NO production by the Griess reagent method with a slight modification (Nathan, 1989). In brief, 50 µL of supernatant was mixed with 1% sulfanilamide/0.1% naphthylethylenediamine/2.5% H_3PO_4 and was incubated at room temperature for 15 min to form a chromophore. The control sample contained the same solution mixture without plant extract. The absorbance of the samples was measured at 550 nm. The percentage inhibition was calculated according to the following equation: % inhibition = (1-Absorbance of the extract ($A_{1)}$/Absorbance of the control (A_0) × 100. The concentration of sample required to inhibit 50% of nitric oxide radical (IC_{50} value) was calculated by plotting the graph. All the above experiments were done in triplicates and ascorbic acid was used as the standard.

2.2.3 Estimation of Biochemical parameters in *in vivo* studies

2.2.3.1 Determination of haematological parameters

2.2.3.1.1 Determination of haemoglobin

The concentration of haemoglobin in blood was determined using Agappe Diagnostic Kit. According to the proposed protocol of Drabkin and Austin, 1932. To 2.5 mL of Drabkin's reagent, 10 µL of fresh blood was added. This was followed by a short incubation for 5 min at room temperature and the absorbance was seen at 546 nm against blank. A standard was set in the same manner. The working principle is that the presence of potassium ferricyanide and potassium cyanide in the reaction mixture converts haemoglobin to cyanmethaemoglobin, whose formation is directly proportional to the concentration of haemoglobin and was calculated using the formula

$$\text{Concentration of Hb (mg/dl)} = \frac{\text{OD of test sample}}{\text{OD of standard}} \times 15$$

2.2.3.1.2 Determining total White Blood Cell (WBC) count

The method described by Chaudhari, 2000 was followed to determine the total white blood cell count (TC). The blood samples (20 µL) were added to 380 µL of diluent (Turk's fluid - 2% acetic acid in distilled water), mixed and allowed to stand at room temperature for 3-4 min. Then, 10 µL of the mixture was loaded into the counting chamber, covered with cover slide, allowed to settle for a minute and viewed under microscope. The acetic acid which is present in the Turk's fluid lyses the red blood cells and all the nucleated cells become intact and stained by crystal violet. The cells present in the 4 large corners were counted and total count was calculated as

Total WBC count/mm^3 = Total no. of cells counted x 50 count/mm^3.

2.2.3.2 Preparation of haemolysate

After the experiment, blood were collected from the animals by cardiac puncture and transferred to heparinised tubes, centrifuged at 1500 rpm for 20 min. After removing the top portion, the bottom layer which contained the packed erythrocytes was washed two times with phosphate buffer saline (pH 7.4). A known volume of red blood cells of mice were lysed with hypotonic phosphate buffer. After removing the red blood cell debris by centrifugation (3000 rpm for 15 min), the haemolysates were recovered and immediately used for biochemical analysis.

2.2.3.3 Preparation of tissue homogenate

For various experiments, different parts and organs of the experimental animals were used for the analysis. After sacrifice, liver, kidney and mucosa of inner stomach were collected, washed in saline and pressed mildly against a blotting paper. Each one was weighed in an analytical balance and a homogenate (10%) was prepared using Tris-buffer (pH 7.0). The homogenate was centrifuged around 9000-10,000 rpm for 25 min at 4°C, supernatant was slowly collected and utilised for the analysis of various biochemical parameters like catalase, superoxide dismutase, glutathione peroxidise, glutathione reductase and malnoldehyde.

2.2.3.4 Determination of superoxide dismutase (SOD) activity in the blood and tissue

SOD activity was determined according to the method of McCord and Fridovich (1969).

Principle

The assay involves a photo-illumination process by which riboflavin gets reduced to flavin in the presence of EDTA. The flavin gets reoxidised and also reduces oxygen to O_2 which further reacts with NBT which get reduced to form a formazan blue. The formation of formazan is proportional to the activity of SOD in the test material.

Method

To haemolyse the packed RBCs (100 µL), 900 µL of cold water was added. Then treated with 250 µL of $CHCl_3$ and 0.5 mL of ethanol and then thoroughly mixed to remove haemoglobin. This was followed by centrifugation at 15,000 rpm for 1 hr at 40°C. To 100 µL of the supernatant, added 200 µL of 0.1 M EDTA (containing 0.0015% NaCN), 100 µL of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.95 mL. The absorbance was immediately measured at 560 nm, soon after 0.05 mL of riboflavin was added. The tubes were illuminated with an incandescent lamp for 15 min and again absorbance was noted at the same wavelength. The concentration required to scavenge 50% was calculated by comparing the control (untreated) with the test sample and expressed in the units, U/g Hb. In the case of tissue homogenate, the volume used was 100 µL and the same procedure was followed. The IC_{50} of the sample for scavenging superoxide anion was considered as 1 unit of enzyme activity and expressed in units, U/mg protein.

2.2.3.5 Determination of catalase activity in the blood and in the tissue homogenate

The method of Aebi (1974) was followed to analyse the catalase activity in blood.

Principle

The decomposition of H_2O_2 catalysed by the enzyme, catalase at absorption maxima of 240 nm was determined to analyse catalase activity. The absorption decreases as the decomposition of H_2O_2 increases. The decrease in extinction per unit time is the measure of the catalase enzyme activity.

Procedure

After washing with saline solution, a stock of the packed RBC lysate was prepared in ice cold water containing approximately 5 g Hb/dL. The haemolysate and sodium-potassium

phosphate buffer (0.05 M, pH 7) was diluted by taking in the ratio1:500 just before the assay. Then 1 mL of buffer and 2 mL of haemolysate served as the reference cuvette and 2 mL diluted haemolysate, and 1 mL buffer served as the test cuvette. The reaction was initiated by adding freshly prepared 1 mL of H_2O_2 (30 mM in the buffer) to the test solution, mixed well and the decrease in extinction was monitored for four times at an interval gap of 15 sec for 1 min. Catalase was expressed in k/g Hb, where k is a rate constant of 1st order reaction.

$$\text{Catalase (k/g Hb)} = \frac{2.303 \times (\log E1 - \log E2) \times \text{dil. Factor}}{15 \times \text{g Hb/mL of blood}}$$

Where, E_1 is E_{240} at t=0 and E_2 is E_{240} at t=15 sec.

Catalase activity in the tissue: It was determined by the method of Beers and Sizer, 1952. To 100 μL of tissue homogenate, 1.9 mL of phosphate buffer (pH 7) was added. Immediately after adding 1 mL of H_2O_2 solution in buffer, the decrease inextinction was measured for 3 times in a total 3 min time, at 1 min gap. In the reference cuvette, 100 μL of tissue homogenate and 2.9 mL of buffer was added and taken as the sample control. Activity of catalase was determined using the molar extinction coefficient of 43.6. Specific activity at 25°C was expressed in mmoles of H_2O_2 consumed/min/mg of protein sample.

$$\text{(U/mg protein)} = \frac{\Delta A \text{ /min} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}$$

2.2.3.6 Determination of reduced glutathione (GSH) in the blood and tissue

Reduced glutathione in blood and tissue was determined according to the method of Moron et al (1979).

Principle

A yellow coloured complex is formed when GSH reacts with dithionitrobenzene (DTNB) and the absorbance was measured at 412 nm.

Procedure

Haemolysate of the heparinised blood was prepared in distilled water. Haemolysate/tissue homogenate (500 µL) was mixed with 125 µL of 25% TCA and cooled on ice for 5 min.

The solution was again diluted with 600 µL of 5% TCA followed by a centrifugation at 3000 g for 5-10 min, to settle down the precipitate. To the supernatant (150 µL), 350 µL of sodium phosphate buffer (0.2 M, pH 8.0) and 1.0 mL of DTNB (0.6 mM in 0.2 M, pH 8.0 phosphate buffer) was added and mixed gently. The resultant mixture produced a yellow colour which was measured at 412 nm. Instead of the supernatant, 5% TCA served as the blank. By plotting different concentrations (10-50 nmoles) of GSH, a standard graph was drawn and the GSH content was deduced and expressed as nmol/mL of blood and as nmol/mg protein for tissue.

2.2.3.7 Determination of glutathione peroxidase (GPx) activity the tissue homogenate

The method of Hafemann et al (1974) was adopted to analyse glutathione peroxidase activity

Principle

In the presence of H_2O_2 and NaN_3, the GSH content in the sample decreases as the incubation progresses. This is utilised to estimate the GSH activity. The GPx enzyme degrades the H_2O_2 in presence of GSH by the following reaction;

$H_2O_2 + 2\ GSH \rightarrow 2H_2O + 2GSSG$

The GSH that remained was measured by its reaction with DTNB

Procedure

To 0.02 mL of heparinised blood/tissue homogenate (100 mL), 100 µL of 1.2 mM H_2O_2, 100 µL of 5 mM GSH, 0.1 mL of 25 mM NaN3 and phosphate buffer (1 M, pH 7.0) was taken in a total volume of 2.5 mL and kept at 37°C for 6-10 min. To this, 2.0 mL of 1.65% HPO_3^{2-} was added to stop the reaction, centrifuged at 3000 rpm for 10 min. To 2 mL of the supernatant, equal volume of 0.4 M Na_2HPO_4 was added, followed by 1 mL of 1 mM DTNB (in buffer). The absorbance of the yellow coloured complex was measured at 412 nm against distilled water after incubation (10 min at 37°C). A sample without the homogenate/haemosylate was kept as blank. The GPx activity was expressed as U/mg protein for tissue homogenate and U/g Hb for blood. This was estimated using the formula,

$$\text{The GPx activity} = \frac{\text{O. D of blank-O. D of sample x 1}}{\text{0.001x g Hb or mg protein x10}} \times 1000$$

2.2.3.8 Determination of the total protein in tissue homogenate

The method of Lowry et al (1951) was followed to determine the protein content in the tissue.

Principle

The Folin-Ciocalteau reagent contains phosphomolybdate and phosphotungstate and they get reduced by the action of tyrosine and tryptophan residues of protein present in the tissue in an alkaline medium to give a bluish purple colour at 660 nm.

Procedure

A reaction mixture consisting of 10 µL homogenate mixed with 990 µL of distilled water, 5 mL of alkaline $CuSO_4$ (0.5 % $CuSO_4$ in 1% sodium potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH was mixed in the ratio 1:50 and kept at room temperature for 10 min. To this, 500 µL of Folin-Ciocalteau reagent was added and waited for 30 min. Then absorbance was measured at 660 nm against the reagent blank. Using different concentrations of bovine serum albumin (BSA), a standard graph was plotted and the protein content in the tissue was estimated from the graph plotted.

2.2.3.9 Estimation of lipid peroxidation in tissue /blood

By following the method of Ohkawa *et al* (1979), the lipid peroxidation level in the tissue was measured as malondialdehyde (MDA)

Principle

The TBA and the tissue malondialdehyde were mixed together for the reaction to take place. MDA-TBAadduct formed in acidic medium was extracted to theorganic layer and the absorbance was measured at 532 nm.

Procedure

A reaction mixture containing 0.4 mL of the tissue homogenate, 1500 μL of 0.80% TBA, 1500 μL of acetic acid (20%, pH 3.5) was made upto a total of 4 mL with distilled water and kept in a boiling water bath at 95^{o}C for 1 h. After cooling, 1 mL of distilled water was

added. A mixture of butanol: pyridine was prepared in the ratio 15:1. From this, 5 mL was added to the reaction tube, shakenand centrifuged at 3000 rpm for 10 min. From the top layer, supernatant was removed and absorbance was measured at 532 nm against butanol: pyridine mixture. Lipid peroxidation was calculated and expressed in units, nmol of MDA/mg protein with the help of a standard graph prepared by using different concentrations (1-10 nmol) of 1'1'3'3'-tetramethoxypropane in 1 mL distilled water.

2.2.3.10 Determination of glutamate oxaloacetate transaminase (SGOT) activity in serum

SGPOT/Aspartate Transaminase (AST) activity was estimated by the method of Reitman and Frankle (1957).

Principle

L-aspartate and α-ketoglutarate reacts to form the products, oxaloacetate and glutamate. This reaction is catalysed by glutamate oxaloacetate transaminase present in the serum. Oxaloacetate, the product formed during the reaction is unstable and gets converted to pyruvate and reacts with 2, 4, dinitrophenyl hydrazine to form a brown coloured phenyl hydrazone. The absorbance is measured at 505 nm.

Procedure

Reagents used were from Span Diagnostic Kit. Added 0.1 mL of serum to 0.5 mL of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-aspartate in 100 mL phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 1 h. After that, 0.5 mL of dinitrophenyl hydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min. Then added 0.4 mL of NaOH and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube was set up in the same manner. The enzyme activity was determined from the (sodium pyruvate, 2 mM) calibration curve of the standard and expressed in IU/L of serum.

2.2.3.11 Analysis of serum glutamate pyruvate transaminase (SGPT) activity

Serum glutamate pyruvate was determined according to the method of Reitman and Frankle (1957).

Principle

The reaction between L-alanine and α-ketoglutarate is catalysed by glutamate pyruvate transaminase present in the serum to form pyruvate and glutamate. Then, pyruvate was treated with 2, 4,-dinitrophenylhydrazine. The absorbance of the resultant brown coloured phenylhydrazone is measured at 505 nm under alkaline condition.

Procedure

Reagents used were from Span Diagnostic Kit. Added 0.1 mL of serum to 0.5 mL of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 mL phosphate buffer 0.1 M, pH 7.4) and incubated at 37°C for 30 min. After incubation, 0.5 mL of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mixed and kept at room temperature for 20 min. After adding 0.4 mL of NaOH, the absorbance was measured at 505 nm after 10 min at using the reagent blank. A control tube containing buffered substrate was treated with serum and followed the same procedure. The enzyme activity (IU/L of serum) was estimated using a standard (sodium pyruvate, 2 mM) calibration curve.

2.2.3.12 Analysis of serum alkaline phosphatase (ALP) activity

Method

ALP was analysed by Kind and King, 1954.

Principle

Under alkaline condition (pH 10), the alkaline phosphatase present in the serum reacts with disodium phenyl phosphate and liberate phenol. A red coloured complex is formed when phenol reacts with 4-aminoantipyrene in thepresence of alkaline oxidizing agent, which can be measured at 510 nm against blank.

Procedure

Reagents were purchased from Span Diagnostic Kit. Serum (50 μL) was incubated with 500 μL of the buffered substrate (1 mL of 0.254 g of disodium phenyl phosphate dihydrate mixed with 1 mL of carbonate buffer, P^H 10), added 1.54 mL distilled water and incubated

at 37°C for 15 min. Soon after, 2 mL chromogen (1 mL of 0.6 g 4-aminoantipyrene\ distilled water and 1 mL of potassium ferricyanide in 2.4 g\dl water) was added and the absorbance was taken at 510 nm. Phenol (10%) was used as standard to prepare the calibration curve. By multiplying with a factor of 7.1, the activity (KA/dl) is expressed in IU/l.

2.2.3.13 Determination of serum creatinine

Method**:** Jaffe's reaction method (Moss *et al.*, 1975)

Principle

Creatinine reacts with picric acid in alkaline conditions to give a yellow to orange coloured compound, having absorption maxima at 500 nm. The concentration of the coloured product over a period of time gives the measure of the creatinine concentration.

Commercially available kit was obtained from Span Diagnostics. Briefly, 2.0 mL of picric acid reagent was added to 200 μL of serum which results in deproteinization of specimen, then mixed well and centrifuged at 3000 rpm to obtain a clear supernatant. Then, 100 μL of buffer reagent was added to 1.1 mL of supernatant, 100 μL of standard creatinine and 100 μL of distilled water to prepare test, standard and blank, respectively. To both blank and standard, 1.0 mL of picric acid reagent was added. The mixture was shaken and kept at maintained at room temperature for 20 min. The absorbance of the coloured product was read at 520 nm spectrophotometrically. Using the following formula, the serum creatinine was calculated.

$$\text{Concentration (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 2$$

2.2.3.14 Estimation of urea in serum

Serum urea was analysed using the kit of Span Diagnostics Ltd.

Principle

In the presence of water and the enzyme urease, urea gets hydrolysed to ammonia and carbon dioxide. Ammonia so formed, reacts with hypocholite and phenolic chromogen

catalysed by sodium nitroprusside to form coloured indophenols under alkaline conditions and this can bemeasured at 578 nm. The intensity of colour is proportional to the concentration of urea present in the sample.

Procedure

A reagent solution comprising phosphate buffer (20 mM), urease (200000 U/L), sodium nitroprusside 3.2 (mM), sodium salicylate (60 mM/L) was provided in the kit. Then, 1.5 mL of this reagent solution was mixed with 10 l of serum and incubated at 37°C for 3 min. To this, 1.5 mL of solution 2 containing 0.2% sodium hypochlorite and sodium chloride was addedand again incubated at 37°C for 5 min. The intensity of chromogen/coloured complex formed was measured at 578 nm.

The concentration was calculated using the formula

$$\text{Urea concentration (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50$$

2.2.3.15 Estimation of blood urea

Method**:** Urea of blood was estimated by the method of Weatherburn, 1967

Principle

In the presence of water and the enzyme urease, urea gets hydrolysed to ammonia and carbon dioxide. The ammonia formed, reacts with hypocholite and phenolic chromogen catalysed by sodium nitroprusside to form coloured indophenols under alkaline conditions and this can be measured at 578 nm. The intensity ofcolour is relative to the concentration of urea present in the sample.

Procedure

The blood urea was estimated using a kit from Span Diagnostics. One millilitre of working reagent-1 (urease reagent, and a mixture of salicylate, nitroprusside and hypochlorite) was added to 10 μL of serum, 10 μL of standard urea (40 mg/dl) and 10 μL of H_2O to prepare

test, standard and blank, respectively. All the tubes were mixed well and incubated at 38°C for 5 min. Then, 1 mL each from reagent 2 containing alkaline buffer was added to all the test tubes and incubated at 38°C for 5 min. The intensity of chromogen/coloured complex formed was measured at 578 nm. The concentration was calculated using the formula,

Blood urea (mg/mL) = Ab of test/ab of standard × 40.

2.2.3.16 Determination of serum protein

Method used: Bradford *et al* (1976). Protein estimation kit was obtained from Bangalore Genei.

Principle

The principle underlying is that a dye, Coomassie brilliant blue G250 can bind to the protein and gets converted to an ionic form (blue colour). The amount of protein can be estimated by measuring the coloured product at 595 or 625nm.

Procedure

Serum (10 μL) was made up to 200 μL using distilled water and added 2 mL of Bradford reagent mixed well and kept for 10 min incubation at room temperature. Absorbance was measured at a wavelength of 595 nm against the reagent blank. Form the standard graph drawn using different concentrations (0.1-0.5 mg/mL) of bovine serum albumin (BSA), the concentration of protein can be calculated.

2.2.4 Histopathological examination

After experiments, the animals were sacrificed and portions of liver, kidney and stomach mucosa were excised using clean sterilised scissors and surgical blade. This was fixed in 10% neutral buffered formalin solution which was then treated with increasing grades of alcohol and cleared in xylene. Sections of thickness varying from 3-4 μm were taken using a microtome by impregnating the tissue in paraffin wax. The sections moulded in DPX after hematoxillin/eosine staining was viewed under light microscope.

2.2.5 Anti-lipoxygenase assay

The *in vitro* assay is based on the peroxidation of polyunsaturated fatty acids by lipoxygenase enzyme in arachidonic acid pathway. The assay system is comprised of a total 1 mL volume of phosphate buffered saline (PBS) containing 50 U lipoxygenase and 100 nmol linoleic acid as substrate. Different concentrations of drug were added and ascorbic acid was used as the standard drug. The reaction was allowed to take place at room temperature for 1 h. The conjugated diene formed was measured at 234 nm using UV spectrophotometer and the 50% inhibition (IC_{50} value) was determined.

2.2.6 Analysis of phytochemical constituents

2.2.6.1 Preliminary phytochemical screening

Phytochemical screening was done by following the standard methods (Harbone, 1973; Sofowora, 1993; Evans *et al*., 2002).

2.2.6.1.1 Test for carbohydrate

The occurrence of carbohydrates was confirmed using by Benedicts test. To 500 μL of the filtrate, Benedict's reagent (0.5 mL) was added. The resultant mixture was heated on boiling water bath for 2 min. Formation of red coloured precipitate indicatesthe presence of sugar.

2.2.6.1.2 Test for alkaloids

Extracts were dissolved in dilute hydrochloric acid and filtered. Dragendroff's reagent (solution of potassium bismuth iodide) was added to the filtrate. Formation of red colour precipitate confirms the presence of alkaloids.

2.2.6.1.3 Test for flavonoids

Few drops of dilute sodium hydroxide solution were added to plant extract (1 mL). A dark yellow colouration was seen which became colourless on slowly adding few drops of dilute acid. This shows the presence of flavonoids.

2.2.6.1.4 Test for tannins

Approximately, 0.5 mg of dried and powdered, test samples was boiled in 20 mL of water, filtered and 0.1% ferric chloride was added as few drops. Formation of brownish green or blue black colour indicates the presence of tannins.

2.2.5.1.5 Detection of saponins

Froth formation assay was used to detect the presence of saponins. Extracts were diluted with distilled water (20 mL) and was vigorously shaken in for 15-20 min. Formation of 1 cm layer of foam indicates the presence of saponins.

2.2.6.1.6 Detection of phytosterols

Chloroform was added to the extracts and filtered. The filtrate collected was treated with few drops of concentrated H_2SO_4, shaken well and allowed to stand. The presence of phytosterols can be identified by the formation of a golden yellow colour.

2.2.6.1.7 Detection of diterpenes

Extracts were dissolved in water and treated with 3-4 drops of $CuSO_4$ solution. The appearance of emerald green colour shows the presence of diterpenes.

2.2.6.2. Estimation of total phenol and flavonoid contents

Total flavonoid content was analyzed by colorimetric method (Chang *et al.*, 2002). Briefly, the plant extract (0.5 mL) was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and made up to 3000 μL by adding 2.8 mL of distilled water. The reaction mixture was maintained at room temperature for 30.0 min and the absorbance of mixture was measured at 415 nm. A calibration curve was drawn using quercetin at concentrations of 50 to 250 µg/mL in methanol. Flavonoid content was expressed as µg/mg quercetin equivalent (QE) of dry extract.

Total phenolic content was determined by using Folin-Ciocalteu reagent (as modified by Ainsworth and Gillespe, 2007). Gallic acid (20-100 µg/mL) was used as a reference standard for preparing calibration curve. A volume of 500 μL of the extract (100 µg/mL)

was mixed with 2 mL of the Folin-Ciocalteu reagent (diluted in the ratio 1:10 with deionized water), neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The mixture was incubated at room temperature (for 30 min) with intermittent shaking and the absorbance of the resulting blue colour was measured at 765 nm. Total phenolic content was determined from the linear equation of a standard curve prepared with Gallic acid. The total content of the l phenolic compounds is expressed as µg/mg Gallic acid equivalent (GAE) of dry extract.

2.2.6.3. GC-MS analysis

The plant extract was subjected to GC-MS analysis. Chromatographic separation was carried out with the instrument GC-MS-QP 2010 (Shimadzu) with Db 30.0 column (0.25 µm diameter × 0.25 µm thickness). The oven temperature -50°C with an increase of 10°C/min to 200°C, then 5°C/min to 250°C, ending with a 35 min isothermal at 250°C. Mass spectra was taken at 70 eV; a scan interval of 0.5 s and scan range from 40 - 1000 m/z. The carrier gas used was Helium at 99.999% pressure with flow 1.0 mL/min and electronic pressure control on. Then, methanol dissolved samples were injected automatically (2 µL). Interpretation on GC-MS analysis was done using the database prepared by National Institute Standard and Technology (NIST). The spectra of the components was compared with that stored in the NIST library to ascertain the name, molecular weight and retention time of the components, present in the sample.

2.2.6.4 Absorption spectra analysis

The absorption spectrum analysis was done using a UV-visible double beam spectrophotometer (P G Instruments 80^{+}, UK). The sample was dissolved in methanol taken at appropriate concentrations. The cuvette containing only methanol was kept as blank. The λ max of was detected by scanning between wavelengths ranging from 200-900.

2.2.6.5 TLC analysis

Using different solvents, separation of the sample was done to find out the most suitable solvent system. After ascertaining the appropriate solvent which gave the maximum separation of constituents, it was further used for the study. Glass plates coated with silica

and silica gel plate (20 × 20 cm^2; Merck, silica gel 60 F_{254}) were used for the study. Approximately, 10 μg of the sample was spotted on silica gel plate (20 × 20 cm^2; Merck, silica gel 60 F_{254}) and was developed using the solvent system. The separation profile was visualised under UV at 366 nm and bands obtained were marked with pencil, carefully scrapped off, eluted with methanol and centrifuged at 5,000 rpm for 8-10 min. The supernatant collected was then filtered using syringe filter (Millex GV filter unit 0.22 μm, Millipore), made silica free and air dried. The whole process was done under dim light conditions. While marking, the Rf value of individual bands were calculated using the formula,

$$Rf = \frac{\text{Distance from the origin to the individual band}}{\text{Distance from the origin to the solvent front}}$$

2.2.6.6 HPTLC analysis

High performance thin layer chromatography (HPTLC) analysis was performed using a CAMAG system. The samples (10 μL each) were spotted onto silica gel plate (20×20 cm^2; Merck, silica gel 60 F_{254}) from the stocks. The samples were dissolved in methanol and developed in the solvent system used for TLC. After separation, the bands were visualised under TL-600 UV at 366 and 254 nm and the R_f value of individual bands were detected.

2.2.6.7 HPLC analysis

The samples were dissolved in methanol and passed through the membrane filter (Nylon 6, 6 membrane 0.20 μm, Pall Life Sciences, Mumbai, India). The samples (20 μL) were injected to the end-capped, RPC-18, 5 μm size, 250 × 4.60 mm column and eluted by the solvent system, acetonitrile/ortho-phosphoric acid (0.01 M, pH 4) in the ratio of 30:70 with flow rate 1 mL/min using 10AT VP, HPLC system equipped with a UV-VIS detector. The column temperature was kept at 25°C during running and the absorbance of the eluted samples was measured at 240 nm.

2.2.6.8 LC-MS analysis

The samples were analyzed by LC-MS (LC-1200 infinity-MS-6120 Quadrapole LC/MS, Agilent, USA). LC conditions of the analysis were as, Column: Eclipse Plus C18 4.60×250

mm, 5 μm, mobile phase: 5 mM ammonium formate: acetonitrile (50:50), flow rate 1 mL /min, wavelength 240 nm, diluent-methanol, column oven temperature -25°C. Range varying from 100 to 700 m/z was scanned. MSD detector was used and the analysis was performed by Chemstation Software (Agilent, USA).

2.2.6.9 Nuclear Magnetic Resonance ^{1}H NMR ($CDCl_3$) analysis

The concentrates of the samples were crystallized in appropriate solvent. A 500 MHz FT NMR Spectrometer was used for obtaining ^{1}HNMR spectrum in deuterated chloroform ($CDCl_3$). The chemical shifts in the peak were recorded as peak shifts in ppm.

2.2.7. Cytotoxicity assays

2.2.7.1. Trypan blue exclusion method

Methods: Gupta and Bhattacharya, 1978.

Principle

Trypan blue is impermeable to live cells because they have intact plasma membrane. Due to the action of any drug/samples the dye gets inside the dead cell. Since the dye is blue in colour, the dead cells appear as blue under microscope.

Procedure

The 1x10^6 number of cells were taken and different concentration of the drug (dissolved in minimum amount of DMSO and made up with PBS) were added and was made to 1 mL using PBS, followed by incubation at 37°C for about 3 h. The dye, trypan blue (100 μl) was added after the incubation period. The total number of stained and unstained cells (live) wascounted separately.

$$\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Total number of dead and live cells}} \times 100$$

2.2.7.2 MTT assay

Principle

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT) is converted by the microsomal enzymes into a formazan product and the amount of formazan formed is an index of live cells present in the cell population (Cole, 1986).

Procedure

Approximately, 1×10^6 cells were seeded in 96 well plates and incubated to achieve 80% confluency. The cells were then exposed to different concentrations of test materials for 48 h and allowed to grow further at 37ºC for 48 h in RPMI medium. MTT was added into the well plates and incubated for 4 h. DMSO (0.1% in PBS) was kept as the vehicle control. After centrifugation, the supernatant was removed and the precipitate of the converted dye was solubilized in DMSO. The absorbance of the coloured product was measured at 570 nm and the percentage viability was calculated. The average absorbance of untreated negative control was taken as a 100% cell survival. The experiment was done intriplicate. The percentage of cell viability was determinedby comparing the percentage death of treated cell population with the untreated control.

$$\% \text{ Dead cells} = 100 - \frac{\text{O.D of drug treated}}{\text{O.D of the control}} \times 100$$

2.2.8 Methods for apoptotic evaluation

2.2.8.1 Morphological staining

Cells ($1x10^6$) were plated in 6-well plate and are incubated with sample concentrations of different ranges for 48 h. After incubation, the plates were observed under inverted microscope to find out the major morphological changes of treated and untreated cells. Then the cells were washed with cold PBS, trypsinized and harvested. The differential staining was performed by adding 10 μL of acridine orange/ethidium bromide (AO/EtBr solution (1:1, v/v) to the cell suspension and waited for 2-3 min. To find out the apoptotic

related morphological changes, the cells wereanalyzed under a fluorescent microscope (Lieca microsystem, Germany) with a blue excitation filter (480 nm) and photographed. For Hoechst staining, an aliquot (5 μL) of cells was stained with 0.5 μg/mL of Hoechst 33342 dye for 10 min and viewed under fluorescence microscope and photographed

2.2.8.2 Caspases expression study using RTPCR

2.2.8.2.1 cDNA synthesis from HeLa cells

HeLa cells were treated with various concentrations of drug for 48 h and the cDNA was synthesised using the kit cDNA DirectTM (GeNei Bangalore). The cells were harvested by adding enough trypsin to detach the cells, incubated for 5 min at 37°C and added 2X volume of serum containing fresh medium to deactivate trypsin. Centrifuged the cell suspension to remove the medium and washed the cell pellet with cold PBS twice. The cell density was adjusted to 5000-7000 cells/µL, and uniformity was maintained. Then, 100 µL of the lysis buffer (provided by the kit manufacturer) is added to 2 µL of cell suspension and incubated at 75°C for 14 min. To this mixture, 4 µL of DNase 1 was added and incubated for 15 min at 37°C to remove thegenomic DNA contamination. This cell lysate (5 µL) was treated with 2 µL of random hexamer provided and the total volume is made up to 10 µL with nuclease free water. The suspension is incubated at 70°C for 4 min in a thermal cycler and transferred to icefor 2 min. The tube is centrifuged briefly to collect the condensation and added the 8 µL of cDNA direct RT mix, 1 µL of RNase inhibitor and 1 µL of AMV RT enzyme. The reaction mixture was mixed gently and incubated at 42°C for 60 min followed by 95°C for 10 min to inactivate reverse transcriptase enzyme. The cDNA can be stored at -20°C until use.

2.2.8.2.2 Apoptotic gene expression profiling using RT-PCR

The cDNA synthesized from treated and untreated HeLa cells were analyzed for the expression of housekeeping gene, ß-actin apoptotic genes like, caspase 3, caspase 8 and caspase 9 using real time PCR (Applied Biosystems 7300). The primer sequence used for these genes are given below

Sequence of the primers of the caspases and ß-actin genes

Caspase 8	**F:** a a g c a a a c c t c g g g g a t a c t
	R: t g c a t c c a a g t g t g t t c c a t
Caspase 9	**F:** g c t t a g g g t c g c t a a t g c t g
	R: t g t c g t c a a t c t g g a a g c t g
Caspase 3	**F:** t t t t t c a g a g g g g a t c g t t g
	R: t c a a g c t t g t c g g c a t a c t g
ß-actin	**F:** g g a c t t c g a g c a a g a g a t g g
	R: a g c a c t g t g t t g g c g t a c a g

PCR reactions are performed using SYBR Premix Ex Taq (Tli RNaseH Plus) from TaKaRa. PCR reaction mix (25 µL) contained 12.5 µL of 2 x SYBR Premix (TaKaRa Ex Taq HS, dNTP Mixture, $Mg2^{+}$, RNase H, and SYBR Green154I), 1 µL (0.2 µM) of both forward and reverse primers of respective genes, 0.5 µL of ROX Reference Dye (50 X), 5 µL of cDNA synthesized and made up to 25 μL by adding 5 µL of nuclease free water. The amplification was done by following the thermal cycling sequence:

Step 1: 95°C for 30 sec (1 cycle),

Step 2: 95°C for 5 sec and 60°C for 34 sec (40 cycles),

Step 3: 95°C for 27 sec, 60°C for 1 min, 95°C for 15 sec and finally 4°C for 5 min.

The experiments were done in triplicate. Following thermal cycling for the relative quantification, the threshold was set to 0.20000 using the SDS software and CT values were calculated automatically.

2.2.8.2.3 RT-PCR quantification/data analysis

In the experiment, ß-actin, the house keeping gene (shows constant level of expression) served as the endogenous control. The apoptotic genes like caspase 3, caspase 8 and caspase 9 serve as the target gene. The CT values of both the target and endogenous control genes for treated and untreated cells were obtained from the analysis software. The target gene from the control cells (untreated) will act as the control, target gene from the treated cells is the test and ß-actin (endogenous control) is treated as the reference gene.

This was done using the Livak-DDCt method based on the normalization of the target gene to the CT of the reference gene for both control and test.

1. ΔCT (Control) = CT (Target of control) - CT (reference gene)
   ΔCT (test) = CT (Target of test) - CT (reference gene)

2. Normalization ΔCT of test sample to the ΔCT of control.
   ΔΔCT = ΔCT (Control) – ΔCT (Test)

3. Calculation of fold difference
   Fold difference = 2^ - (ΔΔCt)

2.2.8.3 Assessment of cell cycle by flow cytometry

The HeLa cells were treated with various concentrations of drug and the cells were harvest by trypsinization after incubation for 48 h. Now they were transferred to vial for centrifugation at 5000 rpm for 10 min at 4°C. Soon the supernatant was discarded. The pellet obtained was resuspend in 250 μL of cold 1X PBS, centrifuged at around 10,000 rpm for 5 min at 4.0°C, discarded the supernatant and resuspend the pellet in 300 μL of ice cold 1X PBS. Then, added 700 μL of ice cold 70% ethanol drop wise to the tubes while vortexing gently for fixing the cells. Incubate the sample in ice for 30-60 min. Centrifuge at 5000 rpm for 10 min at room temperature and discard the supernatant. Again, resuspend the pellet in 1X PBS and centrifuge again at 5,000 rpm for 10 min at room temperature. Discard the supernatant, resuspend the pellet in 250 μL of 1XPBS and then added 5 μL of 10 mg/mL RNase. Incubate at 37°C for 1 h and add 10 μL of 1 mg/mL PI after incubation. Then, filter through a 40 μm filter and keep in dark at 4°C until analysis. DNA-bound PI fluorescence was measured using a flow cytometer (Beckman Coulter, U.S.A.). Analysis was performed with 10000 events for each sample and the data were analyzed by using Coulter Elite 4.5 Multicycle software 20.

2.2.9 Statistical analysis

The results obtained were expressed as mean ± SD. The data were statistically analysed using one-way analysis of variance (ANOVA) methodand the Graph Pad Instat3 software package was utilised. The Kramers multiple test was applied to determine the level of significance between the control (untreated) and treated groups. The P values, $P<0.001$, $P<0.01$, $P<0.05$ is considered as extremely significant, significant and moderate significant from the control. $P>0.05$ is considered non significant.

Chapter 3

Screening of cytotoxic, anti-oxidant and anti-inflammatory properties of allied taxa of Nothapodytes

3.1 Introduction

From time in memorial, plants have been used for treating various ailments like fever, cough, stomach problems, and skin diseases. Recent scientific studies explored its various pharmacological properties like antioxidant, anti-inflammatory, anti-diabetic, wound-healing etc. Oxidative stress and inflammation are considered to play a major role in the initiation and progression of various degenerative diseases including cancer. The antioxidant and anti-inflammatory properties of the plants have been studied extensively and has a wide range of applications in treating almost all human ailments. Specifically, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide are highly reactive and over production of these causes severe damage to the biological molecules like proteins, lipids and DNA (Liu, 2002; Halliwell, 1997). Several studies indicate that the intake of antioxidant substances can reduce the damages caused by free radicals. Since synthetic antioxidants can result in toxicity, their usage has been limited (Valentao *et al.*, 2002) and the search for natural, effective antioxidants is of great significance.

Inflammatory diseases are also thought to be a major worldwide problem (Ravikiran *et al.*, 2012). Inflammation results in the release of endogenous mediators like histamine, serotonin, prostaglandins etc. Prostaglandins amend cell and tissue responses involved in inflammation. The anti-inflammatory drugs now available mostly act by blocking the production of prostaglandins through cyclooxygenase (COX) and lipoxygenase (LOX) pathway of arachidonic acid metabolism (Chung *et al.*, 2009). The products of COX and LOX are involved in the stimulation of many inflammatory diseases including cancer (Backlund *et al.*, 2005). The agents that block the production of both leukotrienes and prostaglandins are believed to give better results than the conventional NSAIDs (Celotti and Laufer, 2001). 5-LOX is the key enzyme that synthesises leukotrienes via the arachidonic acid pathway (Zhang *et al.*, 2002) and has been associated with various diseases including cancer (Ghosh and Myers, 1998). Therefore, agents which can inhibit LOX enzyme are considered promising anti-inflammatory agents (Naveau, 2005).

Chemotherapy is considered as a major treatment method used for the control of cancer malignancies. Chemotherapuetic drugs such as anthracyclines (doxorubicin), alkylating agents (cyclophosphamide, cisplatin), antimetabolites (methotrexate, 5-fluro uracil, 6-) etc (Nobili *et al.*, 2009) are toxic compounds that target the rapidly growing cells. Most of these

drugs target the cell cycle and block DNA replication. Even though these are well-known for their ability to kill rapidly growing cells, they also kill normal dividing cells like haematopoietic precursors, stomach, and hair follicle cells (Kaelin, 2005). This lack of selectivity towards cancer cells is one of the major drawbacks of chemotherapeutic treatment. The resistance shown by tumour cells to chemotherapeutic drugs, better known as multidrug resistance has been another hindrance for cancer therapy (Gottesman and Pastan, 1993). Thus the current situation demands a chemotherapeutic drug of natural origin which can troubleshoot these problems.

Restriction on the use of synthetic drugs (Wang *et al*., 2011) and increasing interest on natural remedies have pushed the scientific community in the search of novel drug with antioxidant and anti-inflammatory potential (Ozsoy *et al*., 2008). Determination of the natural antioxidant compounds from plant extracts will help to develop novel drug candidates (Erdemoglu *et al*., 2006). Thus, it is seen that free radicals and inflammation are thought to play an important role in the carcinogenesis process. To prevent the harmful situation which can lead into cancer, chemopreventive strategies like intake of natural/synthetic products or formulations (dietary substitutes) which are rich in anti-oxidant and anti-inflammatory molecules are well practised. Another therapy is the chemotherapeutic one, which avails the cytotoxic drugs to reduce the proliferation or kill the cancer kills during its initial or progressive stages. Thus a chemotherapeutic and chemopreventive two-dimensional approach is intended through the present study by screening the plant members for its potential antioxidant, anti-inflammatory and cytotoxic properties.

The Western Ghats of India is a rich source of plant biodiversity. However many plants have not been yet explored to study its biological activities. *Nothapodytes nimmoniana* belonging to the family Icacinaceae, is well known as a rich source of the anti-cancer alkaloid, camptothecin. Studies indicate that camptothecin has been isolated from 13 members of the Icacinaceae family (Ramesha *et al*., 2013). Moreover, the family members of Icacinaceae, contains many iridoid type of compounds which has antioxidant and anti-inflammatory activity.The literature evidence suggests that the allied taxa of *Nothapodytes*, found in Western Ghats of Kerala have not been subjected to extensive studies in an anti-cancer background apart from the screening for new sources of camptothecin from the family Icacinaceaea. Since the family Icacinaceae is gaining attention as new sources of an

alkaloid drug, camptothecin, the present study aims screen the allied taxa of *Nothapodytes* like *Apodytes dimdiata*, *Gomphandra tetrandra*, *Miquelia dentata* and *Sarcostigma kleinii* based on its *in vitro* cytotxic, antioxidant and anti-inflammatory activities.

3.2 Materials and methods

3.2.1 Preparation of plant extracts

The petroleum benzene, chloroform, acetone and methanol extract of leaf of *Sarcostigma kleinii*, *Gomphandra tetrandra*, *Apodytes dimidiata* and *Miquelia dentata* was obtained from soxhlet and of this, the most active methanolic fraction (for all the plants) were used for the study (2.2.1 of Chap 2). The methanolic soxhlet extract of *Apodytes dimidiata* was further purified through column chromatography by eluting solvents of different polarity successively and finally the methanolic column fraction was chosen to obtain Active methanolic fraction (AMF) of A. dimidiata (2.2.1.1 of Chap 2). The extracts were dissolved in minimum volume of DMSO (expand), made to desired concentrations with PBS for cytotoxicity assays and dissolved in methanol for *in vitro* antioxidant and anti-lipoxygenase assays.

3.2.2 Cell lines

DLA (Daltons Lymphoma Ascites), EAC (Ehrlich's Ascites Carcinoma) and Vero cell lines were used for the study. The details regarding its maintenance are mentioned in 2.1.5 of Chap 2.

3.2.3 *In vitro* cytotoxicity assay

The cytotoxicity of the plant extracts was screened by trypan blue exclusion method explained (as described in section 2.2.7.1, Chap 2).

3.2.4 *In vitro* antioxidant assays

The assays included determination of superoxide radical scavenging activity (2.2.1. of Chap 2), hydroxyl radical scavenging activity (2.2.2. of Chap 2), lipid peroxidation assay (2.2.3. of

Chap 2), DPPH radical scavenging activity (2.2.4. of Chap 2), ABTS radical scavenging activity (2.2.5. of Chap 2), FRAP assay (2.2.6.of Chap 2).

3.2.5 Anti-lipoxygenase assay

Various concentrations of methanolic plant extracts were used to obtain the IC_{50} values from three consecutive experiments. Ascorbic acid was used as positive control. The detailed procedure is mentioned in 2.2.5. of Chap 2.

3.3 Statistical analysis

The values are represented as mean ± SD of three separate determinations and the IC_{50} values were obtained by plotting the graph.

3.4 Results

3.4.1 *In vitro* cytotoxic activity of the plant extracts

Among the plants screened for analysis, *Apodytes dimidiata* leaf showed greatest cytotoxic activity. The crude methanolic extract of *A. dimdiata* leaf obtained by soxhlet extraction showed significant cytotoxicity with IC_{50} values of 9.30 ± 1.24 and 9.26 ± 0.73 μg/mL for EAC and DLA cells, respectively. *Miquelia dentata* showed the IC_{50}value at 500 ± 2.3 μg for DLA and 480 ± 1.9 μg/mL for EAC cells. *Gomphandra tetrandra* showed only a 28% death against DLA cells and 24.2% against EAC cells at 500μg/mL concentration.Only, 33.5 and 28% death were observed in DLA and EAC cells, respectively by using *Sarcostigma kleinii* extract at the concentration of 500 μg/ml (Table 3.1). Since, crude methanolic extract of *A. dimdiata* showed highest cytotoxic activity among all the plants, a partially purified fraction of the crude extract in methanol (AMF), obtained through column chromatography was isolated and it showed IC_{50} values of 3.0 ± 1.43 and 3.22 ± 0.84 μg/mL for EAC and DLA cells, respectively. Moreover, against a normal cell line Vero, the IC_{50} value of AMF is estimated to be above >102.4± 3.5 μg/ml (Table 3.2).

Table 3.1 The *in vitro* cytotoxic activities of the allied taxa of *Nothapodytes*

Cell line used	IC_{50} values of plant extracts in μg/mL			
	Apodytes dimidiata	*Miquelia dentata*	*Sarcostigma kleinii*	*Gomphandra tetrandra*
DLA	9.2 ± 0.7	500 ± 2.3	>500	>500
EAC	9.3 ±1.2	480 ± 1.9	>500	>500

Table 3.2 The *in vitro* cytotoxic activities of the AMF obtained from *Apodytes dimidita*

Cell line used	IC_{50} values of AMF in μg/ml
DLA	3.22 ± 0.84
EAC	3.0 ± 1.43
Vero	> 102.4± 3.5

3.4.2 *In vitro* antioxidant activity of plant extracts

3.4.2A Free radical scavenging property of *Sarcostigma kleinii*

Superoxide, DPPH, ABTs and hydroxyl radicals were inhibited by *Sarcostigma kleinii* extract at IC_{50} of 210 ± 2.5, 89 ± 3.8, 74 ± 7.9 and 108 ± 2. 3 μg/ml, respectively. The generation of malondialdehyde (MDA) was found to be inhibited by the extract and showed the IC_{50} value at 156± 6.8μg/ml. The ferric reducing activity of 2 μg of methanolic extract of the plant was equivalent to the reducing power of 0.45± 0.11 μmole/ml $FeSo_4 7H_2O$.

3.4.2B Free radical scavenging property of *Miquelia dentata*

The methanolic leaf extract of *Miquelia dentata* shows significant free radical scavenging activity. The concentration required for 50% (IC_{50}) inhibition of superoxide, DPPH and hydroxyl radical and ABTS were 128 ± 10.7, 190 ± 5.4, 175 ± 2.9 and 75 ± 5.9 μg/ml, respectively. The ferric reducing activity of 2 μg of methanolic extract leaf of *Miquelia dentata* is equivalent to the reducing power 0.20 ± 0.01μmol/ml $FeSO_4 7H_2O$. The generation

of malondialdehyde (MDA) and related substances that react with thiobarbituric acid (TBA) were found to be inhibited by these extract and showed IC_{50} value of 96 ± 1.7 μg/ml.

3.4.2C Free radical scavenging property of *Gomphandra tetrandra*

The 50% inhibition of superoxide, DPPH, ABTs and hydroxyl free radicals were 90 ± 7.9, 257 ± 12.3, 65 ± 4.4 and 180 ± 3.1 μg/ml, respectively for methanolic leaf extract of *Gomphandra tetrandra*. The generation of malondialdehyde (MDA) was found to be inhibited by the extract and showed IC_{50} value at 79 ± 3.4 μg/ml. The ferric reducing activity of 2 μg of methanolic extract of the plant was equivalent to the reducing power of 0.32 ± 0.13 μmole/ml $FeSo_4 7H_2O$.

3.4.2D Free radical scavenging property of *Apodytes dimidiata*

The active methanolic fraction of *A. dimidiata* (AMF) obtained by column chromatography showed significant antioxidant activity than the crude extract of *A. dimidiata* obtained from soxhlet in all the free radical scavenging assays. AMF showed significant superoxide scavenging activity and the concentration required for 50% (IC_{50}) inhibition of superoxide radical was 78 ± 6.8 μg/ml. AMF was found effective in inhibiting the lipid peroxidation, induced by Fe^{2+} - ascorbate system in rat liver homogenate. The extract showed IC_{50} value at 90 ± 9.4 μg/ml. AMF also inhibited degradation of deoxyribose induced hydroxyl radical, generated from Fe^{2+}-ascorbate-EDTA H_2O_2 system and at concentration of 85 ± 7.5μg/ml, the extract showed 50% inhibition of hydroxyl radical. The extract effectively scavenged the ABTS radicals and ABTs radical scavenging assay of AMF is 59 ± 3.4 μg/ml. The stable free radical, DPPH (2, 2-diphenyl-1-picryl hydroxyl) was effectively scavenged by methanol leaf extracts of *A. dimidiata*. The extract showed IC_{50} valueat 125 ± 5.6 μg/ml. The ferric reducing activity of 2 μg/ml of AMF was equivalent to the reducing power of 0.65 ± 0.12 μmole/ml $FeSo_4 7H_2O$.

From the above results, it was evidenced that all the plants showed appreciable antioxidant activity. In most of the scavenging assays, the methanolic extract of A. dimidiata, (AMF) showed remarkably much better activity compared to the rest of the plants (Table 3.3).

Table 3.3 The free radical scavenging activity of the allied taxa of *Nothapodytes*

Antioxidant Assays	IC_{50} values of plant extracts in μg/ml			
	AMF of *Apodytes dimidiata*	*Miquelia dentata*	*Sarcostigma kleinii*	*Gomphandra tetrandra*
Superoxide radical	78 ± 6.8	128 ± 10.7	210 ± 2.5	90 ± 7.9
Lipid peroxidation	90 ± 9.4	96 ± 1.7	156 ± 6.8	79 ± 3.4
Hydroxyl radical	85 ± 7.5	175 ± 2.9	108 ± 2.3	180 ± 3.1
DPPH Radical	125 ± 5.6	190 ± 5.6	89 ± 3.8	257 ± 12.3
ABTS radical	59 ± 3.4	75 ± 5.9	74 ± 7.9	65 ± 4.4
FRAP assay	2 μg/ml of AMF was equivalent to the reducing power of 0.65 ± 0.12 μmole/ml $FeSo_4 7H_2O$	2 μg of extract is equivalent to the reducing power 0.20 ± 0.01 μmol/ml $FeSO_4 7H_2O$	2 μg of extract of was equivalent to the reducing power of 0.45± 0.11 μmole/ml $FeSo_4 7H_2O$	2 μg of extract was equivalent to the reducing power of 0.32 ±0.13 μmole/ml $FeSo_4 7H_2O$

3.4.3 Anti-lipoxygenase activity of plant extracts

AMF showed a concentration dependent inhibition of lipoxygenase activity. The IC50 value obtained was 72.56 ± 1.45 μg/ml for AMF and 60.02 ± 2.1 μg/ml for the standard drug, ascorbic acid (Fig. 3.1). In case of other plant extracts, the IC50 values obtained were 75.64 ± 2.2, 106.15 ± 0.9, 98.23 ± 1.6 for Miquelia dentata, Sarcostigma kleinii and Gomphandra tetrandra.

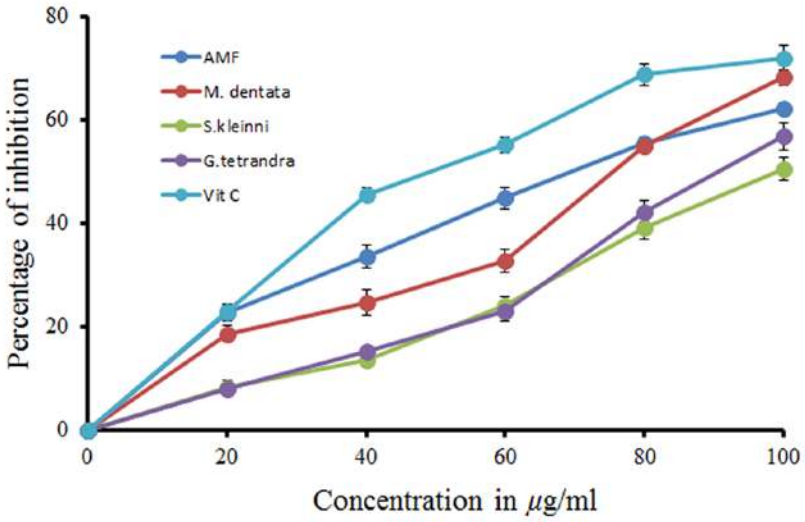

Figure 3.1 The anti-lipoxygenase activity of plant extracts

3.5 Discussion

In the present study, the allied taxa of *Nothapodytes nimmoniana* was screened for its antioxidant, anti-inflammatory and cytotoxic properties. The *in vitro* antioxidant assays such as superoxide, hydroxyl, FRAP, ABTS and ferric reducing ability of plant extracts was employed to examine the antioxidant potential. These are *in vitro* system assays which are easy to follow, set and analyse and have been employed commonly to screen the antioxidant potency of natural and synthetic drug candidates. Various studies indicate that the superoxide anion mediates the formation of the more reactive hydrogen peroxide, hydroxyl radical and singlet oxygen, which causes damage to lipids and DNA (Pietta, 2000). Therefore, studying the superoxide free radical scavenging ability of plant extracts would reveal its potent antioxidant ability. The hydroxyl radicals generated during aerobic metabolism (Harsh, 2010) are thought to be the most reactive and produces lipid hydroperoxides that damages the cell membrane. The DPPH radical is a stable radical and undergoes reduction in the presence of an antioxidant. Since this reaction can be easily studied, they are now widely used for assessing the free radical-scavenging activity (Eyob *et al*., 2008). In FRAP assay, the test sample can react with ferric tripyridyltriazine (Fe3+ TPTZ) complex to produce a coloured ferrous tripyridyltriazine (Fe2+ TPTZ) which can be easily measured and thus, this serves as an important tool of antioxidant measurement. ABTS assay is also utilised as an efficient tool to ascertain the antioxidant activity of hydrogen donating and chain breaking antioxidants (Kumaravel *et al*., 2013).The reducing power of a compound may serve as a important indicator of its potential antioxidant activity and this was tested in the ferric reducing power assay (Oliveira *et al*., 2008). The present study revealed that all the plants chosen for the study showed fine antioxidant activities. It was *Sarcostigma kleinii* which showed the highest DPPH scavenging ability, and *Gomphandra tetrandra* was found best in reducing lipid peroxidation. But, taking into comparison all the assays, it was AMF of A. dimidiata which showed steady and good amount of antioxidant activity for all the *in vitro* free radical scavenging assays.

A cascade of biochemical events is triggered during inflammatory reaction, involving the release of various inflammatory molecules within the injured tissue (Leelaprakash and Dass, 2011). LOX is a key enzyme in the biosynthesis of leukotrienes which is considered to play

a significant role in various inflammatory processes (Sircar *et al.*, 1983, Bhattacharjee, 2007). It is found that antioxidants inhibit the formation of lipid hydroperoxide, the substrate required for LOX catalysis (Rackova *et al.*, 2007). Hence, the anti-lipoxygenase assay is an *in vitro* anti-inflammatory assay which can easily assess the anti-inflammatory potential of any test material and the *in vitro* cytotoxicity assay to know the cytotoxic action of any component. In the current study, these assays were utilised for screening the plant members chosen for the study and we found that the plant, *Apodytes dimidiata* showed the highest amount of cytotoxicity and anti-lipoxygenase activity compared to the other plants chosen for the study. Along with that, the plant also exhibited more anti-oxidant activity than the other members in most of the *in vitro* assays.

Most of the currently used anti-cancer agents are derived from natural sources (Cragg *et al.*, 2005). Novel anti-tumour agents from natural sources were mostly screened based on its cytotoxic activity against cancer cell lines or by using *in vivo* antitumor models (Cragg *et al.*, 2005). Most current assays for measuring cytotoxicity are based on changes of plasma membrane permeability or the uptake of dyes, normally expelled by viable cells. In the current study, the cytotoxicity of plant extracts was estimated by trypan blue dye exclusion method in which the dead cells take up the dye and appear blue under the microscope. The plants *Miquelia dentata*, *Sarcostigma kleinii* and *Gomphandra tetrandra* showed very less cytotoxicity (IC_{50} around 500 μg/ml) towards the cancer cells, DLA and EAC. Whereas, both crude and partially purified form (AMF) of A. dimidiata showed profound activity against the cancer cells studied. Since, AMF was found to be the most cytotoxic amongst all the plant extracts, it was also examined for their effect on normal cell line, Vero and was found that Vero cell line was less sensitive towards AMF. This differential toxicity showed by AMF on cancer and normal cell linesis an important aspect in cancer chemotherapy. One of the major challenges faced by the modern chemotherapeutic drugs is the toxicity exerted to normal cells, which is due to the inability of drugs to differentiate between normal and malignant cells. It is suggested that one of the best ways to be adapted in effective cancer treatment is by eliminating malignant cells through cell cycle inhibition or apoptosis without or with less toxicity in normal cells (Srivastava and Gupta, 2006). Keeping the side effects of chemotherapy in mind it is essential to know whether the compound is harmful to normally dividing cells (Anazetti *et al.*, 2003).

From the present study it is concluded that out of the four plants studied as the allied taxa of *Nothapodytes nimmonina*, it was the active methanolic fraction of *Apodytes dimidiata* (AMF) which showed steady antioxidant activity along with highest anti-inflammatory activity. AMF showed high cytotoxicity towards cancer cells and less toxicity towards normal cells. Thus, it can be considered as attractive candidate for developing effective chemotherapeutic agents based on its antioxidant, anti-inflammatory and cytotoxic properties. Hence, the plant *Apodytes dimidiata* is chosen for the further studies hereafter.

Chapter 4

Preliminary phytochemical analysis and sub-acute toxicity studies of Apodytes dimidiata

4.1 Introduction

The demand for natural products has increased in recent years in spite of the advances that have been made in the field of chemotherapy with synthetic drugs. This is because, the plant materials are either utilised for extraction of compounds which are pharmacologically active or for the preparation of compounds that serve as precursors for the synthesis of effective drugs (Magherini, 1998). Reports suggest that almost 119 chemicals are extracted in pure form from 90 plant species (Sara old field, 1992). Nowadays, there is a growing tendency to budge from synthetic to natural products all over the world. According to World Health Organization, about 80% of the world population depends on natural products for health remedies because they cause minimal side effect and are cost effective (Jagtap *et al.*, 2009). Today, plants are the major sources of natural products and are used as pharmaceuticals, pesticides, agrochemicals, flavours, ingredients, food additives etc (Balandrin and Klocke, 1988) and the different parts used include leaf, root, stem, flower, fruit, exudates etc. Among the estimated 250,000 - 500,000 plant species, only a limited number have been screened phytochemically and the fraction submitted to biological or pharmacological studies is even smaller.

Plants produce secondary metabolites as a protective mechanism against various environmental stress or infections. Many phytochemicals can prevent humans from various degenerative or infective diseases effectively. The impotant phytoconstituents present in plants are flavonoids, carotenoids, alkaloids, anthocyanidins, phenolics, tannins, carboxylic acids, terpenes, amino acids, and inorganic acids etc (Argal and Pathak, 2006). Phenolic compounds are low molecular weight compounds (mol. wt. <2000 amu), derived from phenylalanine and tyrosine and universally existing in all tissues of higher plants (Naczk and Shahidi, 2004). They constitute an important group of bioactive secondary metabolites of plants (Kim *et al.*, 2003) and play an important role in plant growth and defence. The hydroxyl groups present in the phenolic compounds of plants are very important because this confer scavenging ability of the plant. Even though phenolic compounds have been divided into several categories, the chief among them are the flavonoids which have valuable antioxidant activities (Nunes *et al.*, 2012). Flavonoids possess several properties such as anti-oxidant, anti-inflammatory, cancer protective, anti-allergic, cardio-protective and hepato-protective (Di carlo *et al.*, 1999; Montoro *et al.*, 2005).

Several recent studies focuses on the therapeutic uses of secondary metabolites obtained from plants (Nayak and Lexley, 2006). Identification techniques such as gas (GC) and liquid (LC) chromatography is largely been utilised for the determination of phytoconstituents (Uzer *et al*., 2005; Eisenhauer *et al*., 2009). GC-MS is a powerful, highly specific and sensitive technique used for numerous applications and has proved to be a valuable method for the analysis of non polar components and volatile essential oils, lipids and fatty acids (Khare, 2007) and can identify pure compounds present in a substance at a very minute quantity, less than 1 ng (Liebler *et al*., 1996). Using this method, specific compounds can be identified based on the molecular mass in a complex mixture. The secondary metabolites such as alkaloids, phenol, cardiac glycosides, flavonoids, tannins and terpenoids can be determined by gas chromatography and mass spectrum (Lewis and Ausubel, 2006; Adekunle and Adekunle, 2009).

The relative safeness that herbal drug offers has gained the attention of the common people for the treatment of ailments and are indiscriminately used by the public, but they are used for a prolonged period of time without proper knowledge about its side effects (Chaudhary, 1992). In modern pharmacognosy, toxic reactions of drugs can be detected by systematic toxicological testing. When a new drug or formulation is given to a biological system, different types of reactions occur and some of these responses may be disadvantageous to the system. Studies reveal that secondary plant metabolites exert varied biological activities on physiological systems (Olagunju *et al*., 2006). Depending on the duration of drug administration toxicological studies can be categorized in to three main types- acute, sub-acute and chronic studies. In acute toxicity studies, single dose of a drug is given in large quantity to determine immediate toxic effect and also to estimate the LD_{50} (the dose lethal to 50% of the tested group. In sub-acute toxicity study, the tested sample is given at repeated doses in sub-lethal quantity for a period of 15 to 20 days. Sub-acute toxicity is defined as the progressive effects occurring as result of the repeated daily (oral) dosing of a chemical to experimental animal (Lee *et al*., 1978). To analyse the toxic effects of a substance, acute toxicity analysis is considered one of the basic step and involves swift methods to measure the concentration that is harmless to the organism (Yang *et al*., 2012). Whereas in the case of chronic studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of the tested drug (Lipnick *et al*., 1987).

Since the present scenario demands a safe, anti-oxidant rich herbal drug, and as we could infer from the results of the previous chapter that *A. dimidita* exhibited good cytotoxic, antioxidant and anti-inflammatory properties, the aim of the present work was to phytochemically screen the plant metabolites present in the active methanolic fraction (AMF) of *Apodytes dimidiata* qualitatively by applying phytochemical tests and quantitatively by using gas chromatography-mass spectroscopy (GC-MS) analysis. The sub-acute toxicity studies of AMF (100, 250 and 500 mg/kg b wt.) on BALB/c mice were also conducted.

4.2 Materials and methods

4.2.1 *Preparation of plant extract*

Active methanolic fraction of *Apodytes dimidiata*, AMF used for the study was obtained as described in 2.2.1.1. of Chap 2.

4.2.2 *Animals*

The animals were purchased and maintained as mentioned in 2.1.6. of Chap 2.

4.2.3 ***Methods:*** The method used for preliminary phytochemical screening of AMF is mentioned in Chap 2.2.6.1. of Chap 2. The total flavonoid and phenolic contents in AMF was estimated following the procedure mentioned in chap 2.2.6.2 of Chap 2. The GC-MS analysis of AMF is explained in chapter 2.2.6.3. of Chap 2.

4.2.3A *Sub-acute toxicity studies*

Male and female BALB/c mice (20-25 g) of five groups each containing six animals were used for the study. Group 1 served as normal and group 2 categorized as vehicle control, received 0.2 ml of 1% propelyene glycol. Groups 3, 4 and 5 received 100, 250 and 500 mg/kg body weight of AMF, respectively. The drug was administered by oral gavage once daily (0.2 ml/animal) for 15 days. The behaviour of the animals, initial and final body weight, water and food intake and state of stool was observed throughout the experimental period. The animals were sacrificed after 15 days under light ether anaesthesia. Blood was collected by direct heart puncture method and weights of the organs collected were

recorded. A part of the blood was collected in heparinized tubes and used for the estimation of hematological parameters such as hemoglobin, total leukocyte count and the other part was used for serum biochemistry. Kidney function markers such as creatinine and blood urea and liver function markers such as albumin, serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP), total protein and glucose were determined in serum with commercially available kits (Agappe Diagnostics, India).

For histopathological analysis, a portion of the selected tissues (liver, kidney and spleen) of normal and treated animals were fixed in 10% neutral buffered formalin. Sections of paraffin embedded tissues were stained with hematoxylin-eosin and observed under light microscope (2.2.4. of Chap 2).

4.4 Statistical analysis - As mentioned in the 2.2.9. of chap 2.

4.5 Results

4.5.1 *Preliminary phytochemical screening of AMF*

The phytoconstituents evidenced by preliminary phytochemical screening were alkaloids, saponins, terpenes, steroids, carbohydrates, flavonoids, glycosides and tannins. This is shown in Table 4.1.

Table 4.1 Preliminary phytochemical screening of AMF

Sl. No.	Constituents	Presence (+)/Absence (-)
1.	Alkaloids	+
2.	Terpenes and terpenoids	+
3.	Carbohydrate	+
4	Steroids	+
5.	Saponins	+
6.	Flavonoids	+
7.	Tannins	+
8.	Cardiac glycosides	-

4.5.2 *Analysis of total phenol and flavonoid contents in AMF*

An estimated amount of total phenolic compounds in methanolic extract of *A. dimidiata* leaf was found to be 61.22 μg/mg gallic acid equivalent and the total flavonoid compounds in the same was 84.75 μg/mg quercetin equivalent.

4.5.3 *GC-MS analysis of AMF*

GC-MS analysis revealed as many as 76 constituents in AMF as evidenced from the spectrum (Figure 4.1). The name of these compounds with their retention time and area percentage is presented as table 4.2. Out of this, the active components with known anti-oxidant, anti-inflammatory and chemopreventive properties are presented in Table 4.3. along with their compound nature and biological activity. The major phytoconstituents identified were n-hexadecanoic acid (38.33 %), 7-tetradecenal, (Z) - (9.76 %) and octadecanoic acid (6.88 %).

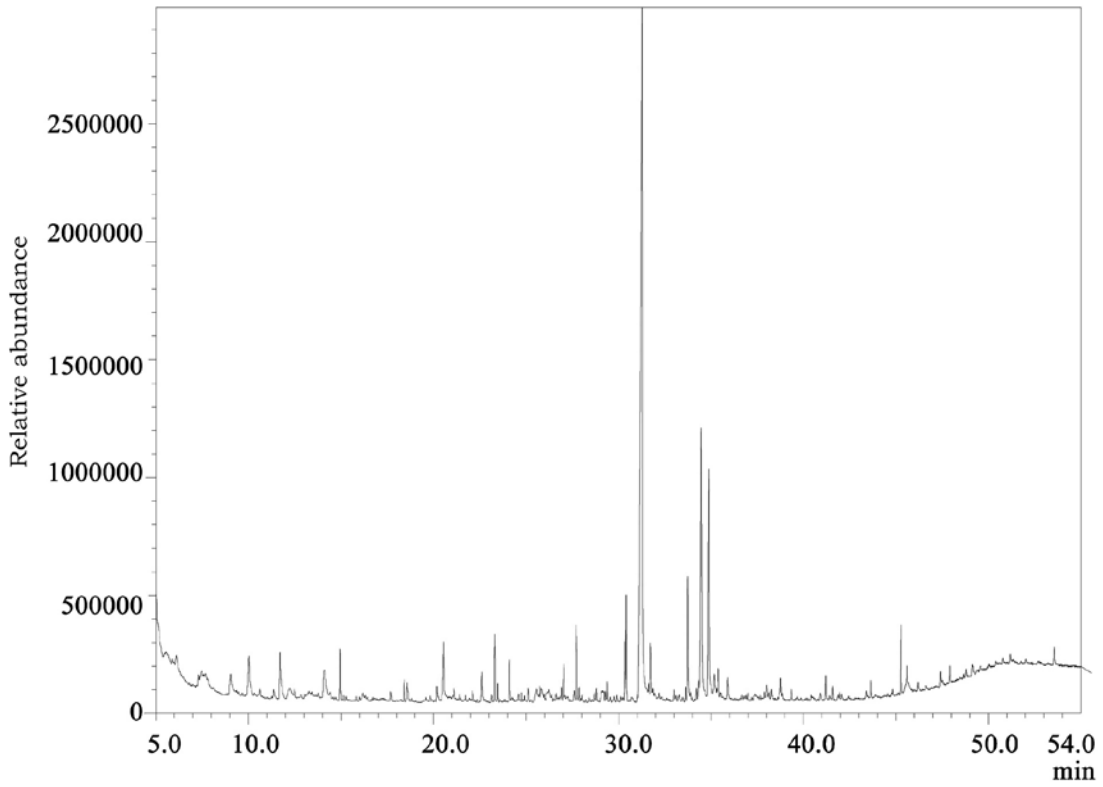

Figure 4.1 The GC-MS spectra of AMF

Table 4.2 The phytoconstituents of AMF identified by GC-MS analysis

R T	Area	Area %	Name
5.875	175224	0.34	2(5H)-Furanone
6.103	391767	0.77	2-Cyclopenten-1-one, 2-hydroxy-
9.039	443219	0.87	Octane
10.024	921618	1.81	Cyclopentane, 1-acetyl-1,2-epoxy-
10.619	72168	0.14	Nonanal
11.704	1110586	2.18	1,5-anhydro-6-deoxyhexo-2,3-diulose
14.095	1082897	2.13	4-Hepten-3-one, 4-methyl-
14.957	575635	1.13	2-Decenal, (Z)-
15.142	70110	0.14	Nonanoic acid
17.678	138842	0.27	1-Heptanol, 6-methyl-
18.415	228574	0.45	3-Tetradecene, (Z)-
18.58	243083	0.48	N-Valeric anhydride
18.625	96730	0.19	Butane, 2,2-dimethyl- E
19.826	54865	0.11	Cyclohexane, hexyl-
20.194	145829	0.29	Undecane, 3,8-dimethyl-
20.555	849024	1.67	2-Butenoic acid, 3-methyl-, 2-methylpropyl ester
21.114	89629	0.18	Tridecane
22.121	108264	0.21	Dodecanoic acid
22.614	406432	0.8	Pentadecanoic acid
23.159	83985	0.17	5-Undecene, 3-methyl-, (E)-
23.307	699591	1.38	3-Octadecene, (E)-
23.478	219687	0.43	Hexadecane
24.108	393112	0.77	Isopropyl dodecanoate
24.597	74608	0.15	9-Octadecenoic acid (Z)-, hexyl ester
24.762	95001	0.19	Cyclohexane, pentyl-
24.919	42933	0.08	Tridecane
25.126	139682	0.27	
25.729	93632	0.18	Heptadecane
25.825	284900	0.56	Benzene, (1-methylnonadecyl)-
26.921	109897	0.22	Tetracosane
27.042	391611	0.77	Tetradecanoic acid

27.607	158327	0.31	1-Dodecene, 2-ethyl-
27.724	822955	1.62	1-Octadecene
27.866	150490	0.3	2,6-Dimethylheptadecane
28.028	82906	0.16	Benzene, [1-[[1-(1-methylethyl)-3-butenyl]oxy]ethyl]-
28.691	128277	0.25	
28.808	130054	0.26	2-Undecanone, 6,10-dimethyl-
29.15	499667	0.98	3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide
29.284	106123	0.21	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
29.396	231007	0.45	8-Octadecanone
29.908	77144	0.15	Pentadecane
30.348	757160	1.49	7,9-Ditert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione
30.421	1127604	2.22	Hexadecanoic acid, methyl ester
31.014	76720	0.15	Oxalic acid, 6-ethyloct-3-yl ethyl ester
31.267	19487807	38.33	n-Hexadecanoic acid
31.628	393684	0.77	3-Octanol, 3,6-dimethyl-
31.735	659453	1.3	1-Tricosanol
31.867	112525	0.22	Heptadecane
32.996	155669	0.31	Hexadecanoic acid
33.629	172634	0.34	
33.746	1591004	3.13	
33.892	97237	0.19	Palmitaldehyde, diallylacetal
34.208	134459	0.26	Oleic acid
34.331	306292	0.6	
34.471	4966191	9.76	7-Tetradecenal, (Z)-
34.875	3498275	6.88	Octadecanoic acid
35.008	144751	0.28	Octane, 3-methyl-
35.175	565908	1.11	2-Aminoethanethiol hydrogen sulfate (ester)
35.394	451107	0.89	
35.893	286344	0.56	9,12-Octadecadien-1-ol)-
36.99	88096	0.17	Silicate anion tetramer
37.882	59217	0.12	2-Oxacyclo[4,4,0]decane, 10-hydroxy-8-cyanomethyl-
38.014	181649	0.36	9,12 Ooctadecadienoic acid (z,z)-

38.249	88025	0.17	Docosanoic acid
38.761	285028	0.56	
39.344	157089	0.31	Silicate anion tetramer
41.196	266645	0.52	
41.562	158581	0.31	
41.937	59500	0.12	Undecane, 3,8-dimethyl-
43.406	70930	0.14	Decane, 2,3,8-trimethyl-
43.645	188314	0.37	Silicate anion tetramer
44.817	51108	0.1	Decanedioic acid, didecyl ester
45.266	739815	1.45	Squalene
45.587	234618	0.46	
46.182	83721	0.16	
47.409	156283	0.31	
47.903	265970	0.52	Iron iodide complex i
48.785	105386	0.21	
49.124	170405	0.33	Silicone polymer
53.551	253435	0.5	Propanoic acid, 2,2-dimethyl-, 2,6-bis(1-methylethyl)phenyl ester

Table 4.3 The important phytoconstituents of AMF detected in GCMS and their properties

Sl. No	Chemical constituents	Compound nature	Biological activities
1	Dodecanoic acid	Carboxylic acids	Antioxidant (Guler at al., 2012)
2	Tetradecanoic acid	Carboxylic acids	Antioxidant, cancer preventive (BodoprostandRosemeyer, 2007)
3	n-Hexadecanoic acid	Palmitic acid	Antioxidant, cancer preventive (BodoprostandRosemeyer, 2007)
4	Oleic acid	Monounsaturated fatty acid	cancer preventive (Win, 2005)
5	Squalene	Triterpene	Antioxidant (Kohno *et al*., 1995), antitumor, chemopreventive (Newmark, 1997; Musakeshavarz*et al*., 2012)

4.5.4 Sub-acute toxicity analysis of AMF in mice

Oral administration of AMF in doses ranging from 100 to 500 mg/kg. b. wt, did not produce mortality or significant adverse effects in the behavioural pattern of both female and male mice. Moreover, there were no significant changes in the body and organ weights and haematological parameters such as haemoglobin and total leukocyte count in the experimental mice (Table 4.4). When the serum profiles were evaluated, the renal function markers such as blood urea and creatinine in treated animals where within the normal range. The level of hepatic function markers such as ALP, SGOT and albumin remained unaltered by the administration of AMF. The vehicle control group also did not exhibit any significant change in all the tested parameters (Table 4.5A and 4.5B). Histopathological analysis did not show any lesions in kidney, spleen and liver of animals treated with AMF with a concentration of 500 mg/kg. b. wt (Figure 4.2).

Table 4.4 Effect of oral administration of AMF on haematological parameters of mice in sub-acute toxicity studies

Groups	Hb (g/dl)(male)	TC of WBC (mill/mm^3) (male)	Hb (g/dl) (female)	TC of WBC (mill/mm^3) (female)
Normal	14.2 ± 2.31	6.8 ± 0.67	13.4 ± 1.35	5.7 ± 0.71
Vehicle Control	14.8 ± 0.67	6.9± 0.56	14.1 ± 1.12	5.2 ± 1.23
100 mg/kg	14.1± 0.50	6.6± 1.80	12.8 ± 0.44	6.1 ± 0.90
250 mg/kg	13.9 ± 0.43	5.8 ± 1.30	12.5 ± 0.89	6.3 ± 0.66
500 mg/kg	13.7 ± 0.39	6.2± 0.83	13.4 ±0.92	5.9 ± 1.37

Values are represented as Mean ± SD (n=6). No significant difference was observed in any parameter. $p \geq 0.05$ was considered as significant.

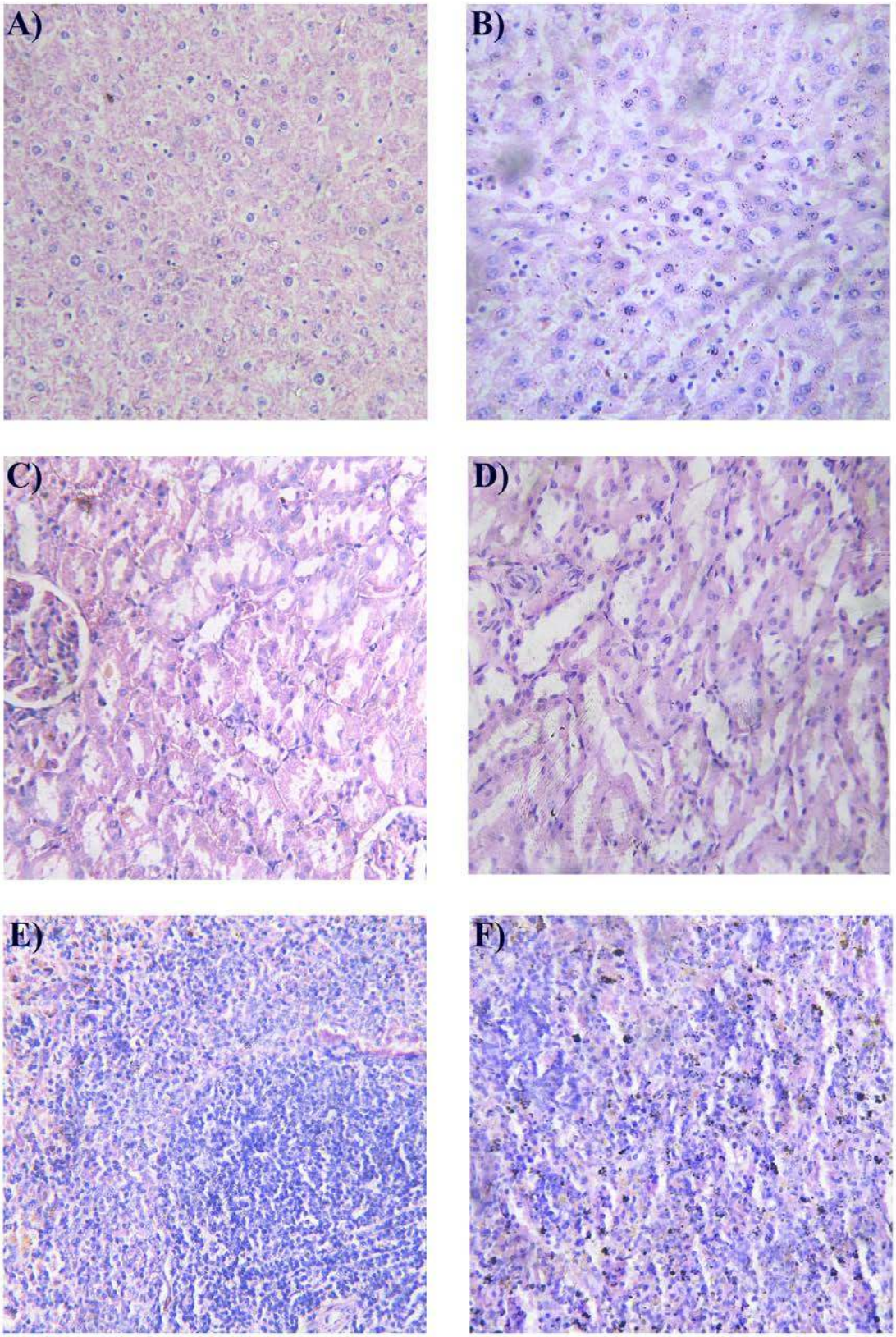

Figure 4.2 Histopathological analysis (400 X) of organs of female BALB/c mice administered AMF (500 mg/kg b.wt.) orally. Normal liver (A), treated liver (B), normal kidney (C), treated kidney (D), normal spleen (E) and treated spleen (F)

Table 4.5A Effect of oral administration of AMF on biochemical parameters in serum of mice (male) in sub-acute toxicity studies

Groups (male)	Glucose (mg/dl)	Total protein(g/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Albumin (g/dl)	SGOT (U/L)	ALP (IU/L)
Normal	63.4±2.34	6.34±0.45	0.33±0.05	42.67±5.2	4.98± 0.05	32.67±2.31	64.12± 3.4
Vehicle Control	65.7±3.42	6.19±0.12	0.31±0.08	44.2±1.3	4.76 ±0.62	34.19±1.12	64.31±3.2
100 mg/kg	65.2±1.38	6.08±0.05	0.28±0.04	42.2±0.38	5.03 ±0.31	33.70±1.43	60.28±1.6
250 mg/kg	62.1±4.51	6.47±0.18	0.29±0.22	42.3±3.40	4.86±0.81	32.76±2.50	58.87± 1.9
500 mg/kg	66.0±2.61	6.35 ±0.12	0.28 ±0.11	41.3±2.10	4.27±0.68	33.84±1.67	54.69±7.2

Table 4.5B Effect of oral administration of AMF on biochemical parameters in serum of mice (female) in sub-acute toxicity studies

Groups (female)	Glucose (mg/dl)	Total protein (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Albumin (g/dl)	SGOT (U/L)	ALP (IU/L)
Normal	62.6±1.48	5.37±0.78	0.31±0.09	41.6±3.20	4.56±0.52	30.34±1.41	63.2±2.67
Vehicle Control	62.2±1.32	5.14±0.18	0.28±0.04	48.3±0.31	4.12±0.38	30.12±1.45	64.66±4.2
100 mg/kg	61.9±3.80	5.02±0.25	0.24±0.04	49.7±2.40	4.93±0.31	31.21±1.39	65.28±4.6
250 mg/kg	68.5±3.32	5.23±0.68	0.32±0.18	45.8±2.30	4.31±0.59	30.89 ±.55	66.01±2.5
500 mg/kg	64.0±5.60	5.67±0.18	0.29±0.17	46.2±3.55	4.66±0.98	29.67±1.43	65.8±3.7

Values are represented as Mean ± SD (n=6). No significant difference was observed in any parameter. $p \geq 0.05$ was considered as significant.

4.6 Discussion

Plants constitute rich sources of secondary metabolites with diverse structural arrangements and remarkable biological activities (de Fatima *et al.*, 2006). Apart from the monographs of plant specimens describing the physicochemical properties alone, a detailed study regarding the active constituents has become inevitable. Moreover, the WHO has emphasized on the necessity to ensure the quality of medicinal plant products using modern controlled techniques and phytochemical characterisation (Chaudhary, 1992).

It is the presence of phytoconstituents which gives specific distinctiveness and properties to plants (Parekh *et al.*, 2007). Therefore, the analysis of these constituents would be helpful in finding out the various biological activities of plants. Knowledge of the chemical constituents of plant secondary metabolite is also advantageous because such information will be helpful for the synthesis of complex chemical substances or for the isolation of active component. The present study revealed that there are various important phytoconstituents in AMF including remarkable amount of flavonoids and polyphenols. Of the phytoconstituents analysed, flavonoids is reported to possess anti-inflammatory, anti-allergenic and anti-carcinogenic activities (Kuhnau *et al.*, 1976; Havsteen *et al.*, 1983; Cody *et al.*, 1986) and phenolic compounds with anti-oxidant (Braca *et al.*, 2002) and anti-cancer properties (Hirvonen *et al.*, 2001).

In the phytochemical studies, appreciable amount of polyphenols and flavonoids were detected in AMF. Several preclinical studies indicate that the total amount of phenolics or flavonoids present in a plant can be correlated with its anti-oxidant activity (Negro *et al.*, 2003; Ramadeep and Geoffrey, 2005).In the GC-MS analysis squalene was identified as one of the major compound in AMF and squalene is reported to possess anti-oxidant (Kohno *et al.*, 1995) and chemopreventive activities (Newmark, 1997; Musakeshavarz *et al.*, 2012). Among other identified components, dodecanoic acid (Guler *et al.*, 2012) is reported to have anti-oxidant and anti-microbial activities. Tetradecanoic acid and hexadecanoic acid is reported to have NPDA anti-oxidant, anti-inflammatory and cancer preventive properties (Bodoprost and Rosemeyer, 2007).

In sub-acute toxicity studies, animals that were treated with AMF (up to 500 mg/kg b wt) did not manifest any significant clinical or macroscopic signs of toxicity and also in the gross examination of internal organs and the body weight it was revealed that there was no significant difference. Studies suggest that that reduction in body weight and also internal organ weights are considered signs of toxicity (Thanabhorn *et al.*, 2006). Our results suggest that the methanolic extract of *A. dimidiata* (AMF) is safe upto 500 mg/kg b.wt dosage. The levels of SGOT, glucose, alkaline phosphatase (indicators of liver function), urea and creatinine (predictors kidney function) of the treated animals, indicated that sub-acute administration of extract neither altered hepatocytes and kidneys of mice nor did it effect the metabolism of the animals. The haematopoietic system is considered as one of the

most sensitive targets of toxic compounds and is a vital index of physiological and pathological status (Adeneye *et al.*, 2006). The haematological evaluation showed no significant difference in the WBC count and haemoglobin level among all AMF treated groups compared to normal group.

In conclusion, the present study revealed that AMF is a rich source of secondary metabolites. Many of the components identified in AMF by GC-MS are reported to have therapeutic uses. These components could be responsible for the anti-oxidant and anti-inflammatory activities of the plant. Moreover, sub-acute toxicity studies underline the safety on the usage of AMF.

Chapter 5

Antioxidant, anti-mutagenic and anti-carcinogenic potential of Apodytes dimidiata

5.1 Introduction

Constant exposure of carcinogenic chemicals and radiation are considered to be the exogenous etiological factors (Gupta and Mukhtar, 2002) in many malignancies. The generation of free radicals, its exerted oxidative stress on cellular events and subsequent mutations are known to be the crucial endogenous mechanisms in the carcinogenic process (Clayson *et al.*, 1994). Hence, the compounds with antioxidant potency can act as revertants of carcinogenesis by blocking the effect of mutagens/carcinogens (Ruan, 1989) or perhaps preventing the initiation, promotion and progression processes (Edenharder *et al.*, 1993). Thus, the chemoprevention strategy looking an effective antioxidant, anti-mutagenic or anti-carcinogenic agents to regulate the development of mutant cell clones (Sporn, 1993; Kim *et al.*, 2001).

Considering the effective scavenging property of free radicals, the phytochemicals has become a primary choice in developing chemopreventive agents. According to Moron and Ames (1983), natural molecules contain factors which act to lower the mutation rate either by inactivating mutagens or interfering in the process of mutagenesis. Antioxidant vitamins such as tocopherols inactivate the free radicals and prevent carcinogen formation from inactive precursors and thereby reduce the cancer risk by 20-30% in humans (Hennekens, 1994). Various plant metabolites such as polyphenols, flavonoids terpenes, steroids, saponins, alkaloids etc. are found to act as free radical scavengers and also exhibit other bioactivities such as anti-mutagenic, anti-carcinogenic, anti-microbial and anti-inflammatory properties (Yen *et al.*, 1993). Curcumin, lutein, folic acid, ursolic acid, β-carotene, genistein, resveratrol etc are examples of some of the active components present in the dietary phytochemicals rendering protection from cancer (Aggarwal and Shishodia, 2006). Substances (natural/synthetic) possessing free radical scavenging, anti-mutagenic, anti-carcinogenic activities are considered as a fine chemopreventive agent (F'guyer *et al.*, 2003). Moreover, reports indicate that the use of anti-mutagenic and anti-carcinogenic substances in our daily life will be the most effective strategy for preventing human cancer and genetic disorders (Ferguson, 1994).

In Southern Africa, Zulu people use *Apodytes dimidiata* as enema for removing intestinal parasites and the leaves are used for ear inflammation (Hutchings *et al.*, 1996; Watt *et al.*, 1962). In a phytochemical analysis, it was evidenced that the acetone extract of *A. dimidiata*

contained various phytoconstituents like saponins, tannins, terpenes, steroids and flavonoids and also found to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals *in vitro* (Masoko and Nxumalo, 2013). The plant is also used as a molluscicide for the control of schistosomiasis in rural communities of South Africa and in acute and sub-acute mammal toxicity studies and it was evidenced that *A. dimidiata* was nontoxic and nonirritating (Brackenbury *et al*., 1997). Six saponins showing antiprotozoal property were elucidated from this plant (Kenn *et al*., 2011). Considering the biological properties, the present study investigated the chemopreventive effect of *A. dimidiata.* Since the free radical mediated mutation are considered to be the prime cause of cancer, the anticarcinogenic potential of A. dimidiata is examined under the limelight of its antioxidant potential against NaF induced oxidative stress and antimutagenic activity against NPDA and NaN_3 induced mutagenicity.

5.1 Materials and methods

5.1.1 ***Preparation of extract*** AMF was the plant extract used. 2.2.1.1. of chap 2 explains the extraction of AMF.

5.1.2 *Animals*

Male BALB/c mice (20-25 g) were purchased and maintained as described in 2.1.6. of Chap 2.

5.1.3 *Bacterial strains*

The strains of *Salmonella typhimurium*, TA 98 and TA 100 were purchased from Microbial Type Tissue Collection and Gene Bank (M.T.C.C), Institute of Microbial Technology Sector, Chandigarh, India in lyophilized forms. TA 102 strain was gifted by Dr. Padma Ambalam, Sourashtra University, Gujarat, India. The strains were sub-cultured in nutrient broth and dispensed in small vials and frozen in presence of 9% DMSO and kept at -70°C. The bacterial cultures were inoculated in fresh nutrient broth and grown for 12 h at 37°C before onset of the experiment.

5.1.4 *Determination of anti-mutagenic activity*

Anti-mutagenic effect of AMF was analyzed using bacterial cultures of *S. typhimurium* TA 98, TA 100 and TA 102. The strains, in a concentration of 1-2 × 10^9 cells/ml mixed with 2

ml of molten agar containing 0.045 mM histidine/biotin solution. Non-toxic concentrations of AMF was added into minimal agar plates along with the direct acting mutagens such as sodium azide (2.5 μg/plates, dissolved in distilled water) and NPDA (20 μg/plate, dissolved in DMSO). Further, it was spread over minimal glucose agar plates and incubated for 48 h at 37°C. Revertant colonies formed spontaneously or by various treatments in plates were counted using a colony counter (Garner *et al*., 1972) and anti-mutagenic effect of AMF was expressed as the difference in the number of colonies between positive control and AMF treated plates. Each data is the mean of 3 plates with standard deviation. The percentage of inhibition of mutagenicity was calculated using the formula $[(R_1\text{-SR}) - (R_2\text{-SR})] \times 100/(R_1\text{-SR})$, where R_1 is the number of revertants in the presence of mutagen alone, R_2 is the number of revertants in the presence of drug and SR is the spontaneous revertants (Moron and Ames, 1983).

5.1.5 *In vivo antioxidant property analysis of AMF against NAF induced oxidative stress in mice*

Male BALB/c mice were divided into eight groups of six each. The first group kept as normal without any treatment. Second, third, fourth and fifth groups were pretreated with propylene glycol (vehicle control), vitamin C (15 mg/kg b wt), AMF (100 mg/kg b wt) and AMF (250 mg/kg b wt), respectively for 4 days followed by the administration of 600 mg NaF/L in water for 7 days. Sixth group was kept as NaF alone treated control. After treatment, all the animals were sacrificed and blood was collected into the heparinized tubes by cardiac puncture for hematological and other biochemical analysis.

The haemolysates were recovered by the method described in 2.2.3.2 of Chap 2.Total WBC and hemoglobin counts were estimated by the method of Chaudhari (2000) and Drabkin and Austin (1932) as described in 2.2.3.1.2 and 2.2.3.1.1 of chap 2. Superoxide dismutase (SOD) activity was carried out by the method of Mc Cord and Fridovich (1969) as in 2.2.3.4 of chap 2. Catalase activity in the blood was determined by Aebi (1974) (2.2.3.5 of chap 2) and the reduced glutathione (GSH) by Moron *et al* (1979) as detailed in 2.2.3.6 of chap 2. Lipid peroxidation of hemosylate was measured by the method of Okhawa *et al.* (1979) as described in 2.2.3.9 of chap 2.

5.1.6 *Determining the effect of AMF on DMB/croton oil induced papilloma in mice*

Male BALB/c mice were used for the study. The hairs on the dorsal side (2 cm diameter) of the mice were shaved with razor at least two days before the application of chemicals. Animals were divided into seven groups having 6 animals each and treatment was as follows. Group I - DMBA + croton oil, group II - DMBA + croton oil + propylene glycol, group III - DMBA + croton oil + 1% AMF, group IV - DMBA + croton oil + 3% AMF, group V - DMBA + croton oil + 5% AMF extract, group VI - DMBA alone and group VII - croton oil alone. DMBA was applied topically in a concentration of 470 nmol/mouse in 200 μl acetone as single dose. Animals in group I - VII were applied with 1% croton oil in acetone (200 μl/animals) as well as AMF (200 μl/animals) dissolved in propylene glycol topically two weeks after DMBA treatment. Croton oil and drug was applied twice weekly for 6 weeks. AMF and propylene glycol was applied 30 min before each croton oil application. The animals in all groups were examined during the entire period of the study for food intake as well as any toxicity such as weight loss or mortality. Skin papilloma greater than 1 mm diameter size persisted for 2 weeks or more was recorded and included in the cumulative total. Delays in the onset of tumors and number of papilloma per cage in various groups were also recorded (Reddy and Failkow, 1983). After the experiments, the animals were scarified.

5.2 Statistical analysis

Statistical evaluation of the data was done by the method described in 2.2.9. of chap 2.

5.3 Results

5.3.1 *Effect of AMF on NaF induced oxidative stress in mice*

NaF treatment induced a significant reduction in antioxidant enzymes, SOD (U/g Hb) and catalase (K/g Hb) and GSH (nmol/ml) levels in mice, while the administration of AMF prior to intoxication with NaF significantly prevented the depletion of antioxidant marker levels. NaF intoxication led to a significant reduction in GSH level (35.23 ± 9.34) and treatment of AMF (higher dose) elevated the level of GSH to 68.5 ± 10.32 and the depleted level of SOD (302.45 ± 45.98) and catalase (28.56 ± 5.34) was enhanced to 589.75 ± 87.32 and 40.98 ± 6.56 by the administration of AMF. In NaF treated animals, the MDA level (nmol/mg protein) was raised to 2.67 ± 0.16 and a significant reduction in lipid peroxidation status was

observed as inferred from the value of MDA (1.13 ± 0.25) by the administration of AMF. AMF (250 mg/kg) increased the serum SOD and catalase activities by 48.71 and 30.3% against the NaF induced drop. GSH level was improved by 48.59% with a concomitant decrease in TBARS (57.67%). The skin papilloma reduction was 79.32% for 5% AMF. The effect of AMF on the normalization of these antioxidant markers were comparable to that of vitamin C (standard drug) treated animals (Figure 5.1). Counts of WBC and hemoglobin were decreased in NaF alone treated control animals. The count of WBC (cells/mm^3) of NaF treated animals (3800 ± 253.56) was significantly increased to 13000 ± 387.61 by the administration of 250 mg/kg b. wt AMF. Similarly, the reduced hemoglobin level in g/dl (9.12 ± 1.34) was significantly elevated to 14.12 ± 1.34 by the administration of AMF (Figure 5.2).

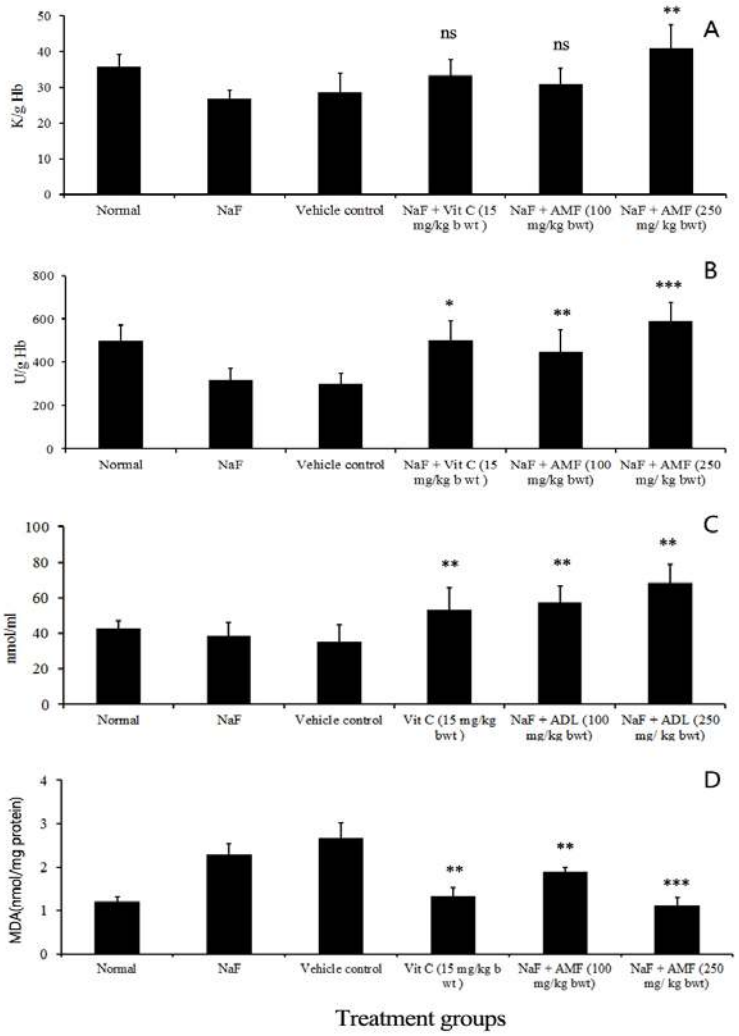

Figure 5.1 Effect of AMF on antioxidant parameters in NaF treated mice. (A) Catalase, (B) SOD, (C) GSH and (D) Lipid peroxidation. Values are mean ± S.D., n=6. *** P<0.001, ** P<0.01, * P<0.05, (Tukey test). Vehicle control group were compared with treated groups

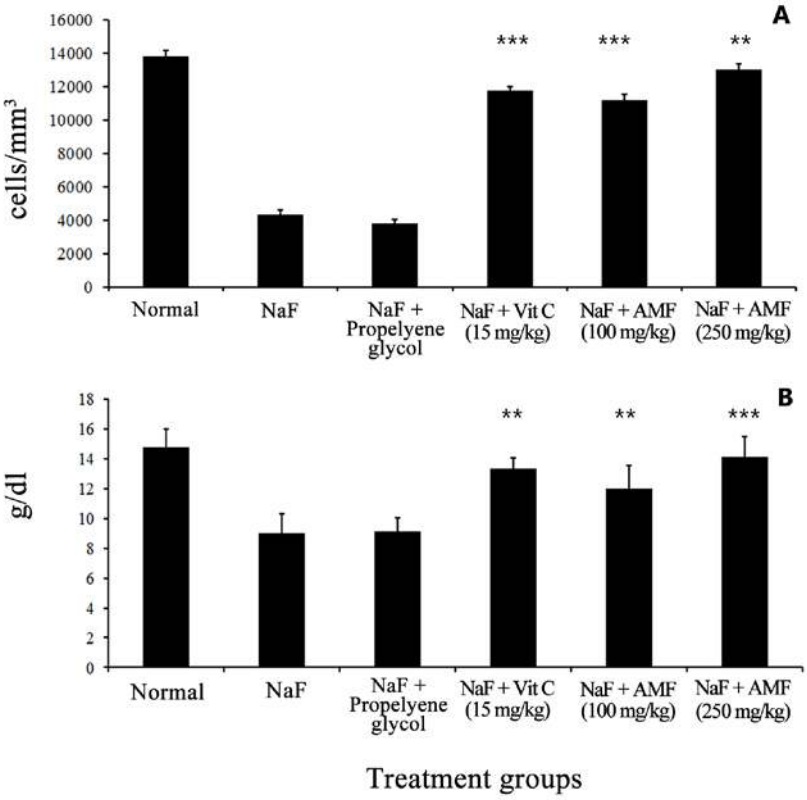

Figure 5.2 Effect of AMF on haematological parameters in NaF treated mice. A- Total WBC count, B- Hemoglobin level. Values are mean ± S.D., n=6. *** P<0.001, ** P<0.01, * P<0.05, (Tukey test). Vehicle control group were compared with treated groups

5.3.2 Antimutagenic effect of AMF in Salmonella typhimurium strains

The mutagenicity induced by NaN_3 and NPD in tester strains TA 98, TA 100 and TA 102 was found inhibited by AMF. The percentage of inhibition of AMF at the concentrations of 25, 50 and 75 μg extract/plate was 66.9, 76.6 and 80.4 with TA 98 strain, 54.2, 68.6 and 71.3 respectively for TA 100, and 47.2, 69.6, 71.3 for the strain, TA 102, respectively against sodium azide (Table 5.1). AMF also showed decrease in the number of colonies of NPDA induced revertant tester strains. The extract was found to be effective in preventing mutagenicity by 61.4, 73 and 74.8% for TA 98 in the respective concentrations of 25, 50 and 75 μg extract/plate. It was found that for TA 100, the inhibition percentage varied from 51 to 72% for the tested concentrations and for TA 102 the percentage inhibition was observed between 61 and 69%. The anti-mutagenic activity of the AMF was found to be concentration dependent (Table 5.2).

Table 5.1 Antimutagenic activity of AMF on mutagenicity induced by NaNa3 in S. *typhimurium* strains TA 98, TA 100 and TA 102

Conc. of AMF/plate	TA 98		TA 100		TA 102	
	ANR/plate	% inhibition	ANR/plate	% inhibition	ANR/plate	% inhibition
NaN3	750 ±29.1	-	645±18	-	626±13	-
NaN3+ DMSO	728±21	-	633±20	-	610±9.5	-
SR	55± 1.5	-	105± 6.8	-	155± 14.6	-
NaN3+AMF (25 μg)	248±22***	66.9	295±24	54.2	330±10***	47.28
NaN3+AMF (50 μg)	175±17***	76.6	202±17	68.6	190±22***	69.6
NaN3+AMF (75 μg)	147±11***	80.4	185±22	71.3	145±7.8***	76.8

The values are mean ± SD of 6 different determinations. SR is spontaneous reversion. ANR is average number of revertants.SR is not substracted from the values.Percentage inhibition calculated from NaN_3 alone treated group. ***P < 0.001.

Table 5.2 Antimutagenic activity of AMF on mutagenicity induced by NPDA in S. *typhimurium* strains TA 98, TA 100 and TA 102

AMF (mg/plate)	TA 98		TA 100		TA 102	
	ANR/plate	% inhibition	ANR/plate	% inhibition	ANR/plate	% inhibition
NPD	752 ± 17	-	638±22	-	636±11	-
NPD +DMSO	733±10	-	612±13	-	631±10	-
SR	55±1.8	-	130±4.1	-	190±17	-
NPD+AMF (25μg)	290±22***	61.4	310±13	51.4	248±23***	61.0
NPD+AMF (50μg)	203±17***	73.0	219±23	65.6	207±20***	67.4
NPD+AMF (75μg)	189±11***	74.8	175±20	72.5	195±16***	69.3

The values are mean ± SD of 6 different determinations. SR is spontaneous reversion. ANR is average number of revertants.SR is not substracted from the values.Percentage inhibition calculated from NPDAalone treated group. ***P < 0.001.

5.3.3 Anti-carcinogenic effect of AMF on DMBA and croton oil induced papilloma in mice

Topical application of DMBA followed by 1% croton oil twice weekly for 6 weeks resulted in skin papilloma which started appearing from the 5th week in the control group. The onset of papilloma was found to be significantly delayed or prevented by AMF treatment. In animals treated with 1% AMF, the papilloma appeared from the 8th week of DMBA application and in 3% AMF group it was found to appear in the 9th week. In 5% AMF treated group, the onset was delayed up to 11th week (Figure 5.3).

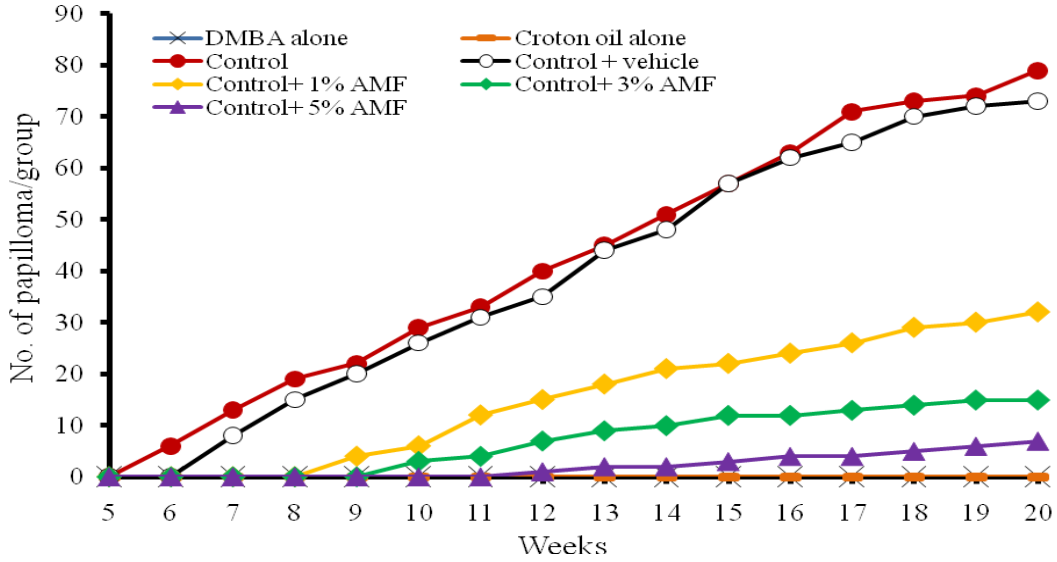

Figure 5.3 Effect of AMF on number of papilloma in DMBA induced papilloma bearing mice. Control group is administered DMBA +Croton oil alone. Dosage - Single dose of DMBA (470 n mol/mouse in 200 µl acetone, Croton oil (1%) in acetone (200 µl/ animal) and treated group is given 1, 3 and 5% AMF in propelyene glycol along with DMBA +Croton oil, twice in a week for 6 weeks. Number of papilloma per animals was recorded up to 20 weeks.

Compared to control, the formation of papilloma was very less in the treatd group (Figure 5.4). Out of the total seven animals in each group, all the animals in the DMBA/croton oil group developed papilloma (7/7) while only three animals developed papilloma in 5% treated AMF group (3/7). Animals in DMBA or croton oil alone treated groups did not produce any papilloma on their skin. At the end of 20th week, the average number of papilloma per mouse was found to be 11.28 ± 1.25 and 10.42 ± 0.97 in DMBA/croton oil

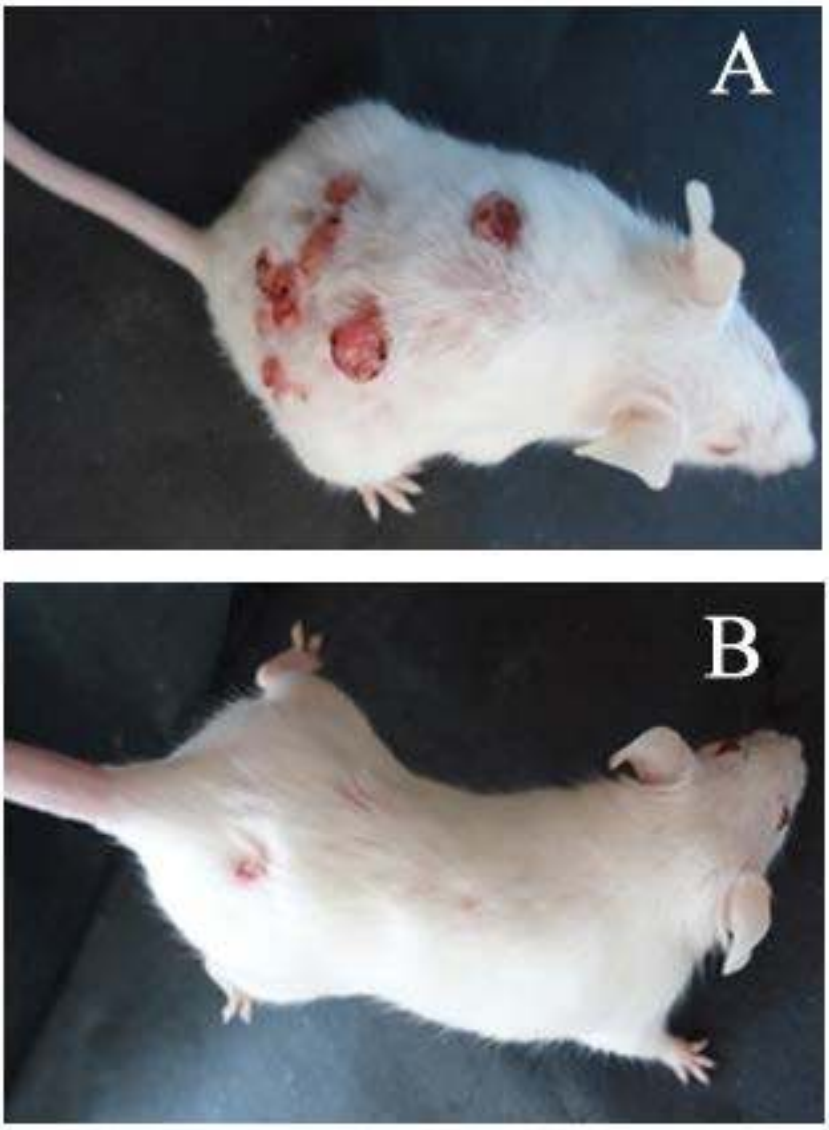

Figure 5.4 The effect of AMF in the formation of pailloma. A - Control group (DMBA + Croton oil alone), B - Treated (DMBA + Croton oil alone + 5% AMF)

group (control) and DMBA/croton oil + propelyene glycol (vehicle control) groups and this number was significantly reduced to 4.57 ± 0.78, 3.75 ± 0.5 and 2.33 ± 0.57 by 1, 3 and 5% AMF treatment, respectively (Figure 5.5). A reduction of 79.32% in papilloma was observed in 5% AMF treated animals when compared to the control.

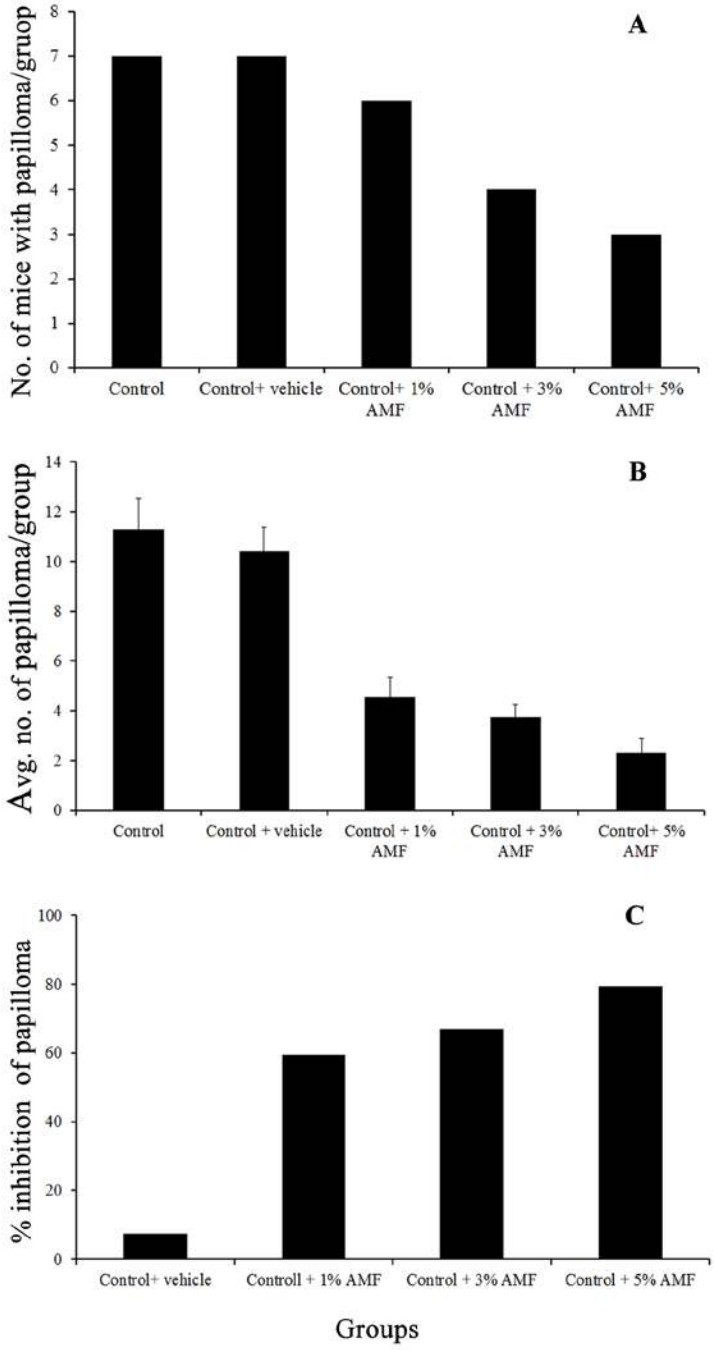

Figure 5.5 The number of mice with papilloma/group, B- Average number of papilloma developed/group, C- The percentage inhibition of papilloma by AMF on DMBA induced papilloma bearing mice

5.4 Discussion

DMBA/croton oil induced mouse skin papilloma is a well-known experimental model to understand the anti-carcinogenic potency of a test material. In this model, DMBA acts as initiator and croton oil which contains the most active phorbol ester, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) promotes further carcinogenic processes. According to Shklar *et al.* (1999), DMBA and croton oil induced carcinogenic process is mediated by oxidative stress. DMBA, when metabolized to its ultimate carcinogenic form dihydrodiol-epoxide initiates the production of excessive ROS which cause subsequent DNA damages and inflammation. Repetitive topical application of croton oil (TPA) has also been leads to the excessive generation of free radicals. This consecutive exposure of free radicals acts as constant bombardment which promotes the mutation of codon 61 of the H-ras gene (Bizub *et al.*, 1986) leading to the conversion of an initiated cell to a malignant form in mouse skin (Huachen and Krystyna, 1991).

In the present study, DMBA/croton oil induced papilloma formation in mice was significantly inhibited by the topical application of AMF in a dose dependent manner. Moreover, the number of mice that developed papilloma was also found to be reduced. The possible reason for this activity of AMF could be explained by the observed antioxidant and antimutagenic properties. In our earlier studies (referred in chap 3) and reports of Masoko and Nxumalo (2013), the free radical scavenging efficacy of this plant has been revealed. The improved activities of antioxidant enzymes and molecules with a concomitant reduction in the lipid peroxidation status of NaF treated mice by AMF administration suggested the strong antioxidant potential of the plant. In initiation phase, the DMBA induced free radical stress could have been alleviated by the antioxidant action of AMF. Similarly, it is also thought that the AMF might have interfered with the overwhelmed generation of free radicals and subsequent mutagenic events often seen in the croton oil mediated progressive phase. This assumption is made from the fact that AMF inhibited the chemical mutagenicity significantly in various strains of *Salmonella* in the present study.

Various recent reports states that the natural antioxidants may reduce or inhibit the mutagenic potential of mutagens and carcinogens. Zahin *et al* (2010) has reported the

anticarcinogenic and antimutagenic activities of medicinal and food plants are executed mainly by scavenging of free radicals. Flavonoids, a class of secondary metabolite has been shown to exhibit the anticarcinogenic activity (Hirano *et al.*, 1989). Previous studies conducted to analyze the total flavonoid content of AMF (detailed in chap 4) has shown that it had appreciably good amount of the same.

GC-MS analysis also had revealed the presence of various antioxidant and chemopreventive constituents in AMF (discussed in chap 4). In our previous studies regarding the anti-cancer activities of AMF, we have isolated the compound, genipin as the most cytotoxic component (Divya *et al.*, 2015a). Genipin is reported to possess anti-oxidant (Lee *et al.*, 2009) and anti-cancer properties (Cao *et al.*, 2010). It is thought that cancer mostly occurs by the inactivation of tumour suppressor genes resulted from a mutation triggered by ROS (Rivlin *et al.*, 2011; Ramsey and Sharpless, 2006). Most of the medicinal phytoconstituents exert their action through this pathway (Li *et al.*, 2012).Thus the role of an anti-mutagen and an antioxidant is inevitable in preventing carcinogenesis signifying that any anti-carcinogen should essentially possess these properties. In conclusion, the study indicates that the antioxidant and anti-mutagenic properties of AMF contributed by its phytoconstituents could have played a major role in the chemopreventive efficacy on skin papilloma formation either by inhibiting DMBA metabolism to its active form or by down regulating reactive oxygen species formation. Since carcinogenesis is a multifactorial event, it is imperative to develop new drugs with multiple targeting actions. Thus AMF, the active fraction of *Apodytes dimidiata* can serve as a new lead in the identification of a drug candidate for the chemoprevention.

Chapter 6

Ameliorative effect of Apodytes dimidiata on cisplatin induced nephrotoxicity in Wistar rats

6.1 Introduction

The most important side effects usually encountered with conventional chemotherapy are free radical mediated toxicity (Conklin, 2000) and multidrug resistance (Gottesman and Pastan, 1993). Chemotherapy effects regenerating tissues such as bone marrow elements, gastrointestinal tract mucosa, hair follicles etc and causes cardiac, nervous and hepatic toxicity (Brenner and Stevens, 2010).

Cisplatin a heavy metal complex, is one of the most effective anti-neoplastic drugs currently available for the treatment of several cancers. But, the administration of cisplatin is conjoined with various side effects including nephrotoxicity. Generally, cisplatin stimulates the ROS production by the damaged mitochondria (Kawai *et al.*, 2006) and release of NO. The consequent development of oxidative stress is considered to be the prime factor for the cisplatin induced nephrotoxicity (Pan *et al.*, 2009). It is seen that the production of several free radicals like, superoxide anion (Davis *et al.*, 2001), hydrogen peroxide (Kadikoylu *et al.*, 2004) and hydroxyl radicals (Shino *et al.*, 2003) are increased in cisplatin treated kidneys of mice and thereby stimulating lipid peroxidation in the kidney tissue (Sadzuka *et al.*, 1992). This causes generation of subsequent reactive oxygen metabolites and inhibits the activity of antioxidant enzymes in renal tissue (Mora *et al.*, 2003), which is responsible for the major side effects of nephrotoxicity (Amptoulach and Tsavaris, 2011). Several lines of evidences suggest that the role of ROS in the pathogenesis of nephrotoxicity (Krishnamohan *et al.*, 2006; Kalyan *et al.*, 2012). Cisplatin induces free radical production causing oxidative renal damage, possibly due to depletion of non-enzymatic and enzymatic anti-oxidant systems.

Plants are rich source of antioxidants because of the diverse phytoconstitutents they possess. Natural antioxidants as potential nutraceuticals have been studied to reduce severe side effects as well as enhance anticancer activities of antitumor drugs (Zhang *et al.*, 2010). To ameliorate the side effects, complementary therapies have recently been investigated (Yang, 2005) and antioxidants are suggested to play a role in protecting against cisplatin (CP) induced nephrotoxicity (Mansour *et al.*, 2002). Reports suggest that CP-induced renal

toxicity in experimental animals could be reduced by the supplementation with well-known antioxidants such as vitamin E and C (Naqshbandi *et al*., 2011; Rajashekar *et al*., 2012).

A.dimidata is used in Zulu traditional medicine as enema for intestinal parasites and leaves are used in the treatment of ear inflammation. As mentioned earlier in the review, recent studies revealed that the plant posses anti-bacterial and anti-oxidant properties. Moreover, the phytochemical screening of the acetone leaf extract showed the presence of various phytoconstituents (Masoko and Nxumalo, 2013). Presence of six new saponins is also reported in the plant. Since there are only preliminary evidences of *A. dimidiata* possessing anti-oxidant properties and containing pharmacologically important constituents, an effort has been made to exploit the antioxidant property of the plant (studied in chap 3 and 5) by evaluate its nephroprotectivity using cisplatin induced nephritic injury model in rats. Since NO free radical plays an important role in causing nephrotoxicity, the NO free radical scavenging property of AMF is also studied *in vitro*.

6.2 Material and methods

6.2.1 Plant extract used

AMF was used for the study. The procedure followed to obtain AMF is mentioned in 2.2.1.1 of Chap 2.

6.2.2 Animals

Wistar rats (120 - 150 g) were used for the study. The maintenance conditions are given in 2.1.6. of Chap 2.

6.2.3 Experimental design

Male Wistar rats were divided into six groups of six animals each. Group 1 - normal (without any treatment), group 2 - control (cisplatin alone), group 3 - vehicle control (cisplatin + propylene glycol (> 99% pure), group 4 - cisplatin + sylimarin (100 mg/kg b.wt), group 5 - cisplatin + AMF (100 mg/kg b. wt), group 6 - cisplatin + AMF (250

mg/kg b. wt). Animals in group 3 were treated with propylene glycol, group 4 with the standard and groups 5 and 6 were treated with AMF by oral gavage for five days before cisplatin treatment. One hour after the drug administration on the 5th day all the mice in groups 2-6 were treated with single dose of cisplatin (16 mg/kg body wt, i.p) to induce renal damage. Drug treatment was continued for 3 more days. After 72 hrs of cisplatin injection (Somani *et al*., 2000) animals were sacrificed using ether anaesthesia; blood was collected directly from the heart of each animal and collected in heparinized vials to determine the total WBC count (2.2.3.1.2 of chap 2) and haemoglobin (2.2.3.1.1 of chap 2) content. Blood collected was in non-heparinized vials to separate serum for the analysis of kidney function parameters urea (2.2.3.14 of chap 2) and creatinine (2.2.3.13 of chap 2) using commercially available kits.

Kidney was excised out, washed in ice-cold saline and a portion of kidney was fixed in 10% formalin for histopathological analysis. Kidney homogenates (10%, w/v) prepared (2.2.3.3. of chap 2) were used for the assay of tissue lipid peroxidation using TBA method (2.2.3.9. of chap 2). The supernatant collected were used for the assay of GSH (2.2.3.6 of chap 2), SOD (2.2.3.4 of chap 2), CAT (2.2.3.5 of chap 2) and GPx (2.2.3.7 of chap 2) activities.

6.2.4 Nitric oxide scavenging assay

Various concentrations of AMF were dissolved in methanol and the NO scavenging ability was determined by following the procedure mentioned in 2.2.2.7. of chap 2.

6.3 Results

6.3.1 *Effect of AMF on haematological parameters in cisplatin induced mice*

Total WBC count was found to be significantly reduced in cisplatin alone treated group. There was a proportionate increase in the WBC count in the AMF administered animals in a dose dependent manner and the significance was high compared to that of vehicle control. There was also a reduction in the haemoglobin content of animals with cisplatin alone treatment and the level was increased by AMF treatment (Figure 6.1). The increase in hemoglobin and leucocyte count was 28.25 and 42.91% when compared to control.

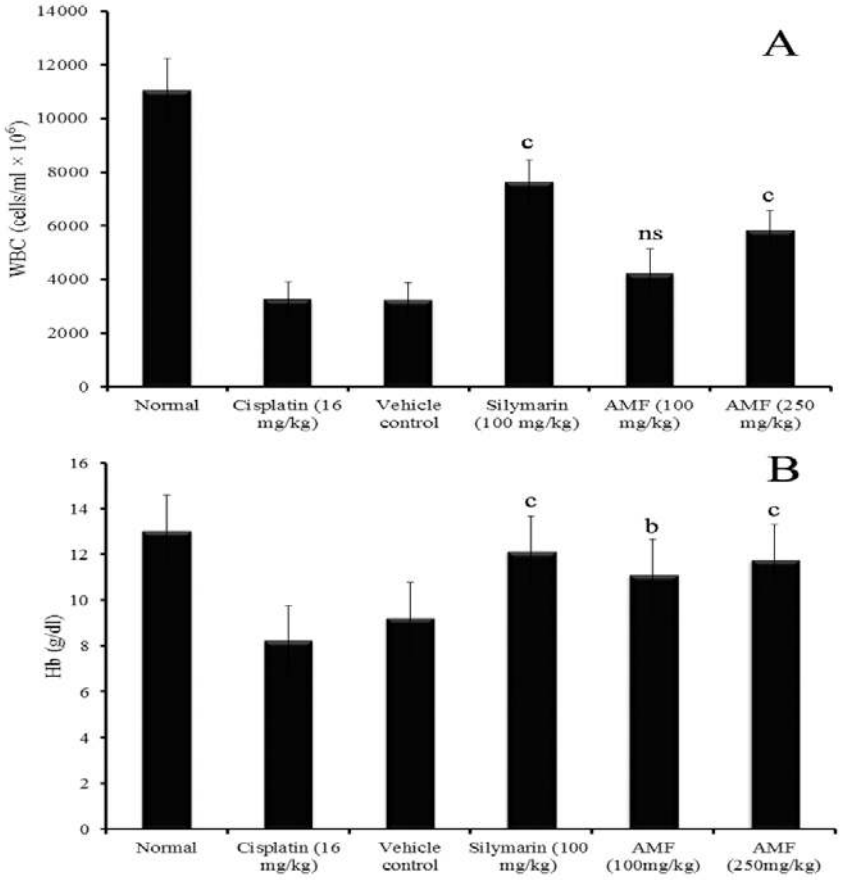

Figure 6.1 Effect of AMF on haematological parameters in cisplatin treated mice. A-Total WBC count, B-Hemoglobin level. Values are mean ± S.D., n=6. c:P<0.001, b: P<0.01, a: P<0.05, (Tukey test). Vehicle control group were compared with treated groups

6.3.2 *Effect of AMF on biochemical parameters in cisplatin induced mice*

Serum creatinine level was increased to 5.14±0.37 in cisplatin alone treated group and was significantly reduced in 250 mg/kg b.wt AMF treated group to 2.47±0.56 ($p<0.001$). Similarly, the urea level was significantly elevated in cisplatin alone treated group and it was reduced in AMF (250 mg/kg. b. wt) to 60 ± 1.92 ($p<0.001$) and 100 mg/kg b. wt to 77.88 ± 2.63 ($p<0.001$) AMF treated group in a dose dependent manner (Fig 6.2). Protective effect of AMF on the normalization of these kidney markers when compared to cisplatin alone control group was found to be significant and the values obtained were comparable to that of sylimarin (standard drug) treated animals.

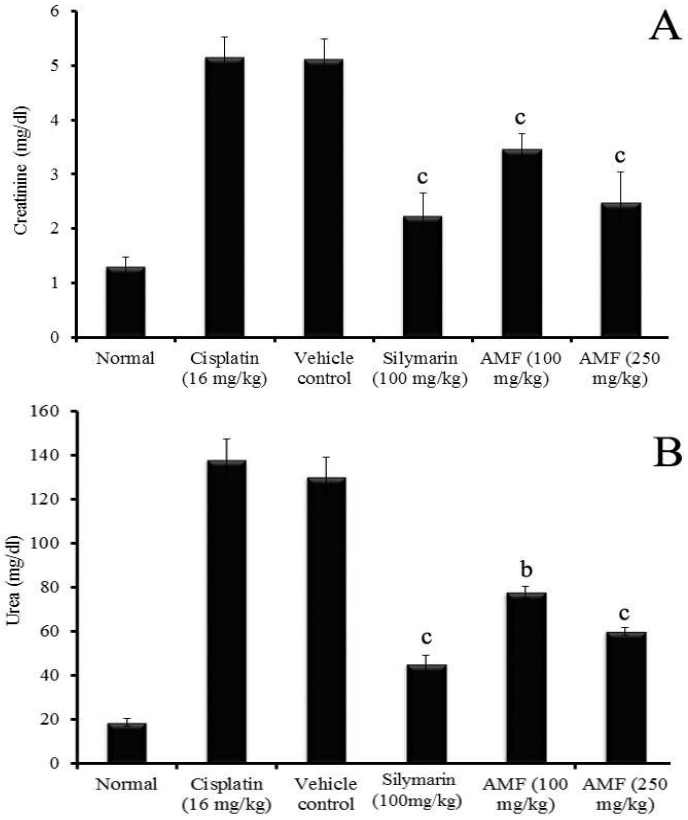

Figure 6.2 Effect of AMF on serum creatinine (A) and urea (B) levels in cisplatin treated mice. Values are mean ± S.D., n=6. Values are mean ± S.D., n=6. c: P<0.001, b: P<0.01, a: P<0.05, (Tukey test). Vehicle control group were compared with treated groups

The antioxidant enzyme, superoxide dismutase was found to be reduced with cisplatin treatment and the SOD activity was increased in AMF treated groups (100 and 250 mg/kg. b. wt). The GSH was found to be depleted in cisplatin alone treated group to 41.51±1.07 and it was elevated to 50.6 ± 2.32 in AMF high dose treated group indicating significant enhancement in the GSH activity ($p<0.001$). Similarly, the GPx content in AMF treated groups of animals were found to be significantly higher ($p<0.001$) compared to cisplatin alone control groups which indicates the triggering of antioxidant enzyme GPx by AMF as a defence mechanism against free radicals generated by cisplatin. The antioxidant enzyme catalase was found to be significantly decreased to 3.3±0.34 with cisplatin treatment and the enzyme activity was increased in AMF treated 100 and 250 mg/kg b. wt groups to 4.15±0.97 ($p < 0.01$) and 5.16±0.40 ($p<0.001$), respectively (Fig 6.3).

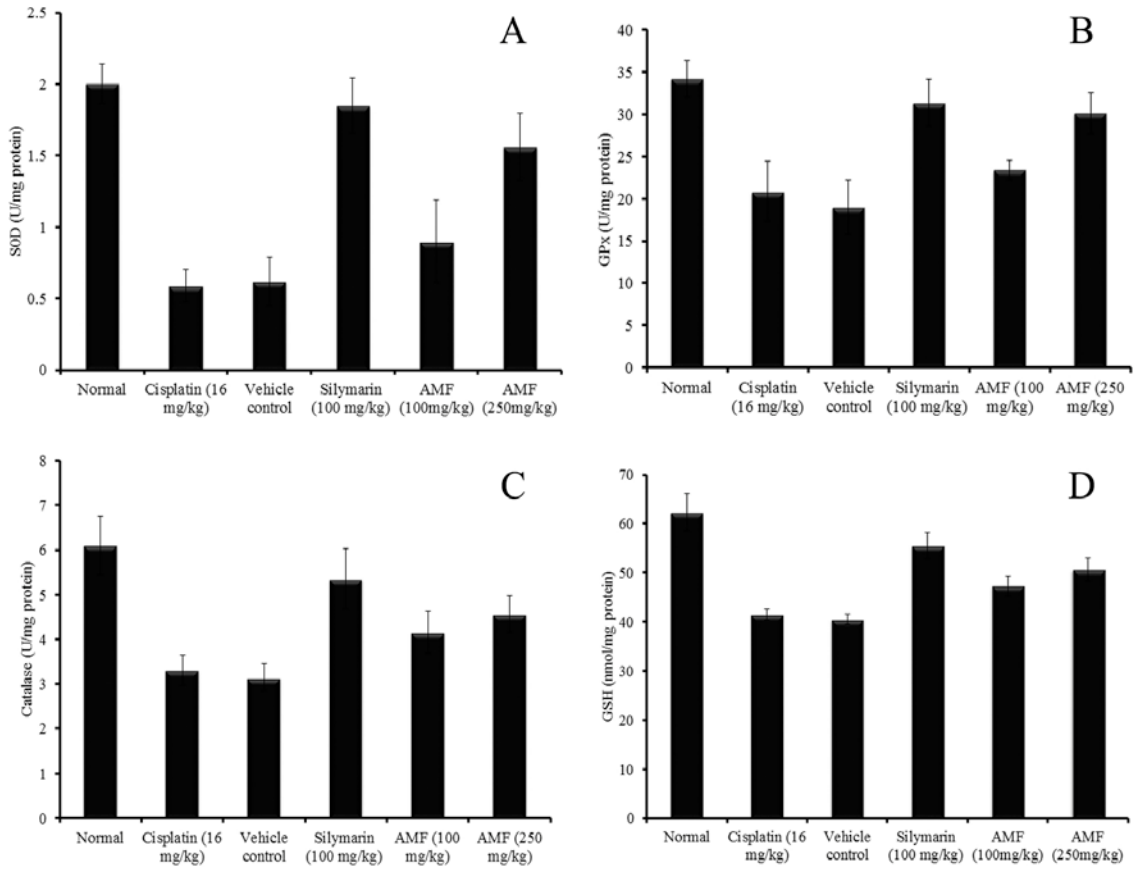

Figure 6.3 Effect of AMF on kidney antioxidant enzymes in cisplatin treated mice. (A) SOD, (B) GPx, (C) catalase and (D) GSH. Values are mean ± S.D., n=6. Values are mean ± S.D., n=6. c: P<0.001, b: P<0.01, a: P<0.05, (Tukey test). Vehicle control group were compared with treated groups

There was a significant reduction in lipid peroxidation activity by the administration of AMF in a dose dependent manner, which was highly elevated with cisplatin treatment in the control group (Fig 6.4). The percentage reduction of urea, creatinine and lipid peroxidation was 58.31, 42.19 and 60 when compared to control. The hike calculated for GSH, $GP_{x,}$ SOD and catalase was 35.64, 18.14, 74.42 and 35.46%, respectively.

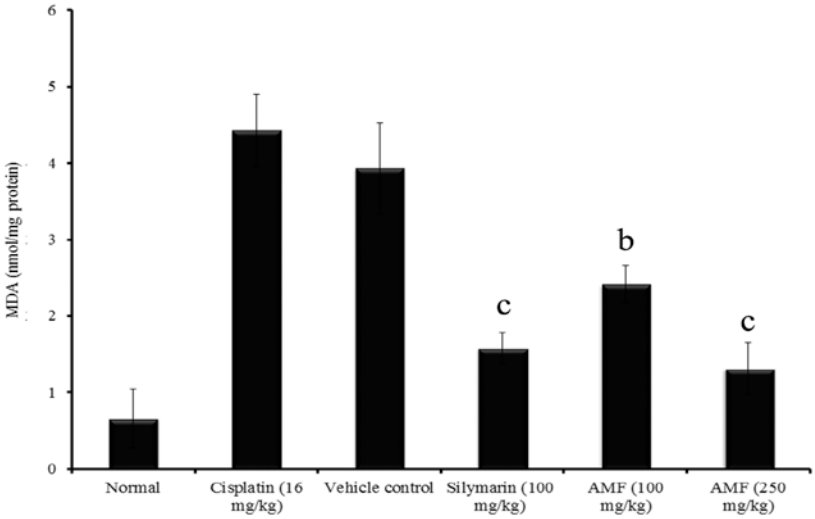

Figure 6.4 Effect of AMF on lipid peroxidation in the kidney of cisplatin treated mice. Values are mean ± S. D., n=6. c: P<0.001, b: P<0.01, a: P<0.05, (Tukey test). Vehicle control group were compared with treated groups.

6.3.3 *Effect of AMF on cisplatin induced kidney damage*

Section of the kidney from cisplatin treated group showed moderate to severe degree of glomerular atrophy and they showed decreased cellularity. The histopathology of tissue sections suggest that the control group had encountered vast histological damages as evidenced by the glomerular and tubular congestion with abnormal Bowman's capsule, blood vessel congestion, vacuolation in cytoplasm, necrosis and haemorrhagic areas. Inflammatory cells were also seen in kidney section from the cisplatin treated group. Concurrent treatment with the AMF extract was found to reduce such changes in kidney histology induced by cisplatin. Very few collections of lymphocytes are seen in the interstitial tissue. The histological features of the AMF 100 mg/kg b.wt treated group showed minimal cellular damage in contrast to the control group. The AMF 250 mg/kg b.wt treated group showed almost normal glomerular and tubular arrangements with minimal blood vessel congestion, very few inflammatory cells. The recovery was comparable to that of the standard drug. The nephrotoxicity was considerably reduced in AMF treated groups as evidenced from the histopathological results. There was almost a complete normalisation in the damage and haemorrhage caused by cisplatin induced AMF treated animals (Figure 6.5).

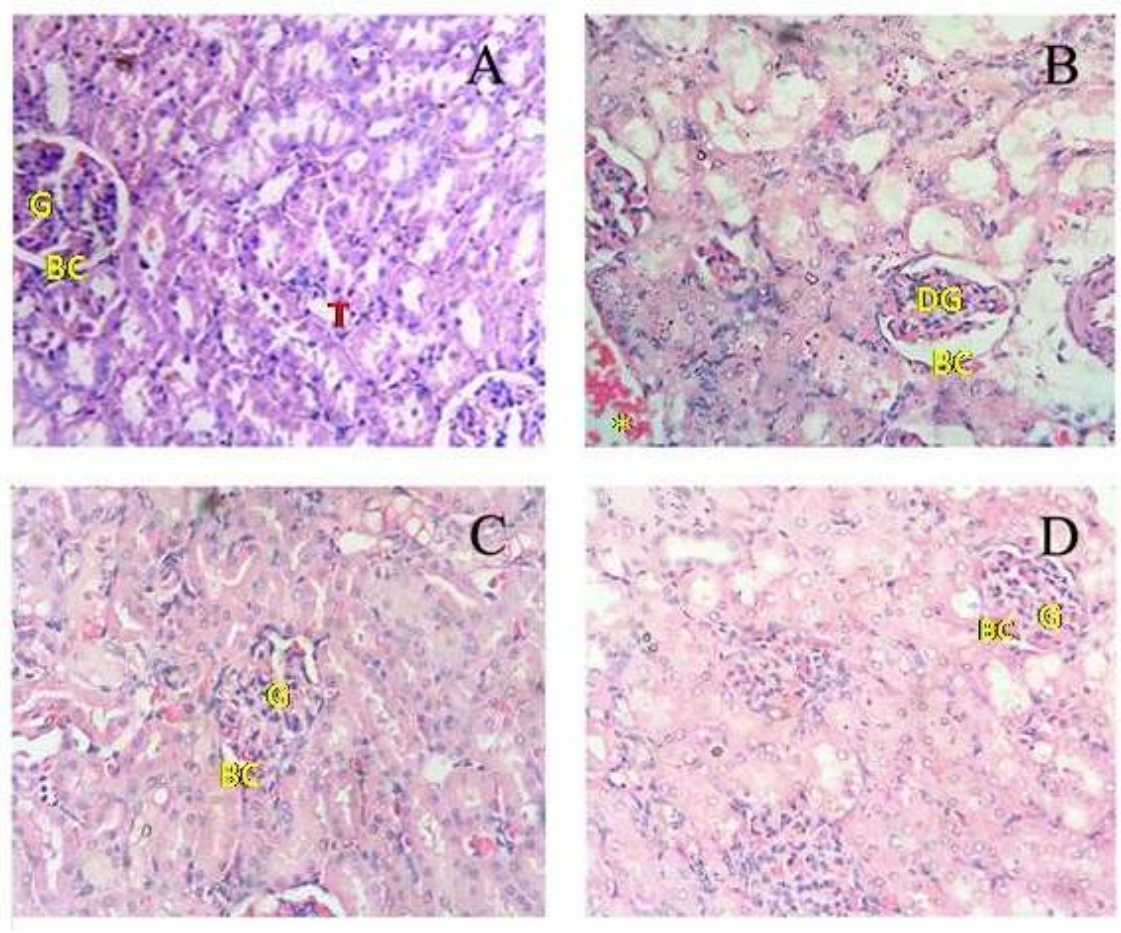

Figure 6.5 Histopathology of kidney treated with cisplatin (400X). (A) Kidney section of normal rats showing normal glomeruli (G), Bowman's space (BC) and normal tubules (T); **(B)** Cisplatin (16 mg/kg) treated group showing severe glomerular degeneration (DG), dilatation in Bowman's space and tubular necrosis invaded by inflammatory cells (*) **(C)** Silymarin (100 mg/kg) and **(D)** AMF 250 mg/kg treated showing normal glomeruli (G), Bowman's space (BC) and renal tubule more or less like a normal structure

6.3.4 Nitric oxide scavenging effect of AMF

Nitric oxide is a very unstable species and reacting with oxygen molecule produce stable nitrate and nitrite which can be estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease which can be measured at 546 nm. AMF exhibited nitric oxide scavenging activity in dose dependent manner and the IC_{50} value is estimated at 121.8 μg/mL). The standard drug, ascorbic acid exhibited IC_{50} value at 68.9 μg/mL (Fig 6.6).

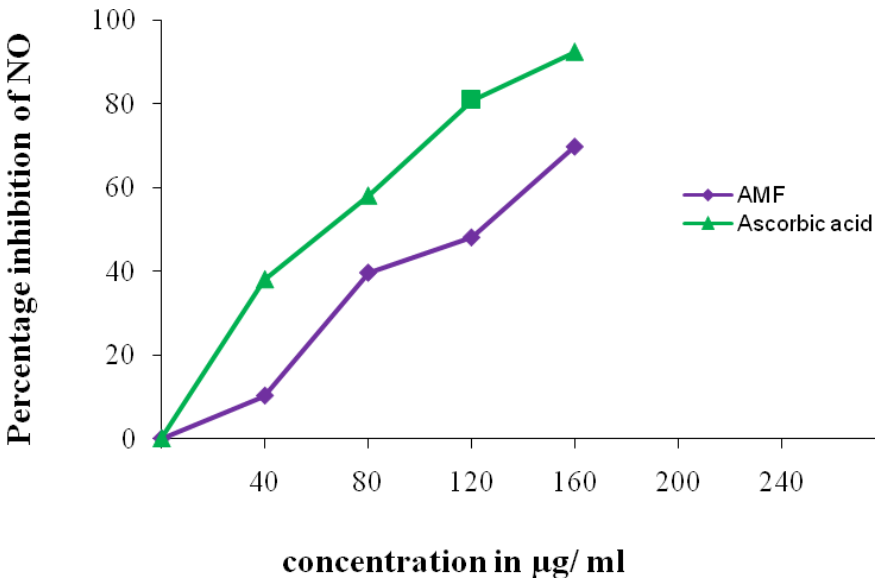

Figure 6.6 Comparison of the scavenging effect of AMF and ascorbic acid on nitric oxide radical. Values are mean ± standard deviation of three independent replicates.

6.4 Statistical analysis- As explained in 2.2.9 of Chap 2.

6.5 Discussion

Chemotherapy and radiotherapy are the important and most common methods of cancer treatment. Cisplatin is currently one of the most important chemotherapeutic drugs used in treatment of wide range of cancers. However, the clinical usefulness of this drug is limited due to the induction of nephrotoxicity. In the present study, the rats treated with single dose of cisplatin shown marked increase in serum creatinine and urea levels indicating induction of acute renal failure. The anti-oxidant enzymes like superoxide dismutases, peroxidases and catalases in the kidney were inhibited in cispaltin alone control group. There renal toxicity was accompanied by increase lipid peroxidation and marked depletion of GSH levels.

Cisplatin is known to accumulate in mitochondria of renal epithelial cells and induces ROS primarily by decreasing the activity of antioxidant enzymes and by depleting intracellular

concentrations of GSH (Naqshbandi *et al.*, 2011; Rajashekar *et al.*, 2012; Sara *et al.*, 2010). Cisplatin can directly interact with the kidney to generate superoxide anion in the free cell system. Moreover, it also enhances the activity of Ca^{++} independent nitric oxide synthase and thereby increases the production of nitric oxide through arginine metabolism (Srivastava *et al.*, 1996). This increased oxidative stress causes cell membrane damage due to oxidation leading to the elevation of LPO (lipid peroxidation) levels. The renal tissue stocks of GSH get depleted while overcoming this oxidative stress.

Treatment with the AMF lowered the level of creatinine and urea, when compared to the toxic vehicle control group. The cisplatin-induced malondialdehyde (MDA) production was decreased by the administration of AMF *in vivo* and it also attenuated cisplatin-induced GSH depletion in mice. Further, the reduced activities of other anti-oxidant enzymes were increased with cisplatin were restored in a dose dependent manner by the administration of AMF to a considerable extent indicating its ability to counteract oxidative stress. These biochemical results were supported by histopathological data. The statistical significance of the nephroprotective activity of AMF treated group and sylimarin (standard group) treated group were found almost equal as both groups gained same level of significance against the toxic group in most of the parameters. The comparison between control and vehicle control group was found to be non-significant in all the assays. Treatment with AMF resulted in the almost complete regeneration of the renal architecture.

Cisplatin treatment has been shown to induce loss of copper and zinc in kidneys. The decrease in SOD activity in renal tissues following cisplatin administration is manifested to be due to the loss of copper and zinc (Sharma, 1985). After cisplatin administration, the ability of the kidney to scavenge toxic hydrogen peroxide and lipid peroxides decreases and thereby the activity of catalase and GPx is also found to decrease. Although the mechanisms underlying the cisplatin-induced acute renal failure have not been fully understood, several investigators have shown that the ROS or free radicals are closely related to the acute renal failure induced by cisplatin (Badary *et al.*, 2005). Among the main approaches used to ameliorate or protect the cisplatin induced nephrotoxicities, the most consistent effects have been observed with the use of antioxidant agents (Jude *et al.*, 2011; Majid *et al.*, 2012).

NO is a free radical which is an effective inhibitor of several physiological processes such as smooth muscle relaxation and neuronal signaling (Hagerman *et al*., 1998). Studies on reactive nitrogen species in cisplatin-induced nephrotoxicity shows that that excess nitric oxide can generate peroxynitrite anions by reacting with oxygen (Siddhuraju *et al*., 2003). It is also reported that cisplatin induces the formation of NO mainly by activating NF-ϰB (Li *et al*., 2006). This, NO then reacts with superoxide anion, forming peroxynitrite which disrupts normal cell metabolism (Roberts *et al*., 2010). It is reported that flavonoids plays an important role in the inhibition of NO release when they are used as antioxidants (Shutenko *et al*., 1999). The *in vitro* antioxidant capacity of *Apoytes dimidiata* was revealed by its appreciable NO scavenging ability.

In a study conducted to evaluate the anti-inflammatory activity of gardenia extract, geniposide and genipin, it was reported that genipin inhibited production of and nitric oxide (NO) in the rat air pouch edema model (Koo *et al*., 2006). It is reported that an iridioid glycoside, genipin was isolated from the bark of *A. dimidiata* in association with its molluscicidal activity (Drews *et al*., 1996). In the cytotoxicity related studies, the presence of genipin in the AMF fraction of *A. dimidiata* leaf was detected (Chap 8). The preliminary phytochemical screening of AMF (chap 4) had revealed the presence of many antioxidant phytoconstituents during like saponins, tannins and steroids, carbohydrates, tannins and the same showed appreciable quantities of polyphenols and flavonoids during quantitative estimations. Here, AMF which is rich in flavonoids content and genipin showed scavenging property against NO free radicals in the inhibition assay in a dose dependent manner. Altogether, the results showed that AMF can serve as a complementary therapy to ameliorate free radical induced damages associated with chemotherapeutic drugs.

Chapter 7

Evaluation of anti-inflammatory and gastro-protective effects of Apodytes dimidiata

7.1 Introduction

Peptic ulcer, the sore on the inner lining of the stomach, duodenum or occasionally on the lower esophagus is a serious concern in gastroenterology (Najim, 2011). Globally, around 4% of the population is reported to have peptic ulcers occasionally and 10% of it develops a diseased condition as chronic or acute (Snowdown, 2008). Etiological studies have revealed that various factors such as stress, alcoholism, smoking, regular use of medications and infection of *Helicobacter pylori* are involved in developing gastric ulcers. However, an inappropriate proportion of digestive fluid in the stomach is considered to be the definite reason for the ulceration. In addition, associated reactive oxygen species (ROS) mediated infiltration of neutrophils (Wallance, 1997) and related inflammatory changes (Leibrisalo-Repo *et al.*, 1993, Jainu *et al.*, 2006, Wallance, 2011) are also known to augment the processes of gastritis. The prolonged gastric lesion and ulcers are reported to play a pivotal role in developing gastric cancer (Anand *et al.*, 2012, Oyagi *et al.*, 2010). On global scale, stomach cancer remains one of the most common cancer and one of the prime causes of gastric cancer includes *Helicobacter pylori* infection, dietary habits and genetic susceptibility (Dikshit *et al.*, 2011).

Many products are currently used for treating gastric ulcers including, steroidal (SAID) and non-steroidal anti-inflammatory (NSAID) drugs and reciprocal antiulcer drugs such as the antacids, anti-cholinergics and proton pump inhibitors etc. Although these drugs have brought about remarkable changes in ulcer therapy, their efficacy is still debatable and some are out of success in certain clinical trials due to their side effects that limit their usage (Ji *et al.*, 2012). Therefore, it is imperative to find out natural remedies with low side effects (Boakye-Gyasi *et al.*, 2008).

Today, a number of modern drugs are derived from the plants which are used as traditional medicines (de Souza Almeida *et al.*, 2011). *Apodytes dimidiata*, a common tree in South Africa is used in traditional medicine of Zulu for treating gastrointestinal ailments, and parts like leaves are used as a medication for ear inflammation. Based on a scientific study on the anti-mycobacterial uses of the plant, it was evidenced that the leaf extract (in acetone) was found to contain various phytoconstituents and showed antioxidant properties (Masoko and Nxumalo, 2013). Considering various reports, it is assumed that substances with strong antioxidant properties may inhibit the gastritis on mucosa (Dekanski *et al.*, 2009). Since long-time gastric ulcer increases the risk of stomach cancer, the present study aimed to

evaluate the anti-inflammatory and gastroprotective efficacy of *A. dimidiata* in this perspective. Since ethanol induces petechial lesions quickly (Ramirez and Rao, 2003), the study was performed in ethanol-induced gastric ulcer model in rats (Santos and Roa, 2001).

7.2 Materials and methods

7.2.1 Preparation of extract

The active methanolic fraction (AMF) which was found most effective in scavenging free radicals was used for further studies. The preparation is detailed in chap 2.2.1.1. of Chap 2.

7.2.2 Animals

Female BALB/c mice (20-30 g size) and male Wistar rats (180-200 g) were maintained as mentioned in 2.1.6. of Chap 2.

7.2.3 Anti-inflammatory activity on carrageenan induced paw oedema

Acute inflammation was induced by carrageenan by the method of Winter *et al* (1962). Male BALB/c mice were divided in to 6 groups comprising six animals each. One group served as positive control by giving carrageenan alone. Group II which served as standard reference drug diclophenac (10 mg/kg b.wt.) was given, intraperitoneally. Group III and IV were treated with different concentrations of AMF (100 and 250 mg/kg. b. wt.) orally for five consecutive days and group V were treated with propylene glycol (vehicle control). On fifth day, acute inflammation was induced by the sub-plantar injection of 0.02 ml freshly prepared 1% suspension of carrageenan in 0.1% CMC (carboxymethyl cellulose) on right hind paw in all groups of animals. Following administration, the footpad thickness was measured using Vernier calipers for every 5 h. The percentage of inhibition in paw volume swelling was calculated according to the following formula $[(V_t\text{-}V_o)$ control$\text{-}(V_t\text{-}V_o)$ treated group$/(V_t\text{-}V_o)$ control$] \times 100$, were V_t is the paw oedema at various time intervals and V_o is the initial paw oedema.

7.2.4 Anti-inflammatory activity on formalin induced paw oedema

Male BALB/c mice (n=6) were divided as group I - control, group II served with standard drug diclophenac (10 mg/kg. b. wt), group III and group IV- AMF (100 and 250 mg/kg b.

wt), respectively and group V treated with propylene glycol (vehicle control). Chronic inflammation was induced by sub-plantar injection of freshly prepared formalin (0.02 ml of 2% formalin) on the right hind paw in all groups (Chau, 1989). The drug was given orally, one hour before the formalin injection and continued for six consecutive days. The percentage of inhibition in paw volume was calculated according to the following formula $[(V_t-V_o)$ control-(V_t-V_o) treated group/(V_t-V_o) control$] \times 100$, were V_t is the paw oedema at various days and V_o is the initial paw oedema.

7.2.5 Effect of AMF on ethanol induced gastric ulcer

Male Wistar rats (n=6 and approximately180-200 gm b. wt) were divided into 5 groups. Group I was kept as normal without any treatment. Group II kept as control (ethanol); group III as standard (Ranitidine, 50 mg/kg b. wt); group IV as AMF (100 mg/kg b. wt.) and group V as AMF (250 mg/kg b. wt.) treatment groups. After 36 h of fasting, the respective groups of animals received 1 ml of ranitidine and AMF. After 1 hr, all the animals, except group I received 1 ml of 70% ethanol orally, to induce gastric ulcer. The animals were sacrificed after 4 h of ethanol administration. The abdomen of animals were opened, stomach dissected along the greater curvature, washed with ice cold normal saline and the damages were analysed.

7.2.6 Calculation of Ulcer Index (U.I.) and Percentage Inhibition (I %)

The ethanol treatment induced gastric lesions were seen as red striations. For determining the ulcer index, the lesions were graded based on the severity as follows - 1 mm or less - grade 1; 1-2 mm- grade 2 and more than 2 mm- grade 3. The U.I was calculated by using the formula, U.I = 1 x (number of lesions of grade 1) + 2 x (number of lesions in grade 2) + 3 x (number of lesions in grade 3). Then the overall score obtained was divided by a factor 10 which was designated as ulcer index (Main and Whittle, 1975). The percentage inhibition (I) of ulcer formation was calculated by the formula, I% = [(UI of control – UI of test) / UI of control] x 100.

7.2.7 Histopathological analysis

The excised stomach was thoroughly washed in PBS, fixed in 10% buffered formalin and embedded in paraffin wax. Sections of 5 μM thickness were stained with hematoxylin-eosin and viewed under a microscope.

7.2.8 Analysis of biochemical parameters

The glandular mucosa contained in inner stomach was collected by scraping with a knife. A 10% homogenate was prepared using the mucosa in Tris-buffer (pH 7.0). Lipid peroxidation of the tissue homogenate was measured by the method described in 2.2.3.9. of chap 2. SOD (2.2.3.4 of chap 2), catalase (2.2.3.5 of chap 2), glutathione peroxidase (GPx) (2.2.3.7 of chap 2) activities and the level of reduced glutathione (GSH) (2.2.3.6 of chap 2) were also measured. The protein content was determined according to the procedure described in 2.2.3.8 of chap 2.

7.3 Statistical analysis - The method followed for the statistical calculation is referred in 2.2.9. of chap 2.

7.4 Results

7.4.1 *In vivo* anti-inflammatory activity

The paw oedema induced by carrageenan in the hind limb of mice was increased acutely and attained a maximum size at 3rd hour. The oedema was found reduced by the administration of AMF and diclophenac treated groups. The reduction was estimated to be 55.17 and 30.8% for 250 and 100 mg/kg b. wt. of AMF, respectively. The results were comparable to that of the reference drug, diclophenac (Figure 7.1).

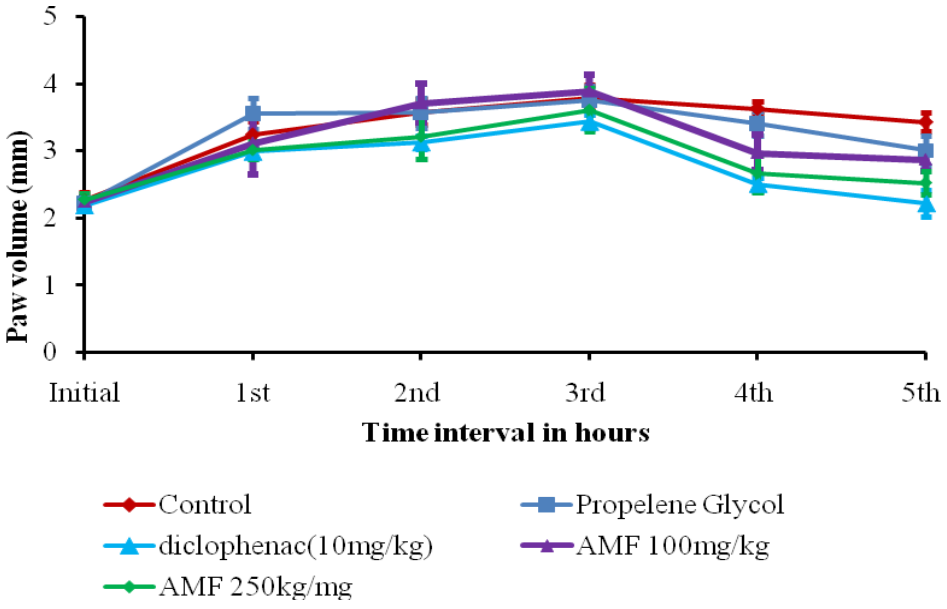

Figure 7.1 Effect of AMF on carageenan induced acute paw oedema

A gradual increase of oedema was observed by the injection of formalin and attained a maximum size at day 6. Upon AMF administration, a reduced volume of paw oedema was observed with a percentage reduction of 60.56 and 23.94% at 250 and 100 mg/kg b wt., respectively. Diclophenac exhibited a decrease of 70% against the formalin induced oedema (Figure 7.2).

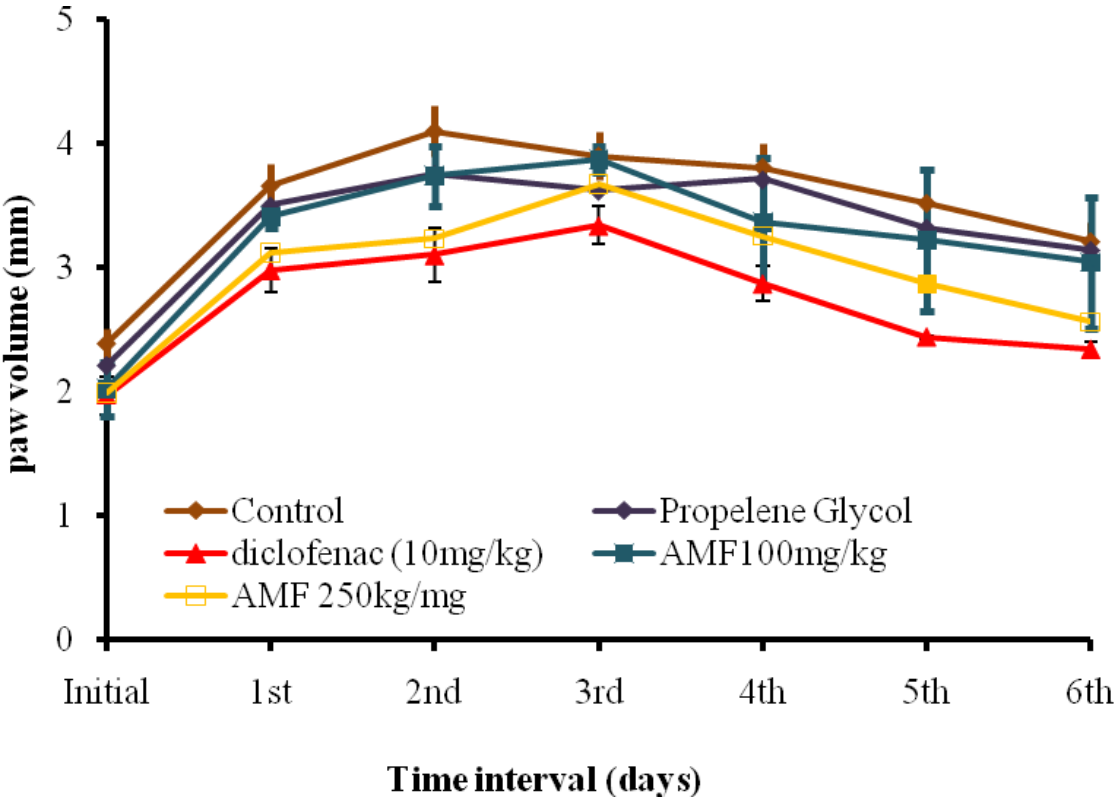

Figure 7.2 Effect of AMF on formalin induced chronic paw oedema

7.4.2 *Examining the gastric lesions*

Elongated hemorrhagic lesions of various sizes were observed on opening of the stomach by the induction of ethanol. The lesions of the control group animals were visible as long, thickened dark bands. AMF significantly reduced the severity of the lesions and only slight marks were seen in most cases and many were devoid of it (Figure 7.3).

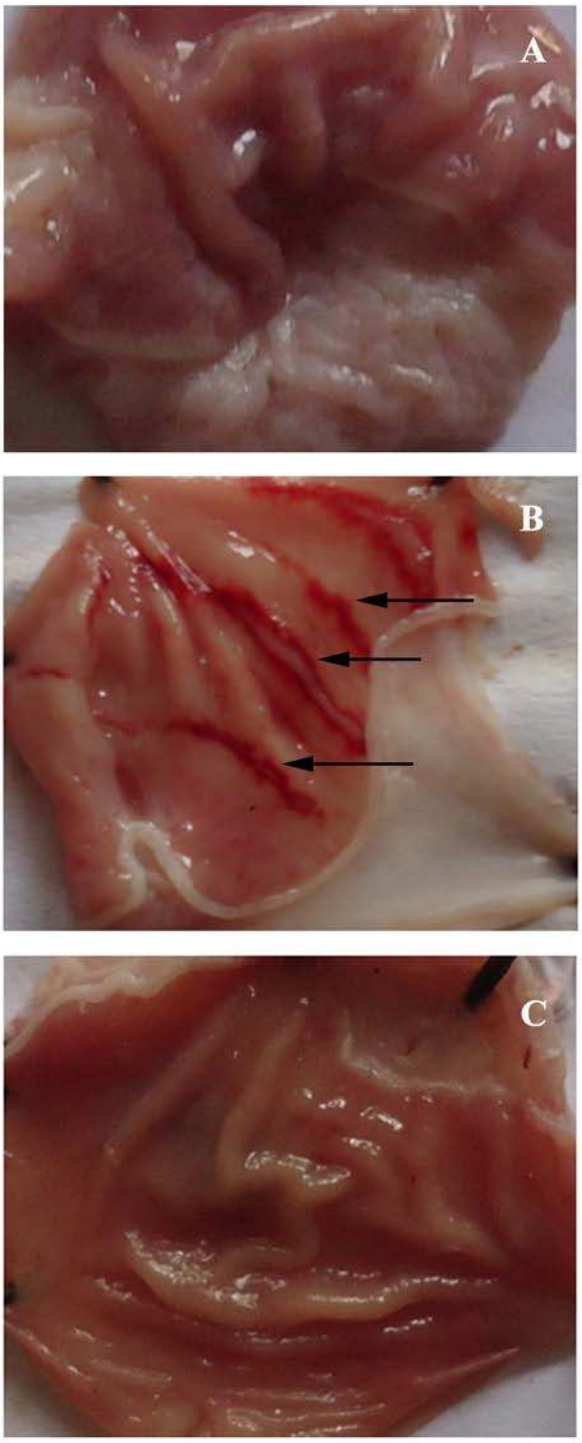

Figure 7.3 Morphology of rat stomach showing gastric lesions induced by 70% ethanol. (A) Normal without any treatment. **(B)** Control group treated with ethanol alone showed severe disruption to the surface epitheliumand necrotic lesions penetrate deeply into mucosa . **(C)** AMF 250 mg/kg treated rats, there is no disruption to the surface epithelium with no edema and no leucocytes infiltration

The gastroprotective properties of AMF against the damage were found slightly higher than that of the standard drug.

7.3.3 *Determination of ulcer index and percentage inhibition*

It was evidenced that were severe gastric lesions in ethanol treated animals and the Ulcer Index value calculated was 3.85 ± 0.17. This was significantly reduced to 0.12 ± 0.02 for AMF 100 mg/kg treated group (96.7 % inhibition) and no lesions were observed at 250 mg/kg AMF dose (Table 7.1).

Table 7.1 The percentage inhibition of AMF in the gastric ulcer formation in rats induced with ethanol

Groups	Dosage	Ulcer Index (U. I)	% of Inhibition (I %)
Normal		------	------
Alcohol Control	1 ml 80 % EtOH	3.98± .08	------
Vehicle Control	1 ml 80 % EtOH +0.1% Propelyene glycol	3.85 ± 0.17	3.2 %
Standard	1 ml 80 % EtOH + 50 mg/kg Ranitidine	0.35± .05	91.2 %
AMF Low	1 ml 80 % EtOH + AMF (100 mg/kg)	0.12 ± .02	96.7 %
AMF High	1 ml 80 % EtOH+AMF (250 mg/kg)	Nil	100 %

7.4.4 *Effect of AMF on gastric mucosal lipid peroxidation*

The level of MDA was found elevated in alcohol treated animals (1.34 ± 0.13 nmol/mg of protein). This enhanced level was restored by the administration of AMF. The reduction was calculated as to 0.85 ± 0.03 ($p < 0.001$) and 0.39 ± 0.06 ($p < 0.001$) for 250 and 100 mg/kg b. wt, respectively (Figure 7.4).

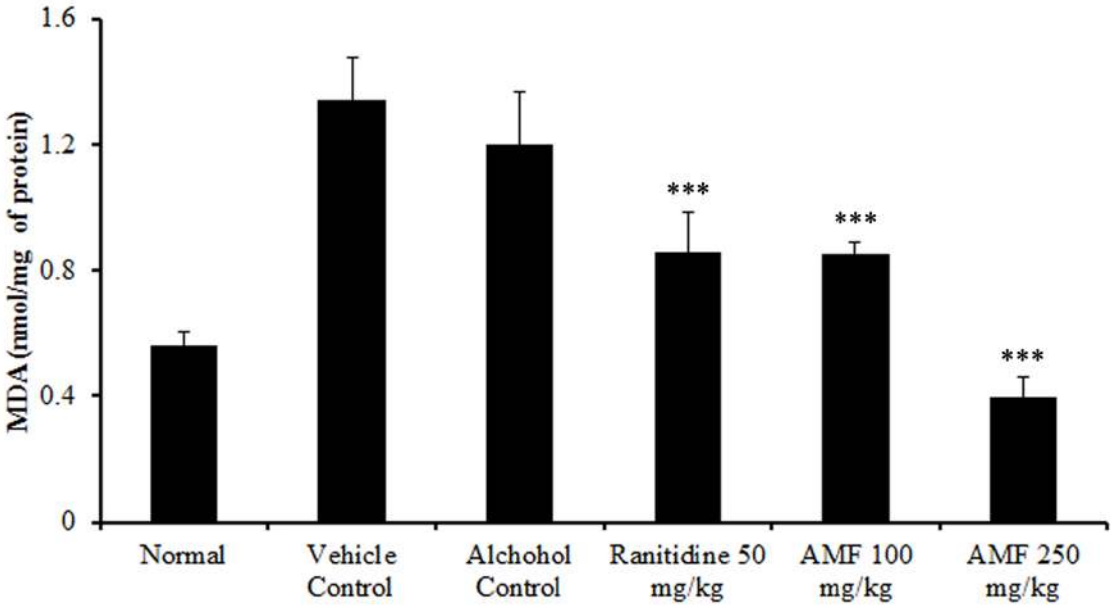

Figure 7.4 Effect of AMFon lipid peroxidation in ethanol induced gastritis. Values are expressed as mean ± SD for 6 animals; (*) $p < 0.05$, (**) $p<0.01$, (***) $p<0.001$ compared to vehicle control.

7.4.5 *Effect on gastric mucosal antioxidant status*

The ethanol mediated reduction in the antioxidant status of rat was found to be regained by the administration of AMF. Ethanol led to significant reduction in GSH levels (22.46 ± 1.8 nmol/mg protein) and treatment of AMF 250 mg/kg b. wt. elevated the level of GSH to 31.87 ± 1.34 ($p<0.001$). The level of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase were drastically reduced in alcohol treated group of animals to 0.67 ± 0.07, 4.74 ± 0.67 and 23.89 ± 1.09 U/mg protein, respectively. AMF treatment restored all the enzyme activities to the normal level- 0.92 ± 0.05 ($p<0.001$), 8.98 ± 0.37 ($p<0.001$) and 30.61 ± 1.09 ($p<0.001$), respectively (Figure 7.5). The effect of AMF on normalization of these antioxidant markers was comparable to that of the standard drug, ranitidine. Moreover, it was observed that AMF treatment gave better gastroprotective results compared to the standard group.

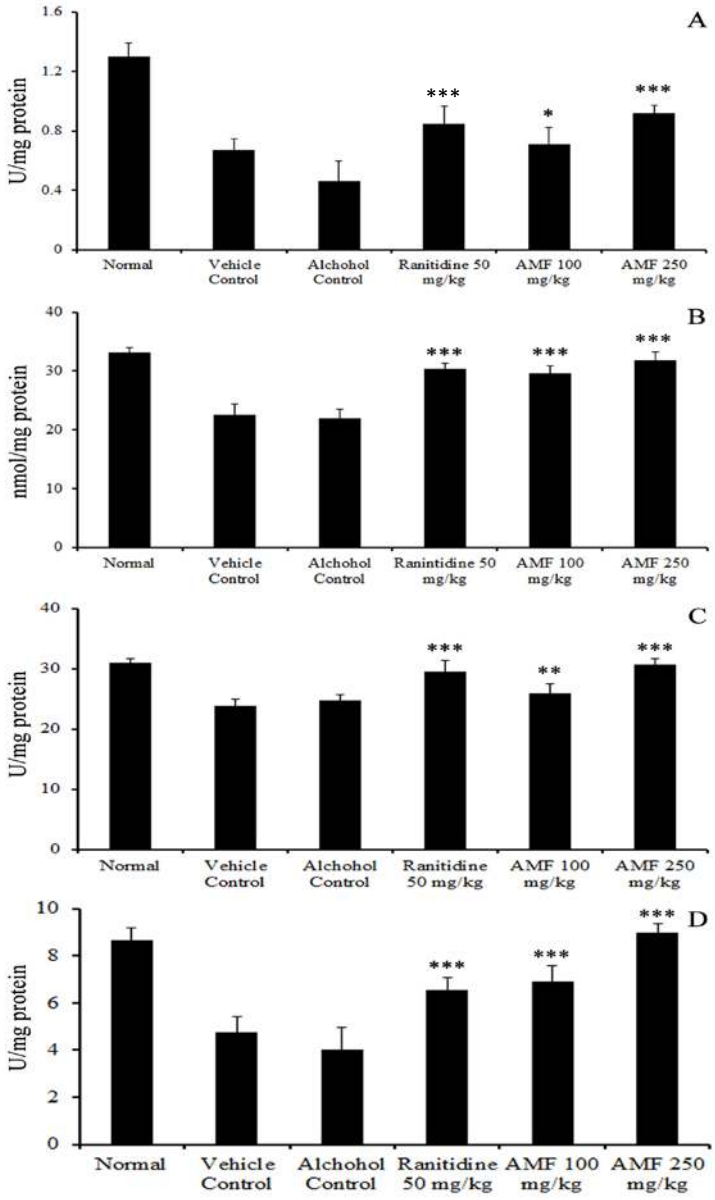

Figure 7.5 Effect of AMF on antioxidant parameters in gastric mucosa of rats treated with ethanol. SOD (A), GSH (B), GPx (C) and catalase (D). Values are expressed as mean ± SD for 6 animals; (*) $p< 0.05$, (**) $p<0.01$, (***) $p<0.001$ compared to vehicle control.

7.4.6 *Histopathology*

Effect of AMF on ethanol-induced gastric damage was further validated by the histological observations. The alcohol treated group of animals exhibited severe cell damages, disorganisation of cell nuclei and gland morphology and erosion of gastric mucosa. Pre-treatment with AMF (100 and 250 mg/kg) minimized these damages as shown in Figure 7.6.

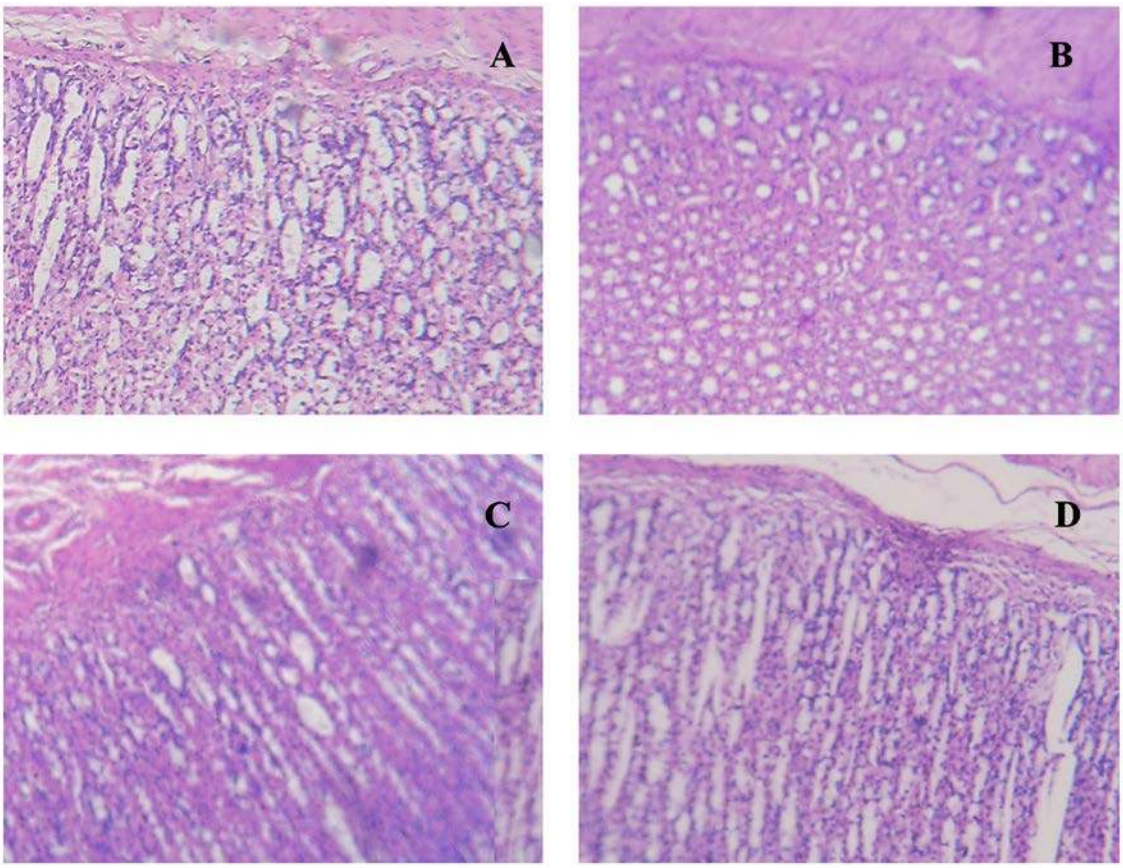

Figure 7.6 Histopathology of rat stomach (400) treated with ethanol. Normal (A), control - 1 ml 70% ethanol (B), standard - ranitidine 50 mg/kg b.wt (C), AMF - 250 mg/kg b.wt (D)

7.5 Discussion

High concentration of ethanol destroys the integrity of the gastric mucosa quickly through its toxic effect by reducing the bicarbonate production and mucus secretion (Pedernera, 2006). Consequently, the ethanol obstructs the blood flow and leads to the development of haemorrhage lesions and tissue injury in stomach. In the present study, the administration of ethanol results in severe damage to the gastric mucosal membrane as manifested in hemorrhagic ulcers. These haemorrhagic lesions are found to be abolished by the oral administration of *A. dimidiata* leaf extract (AMF) with a comparable effect to that of standard drug, ranitidine.

Ethanol intake is reported to stimulate the over production of reactive oxygen species (ROS) which trigger the associated inflammatory changes in gastrointestinal tract

(Cederbaum, 2001). According to Susanti et al (2007), the severity of damages is associated with the oxidative stress and subsequent lipid peroxidation. The role of antioxidants in prevention and treatment of gastric lesions is reported in a number of studies (Takeuchi *et al*., 1991). Accordingly, antioxidants have been proposed as therapeutic agents and co-adjuvants to prevent gastric injury (Jeon *et al*., 2014). Several plant extracts with antioxidant property have been reported to possess gastroprotective effect in experimental animals (Al-Hashem, 2010). In this study, administration of AMF is found to enhance the ethanol-induced depletion of antioxidant enzymes such as SOD, catalase and GPx in a dose dependent manner. It is importantly noticed that there was a significant rise in GSH content in the mucosa and MDA level was lowered by AMF administration. According to Body *et al* (1979), the presence of high amount of GSH in gastric mucosa which is the chief component of endogenous non-protein sulfhydril (NP-SH) pool might enhance scavenging of oxygen derived free radicals and the production of mucus (Salim, 1992). It is suggested that the harmful effects of ethanol on the gastric mucosa are the combined result of increased lipid peroxidation and a decreased glutathione level (Zamora *et al*., 2007). In view of these results, the present study clearly indicates the ability of AMF to counteract oxidative stress during ethanol mediated ulceration.

A variety of diseases are characterized by inflammation including ulcer and the search for a novel anti-inflammatory drug is still under pursuit. To know the anti-inflammatory effect of *A. dimidiata*, well-known *in vivo* models are being used. In these models, the oedema develops in two phases; the first phase characterized by the release of histamine, 5-HT and kinens, while in the second phase, prostaglandins (Brooks and Day, 1991) which subsequently leads to severe inflammation which involves vasoconstriction, vasodilation, swelling, redness and infiltration of plasma proteins. Since the inflammatory mediators have short half-lives, once the stimulus is removed, the inflammation ceases (Collins *et al*., 1998). Here in, AMF inhibited both acute and chronic inflammations significantly. The odema formation was reduced in a similar manner as that shown by the reference compound, diclophenac. Additionally, AMF was found to inhibit LOXs enzyme in an *in vitro* study. LOX is a key enzyme in the biosynthesis of leukotrienes which is considered to play a significant role in various inflammatory processes (Sircar *et al*., 1983, Bhattacharjee, 2007). It is found that antioxidants inhibit the formation of lipid hydroperoxide, the

substrate required for LOX catalysis (Rackova *et al.*, 2007). Earlier studies have reported the free radical scavenging property of *A. dimidiata* (Masoko and Nxumalo, 2013). Thus, the anti-LOX activity of AMF could be aided by its antioxidant property. Considering these results, the anti-inflammatory activity of AMF might be contributing to the protection of gastric mucosa from the damage.

It is reported that persons infected with *H. pylori* have a 10 to 20% lifetime risk of developing peptic ulcers and a 1 to 2% risk of acquiring stomach cancer (Chang and Parsonnet, 2010, Kusters *et al.*, 2006). The gastric damage induced by *H. pylori* is considered to be an etiological factor in gastric cancer. In a study conducted to investigate the protective role of *Gardenia jasminoides* Ellis (GJE) from gastritis, the ethanolic extract has been found to inhibit the growth of *H. pylori*. The presence of two compounds, ursolic acid and genipin in the extract with acid-neutralizing and antioxidant activities (Lee *et al.*, 2009) is suggested to be the active components. In the cytotoxicity related study, we could detect an iridoid glycoside, genipin in AMF (chap 8).

In conclusion, it is conceivable that the gastroprotective efficacy exhibited by AMF could be mediated by its anti-inflammatory and antioxidant mechanisms and this could be largely attributed to the various phytoconstituents identified in AMF. On further development, AMF can be considered as an attractive candidate for peptic ulcer and in advanced conditions for stomach cancer disease also. The study thus provides a scientific validation for the ethonopharmacological use of *Apodytes dimidiata* by the tribals of Zulu for treating various inflammatory and gastric ailments.

Chapter 8
Evaluation of cytotoxic and anti-tumour properties of Apodytes dimidiata and characterisation of the bioactive component

8.1 Introduction

Considering the side effects of synthetic products, even at the genomic level of healthy cells, natural products are currently been exploited as effective chemotherapeutic drugs. In modern medicine, approximately 50% of the prescriptions are phytochemicals or its analogues. Recently, various plant metabolites came out with exciting pre-clinical effects that could be a non-toxic alternative to the present day chemotherapies. Hence, the search for bioactive compounds from natural resources is more imperative (Suttana *et al.,* 2010; Hamedeyazdan *et al*., 2012)

Natural products owing to their chemical diversity provide unconstrained opportunities for new drug discoveries because of the supreme availability (Cos *et al.,* 2006). For this, extraction is the most important step in the analysis of constituents present in standardised plant extracts and herbal preparations. Studies indicate that the analysis of bioactive compounds present in the plant extracts involves usage of various techniques like phytochemical screening assays, HPLC, TLC as well as other non-chromatographic techniques like Fourier Transform Infra Red (FTIR) and the step-wise procedures for isolating the bioactive component includes extraction, pharmacological screening, isolation and characterization of bioactive compound and toxicological studies (Sasidharan *et al.,* 2011).

TLC is considered very effective in the isolation procedure because the crude extract is resolved into its different components and therefore simplifies the process (Rahalison *et al.,* 1991). High performance liquid chromatography (HPLC) is another widely used technique for the isolation of components and is used as a main tool for fingerprinting studies of plants (Fan *et al.,* 2006). NMR is used for structural and purity determination of compounds. Narrow chemical shift with sharp signals are produced in Proton NMR. The ^{1}H signal has been one of the analytical nucleus used for clinical magnetic resonance imaging.

Several chemotherapeutic agents have been developed from natural or synthetic sources by stepwise analysis and extraction and have played a significant role in reducing mortality/morbidity and in increasing patient's quality of life (Suggit and Bibby, 2005). Extensive search for anti-cancer agents as early as in 1950s, has led to the discovery of several drugs like the vinca group of alkaloids - vinblastine and vincristine, and cytotoxic podophyllotoxins (Cassady and Douros, 1980). Another important anti-cancer alkaloid, camptothecin derived from *Camptotheca acuminata* (Rahier *et al.,* 2005) and later on clinically

modified derivatives of camptothecin- topotecan and irinotecan (Cragg and Newman, 2005) are all world renowned anti-cancer drugs. The major side effects usually encountered with these conventional chemotherapeutics are toxicity (Brenner and Stevens, 2010) and multidrug resistance (Gottesman and Pastan, 1993). A multinational survey revealed that 35.9% of cancer patients were either past or present users of complementary and alternative medicine (CAM) (Molassiotis *et al.,* 2005). Nowadays, scientific community is in extensive search to find out agents from natural sources that has anti-tumour and cytotoxic properties and which can counteract the unwanted side effects, toxicity and multi drug resistance.

Plant products stands the basis of modern medicine and most chemotherapeutic drugs for cancer treatment are molecules identified and isolated from plants or their synthetic derivatives. Therefore, extensive studies are needed to focus on this dimension of chemotherapy. Apart from the screening of camptothecin from the endophytic fungi of *A. dimidiata*, no biological studies emphasizing on its anti-cancer potential have been done so far. In the initial cytotoxicity screening, the crude and partially purified fraction, AMF of *A. dimidiata* showed significant cytotoxicity against DLA and EAC cell lines. Hence, the present study investigated the cytotoxic and anti-tumour properties of *Apodytes dimidiata* leaf with emphasis on the isolation of active principle.

8.2 Materials and methods

8.2.1 Preparation of AMF

Using the leaves of *A. dimdiata*, AMF was obtained by partial purification as detailed in 2.2.1.1. of chap 2.

8.2.2 Cell lines

DLA, EAC, Jurkat and SK-BR-3 cells were maintained as described in 2.1.5. of chap 2.

8.2.3 Animals

Swiss albino mice were used for the study. Their maintenance is as mentioned in 2.1.6. of chap 2.

8.2.4 Cytotoxicity assays

Short-term cytotoxicity was assessed by trypan blue dye exclusion method (2.2.7.1. of chap 2). The cell viability was assessed by MTT assay, detailed in 2.2.7.2. of chap 2. The cells were exposed to different concentrations of test materials (AMF, fraction B) in MTT. The results obtained were compared to the standard anti-cancer alkaloid, camptothecin which was kept as the positive control under same conditions. An aliquot (5 μL) of treated EAC cells was stained Hoechst dye and acridine orange/ethidium (Ao/EtBr) bromide for morphological observations. The detailed procedure is in 2.2.8.1 of chap 2.

8.2.5 Effect of AMF on ascites tumour in mice

Ascites tumour was developed by injecting 1 x 10^6 EAC cells into the intra-peritoneal cavity of mice. Male Swiss albino mice were divided into 5 groups of 6 each. Group 1 served as untreated control; group 2 received a single intra-peritoneal injection of standard drug, and cyclophosphamide (25 mg/kg body weight) for 10 consecutive days. Group 3 was given 0.20 mL of propylene glycol orally (> 99% pure) and served as vehicle control. AMF, 100 and 250 mg/kg b wt was given orally (0.20 mL) for group 4 and 5, respectively. The drug treatment was started next day following injection of cells for 10 consecutive days. The animals were observed for death due to the tumour burden for 30 consecutive days. The life span of animals was calculated using the following formula: Percentage increase in life span (ILS) = (T-C)/C ×100, where T and C are mean survival of treated and control mice.

8.2.6 Solid tumour analysis

Solid tumour was developed by injecting 1 x 10^6 DLA cells intra-muscularly in to the hind limb of mice. The grouping of the animals and the administration of drug was same as that of ascites tumour assay. The tumour development in each group of animals was determined by measuring the diameter of tumour in two perpendicular planes using Vernier calipers every third day for 32 days. The tumour volume was calculated using the formula: $V = 4/3\ \pi\ r_1^2 \times r_2$ where r_1 is the minor radius and r_2 is the major radius (Jefferson *et al.*, 1996).

8.2.7 Thin layer chromatography

AMF was subjected to thin layer chromatography (TLC) using the solvent system- ethyl acetate: n-hexane (5:5 v/v). The six bands obtained after separation were marked as A-F. The detailed steps are given in 2.2.6.5. of chap 2.

8.2.8 Absorption spectrum analysis

The λ max of cytotoxically active fraction B (confirmed by trypan blue dye exclusion method) obtained after elution from TLC (1.20 mg/3 mL methanol) and the standard genipin (1 mg/3 mL methanol) was detected. The steps are given in 2.2.6.4. of chap 2.

8.2.9 HPTLC analysis

The samples, (10 μL each) was spotted from the stocks (AMF 20 mg/mL), fraction B (1 mg/5 mL) and standard genipin (0.50 mg/mL) on the plate, developed in the solvent system- ethyl acetate: n-hexane (5:5 v/v) and visualized as detailed in 2.2.6.6. of chap 2.

8.2.10 HPLC analysis

The crude methanolic extract (40 mg/mL), AMF (30 mg/mL), standard genipin (0.50 mg/mL) and fraction B (0.40 mg/mL) were used for the HPLC analysis as given in 2.2.6.7. of chap 2.

8.2.11 LC-MS analysis

The active fraction B and standard genipin was analyzed by LC-MS (2.2.6.8. of chap 2).

8.2.12 Nuclear Magnetic Resonance ^{1}H NMR ($CDCl_3$) analysis

The concentrates of isolated fraction B, was crystallized in ether and white crystals was obtained. Fraction B and genipin were analysed as given in 2.2.6.9. of chap 2.

8.3 Statistical analysis

Detailed in 2.2.9. of chap 2.

8.4 Results

8.4.1 Cytotoxic effect of AMF on cancer cells

Upon incubation with various cancer cell lines in long term MTT assay, AMF exhibited cytotoxicity in a dose dependent manner and the IC_{50} values calculated was 0.92 ± 1.43, 2.48 ± 0.91 and 3.95 ± 1.57 μg/mL for EAC, Jurkat and SK-BR-3 cells, respectively (Figure 8.1). On statistical analysis, the cytotoxicity of AMF was found significant as much of standard drug, camptothecin (Table 8.1).

Table 8.1 Cytotoxicity of different components ($R_f$values of the components are also inserted) of AMF obtained after separation by TLC using the solvent system ethyl acetate:n-hexane (5:5 v/v) to DLA and EAC cell lines (Trypan blue dye exclusion method).

Components (bands with R_f values)	IC_{50} (μg/ml)	
	EAC	DLA
A (0.76)	48.7 ± 2.43	52.4 ± 0.65
B (0.56)	4.8 ± 1.43	5.2 ± 0.32
C (0.37)	22.6 ± 0.67	19.5 ± 1.65
D (0.31)	20.3 ± 1.46	19.3 ± 1.72
E (0.22)	28.6 ± 1.98	24.4 ± 2.22
F (0.1)	34.1 ± 2.32	32.5 ± 1.59

On Hoechst staining of AMF treated EAC, remarkable morphological changes like cell shrinkage and nuclear condensation, characteristic of apoptosis were observed (Figure 8.2). Moreover, Ao/EtBr staining showed the live cells as green and the treated dead cells as orange. This change was observed in a concentration dependent manner. The gradual changes of nuclear condensation and finally the formation of apoptotic bodies was very evident (Figure 8.3).

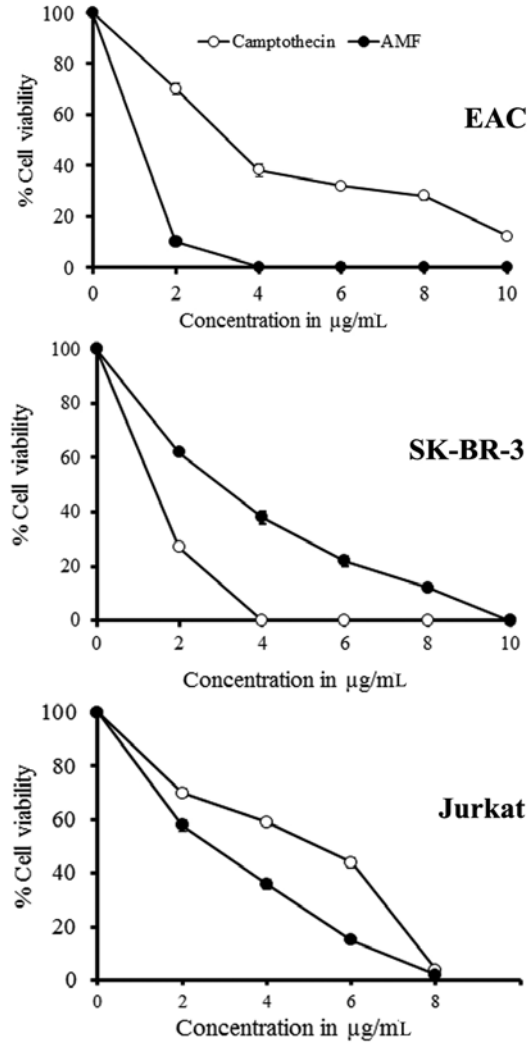

Figure 8.1 Effect of AMF on various cancer cell lines in MTT assay. All values are recorded as Mean ± SD based on three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment

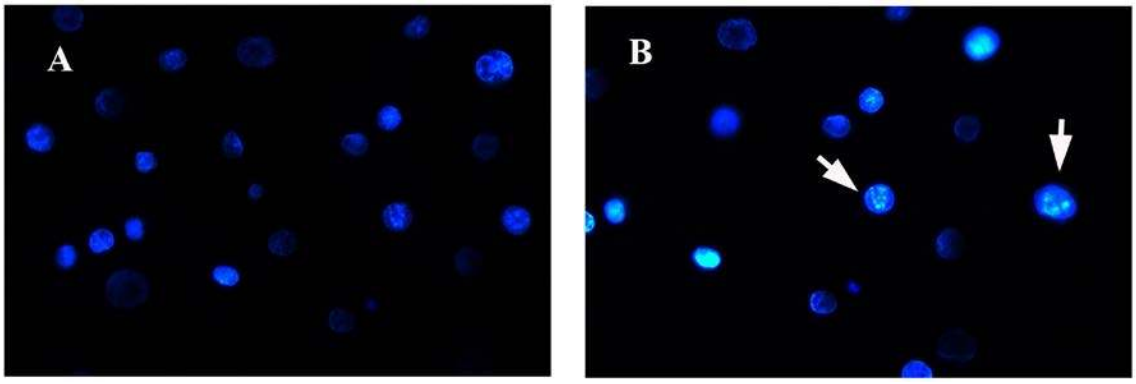

Figure 8.2 Morphology of AMF treated EAC cells. Cells were stained with Hoechst dye and viewed under fluorescence microscope (Leica Microsystem, Germany). (A) Control (B) Cells treated with 5 μg/mL of AMF and the cells marked with the arrow head indicate apoptotic bodies

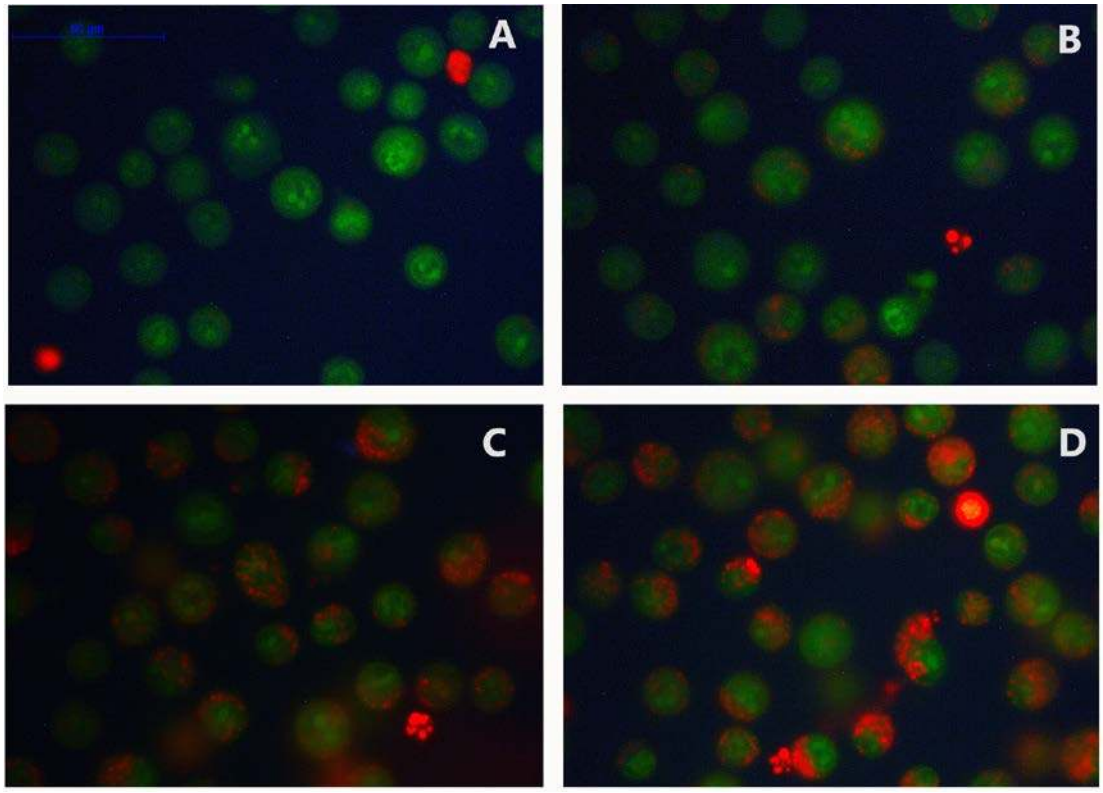

Figure 8.3 Morphology of AMF treated EAC cells. Cells were stained with Ao/EtBr and viewed under fluorescence microscope (Leica Microsystem, Germany). Control (A) Cells treated with 0.5 μg/mL(B) 2μg/mL (C) and 5 μg/mL (D) of AMF

8.4.2 Separation of AMF by TLC

On TLC profiling, AMF separated into 6 bands (A-F) and the individual fractions obtained showed varying levels of cytotoxicity (Table 8.2). Among these, the band with R_f value 0.56 (Fraction B) exhibited highest level of cytotoxicity with IC_{50} of 5.83 ± 1.92, 12.43 ± 3.35 and 14.82 ± 3.50 μg/mL for EAC, Jurkat and SK-BR-3 cells, respectively (Figure 8.4).

Table 8.2 A comparison of the cytotoxicity results of camptothecin against AMF on various cell lines using MTT assay

Treatment	Cell lines (IC_{50} values in μg/ml)		
	EAC	Jurkat	SKBr3
Camptothecin	3.6 ± 2.1	4.4 ± 1.2	1.8 ± 1.6
AMF	0.9 ± 1.4 ^{ns}	2.4 ± 0.9 ^{ns}	3.9 ± 1.5 ^{ns}

Values are mean ± SD of three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment. .'ns'- non significant. Results were considered statistically significant when $p<0.05$ compared to the standard drug, camptothecin.

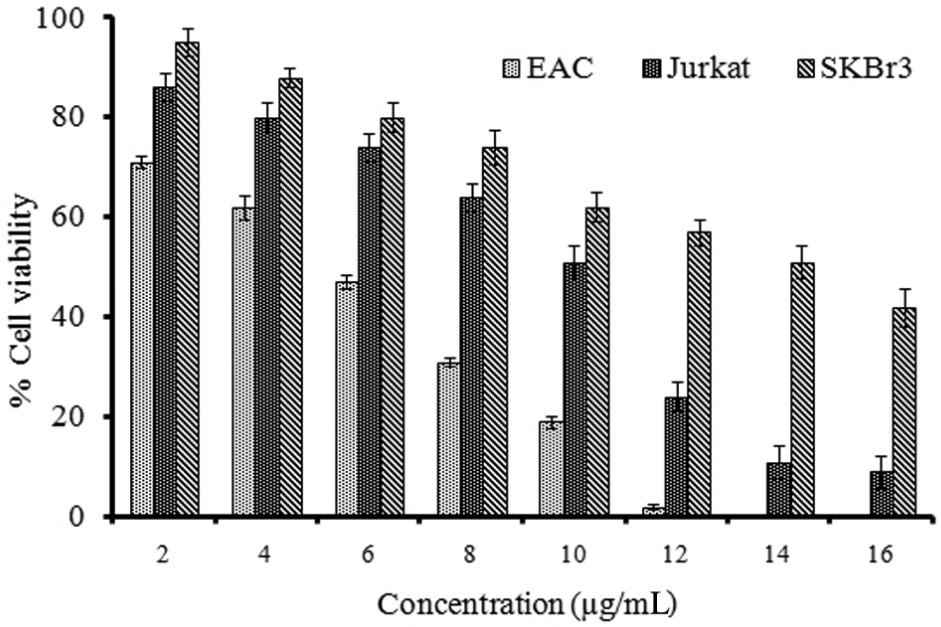

Figure 8.4 Effect of fraction B on various cancer cell lines in MTT assay. All values are recorded as Mean ± SD based on three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment.

8.4.3 *In vivo* anti-tumour analysis of AMF

In *in vivo* anti-tumour studies, treatment with AMF increased the life span of tumour bearing animals. The mean survival for the vehicle control group were 18.82 ± 2.27 days, whereas in the treated group, it was elevated to 24.24 ± 1.76 and 27.40 ± 3.85 days for 100 and 250 mg/kg b. wt. dose, respectively (Figure 8.5). The percentage increase in life span of the drug treated animals was calculated as 28.72 and 44.65% for 100 and 250 mg/kg b. wt., respectively. An elevated survival rate of 60.13% was exhibited in animals with the administration of cyclophosphamide, which was used as the standard.

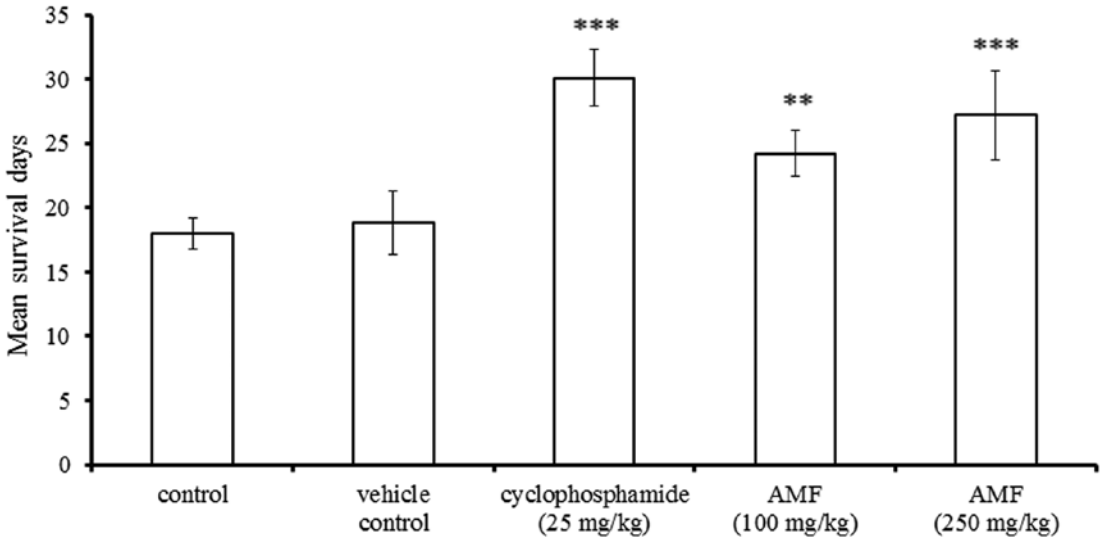

Figure 8.5 Effect of AMF on ascites tumor in mice induced by EAC. Values are mean ± SD (n= 6). *p< 0.05, **p < 0.01, p<0.001 compared to vehicle control.

The tumour volume was reduced in drug treated animals compared to control in the solid tumour assay. On 32nd day of measurement, the volume of tumour was 3.25 ± 1.17 cm^3 in the case of control animals while it was significantly reduced to 1.42 ± 0.56 and 1.84 ± 0.38 cm^3 for 100 and 250 mg/kg b. wt. AMF treated groups (Figure 8.6). The animals which received cyclophosphamide showed a significant reduction in tumour volume to 1.13 ± 0.25 cm^3. The percentage inhibition of tumour volume was calculated as 64.73 in cyclophosphamide and 55.14 in the case of AMF (250 mg/kg b. wt.) treated group.

8.4.4 HPTLC and HPLC analysis of AMF

The fraction B obtained from TLC was scanned in the wavelength range of 200-700 nm and showed the absorption maxima at 240 nm. The iridoid glycoside, genipin also showed the maximum absorption at 240 nm (Figure 8.7). On HPTLC analysis, AMF resolved into a total of six bands named A-F from the solvent front. The most active fraction B had the same R_f value 0.56 as that of the standard, genipin (Figure 8.8).

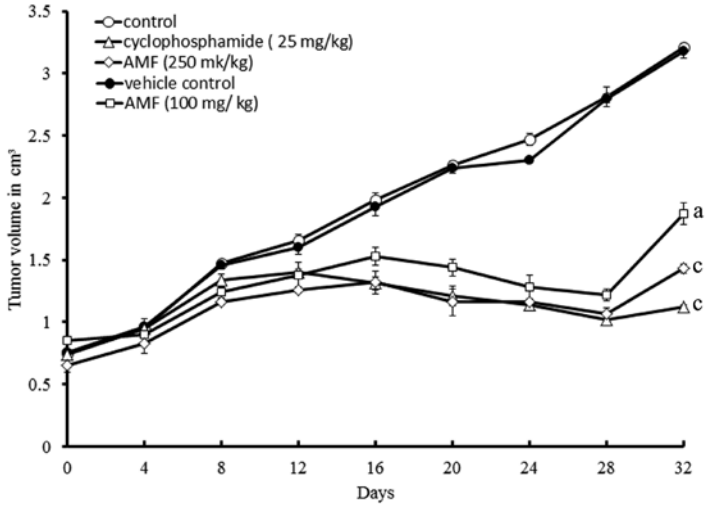

Figure 8.6 Effect of AMF on solid tumor in mice induced by DLA. Values are mean ± SD (n= 6). a ($p< 0.05$), b ($p < 0.01$), c ($p<0.001$) compared to vehicle control.

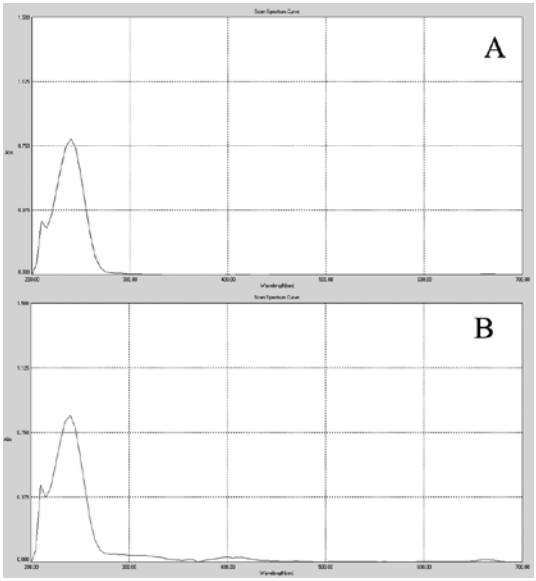

Figure 8.7 UV-visible spectra of (A), standard genipin and (B) Fraction B of *A. dimidiata*. The standard genipin (1 mg/3 mL) and fraction B (1.2 mg/3 mL) were dissolved in methanol, scanned the absorbance from 200 to 700 nm by spectrophotometer and analyzed the data with UV win5 software

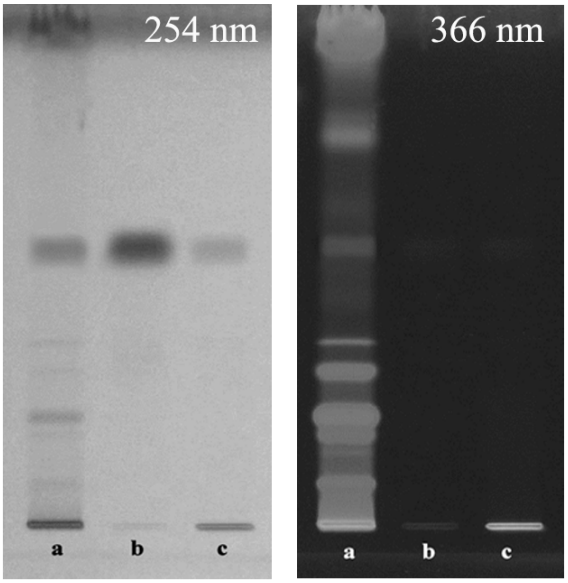

Figure 8.8 High performance thin layer chromatography (HPTLC) of (a) AMF (20 mg/mL) showing six fractions (A-F), (b) standard genipin (0.5 mg/1 mL), (c) fraction B (1 mg/5 mL).

The R_f values of the other fractions A, C, D, E and F are shown in table 1. On HPLC analysis, the crude methanolic extract and the AMF showed 21 and 14 peaks, respectively. Both AMF and crude methanolic extract showed a peak corresponding to that of the standard genipin, at a similar retention time 5.03. The retention time, 5.03 min detected for the fraction B by HPLC analysis was same as that of standard, genipin (Figure 8.9).

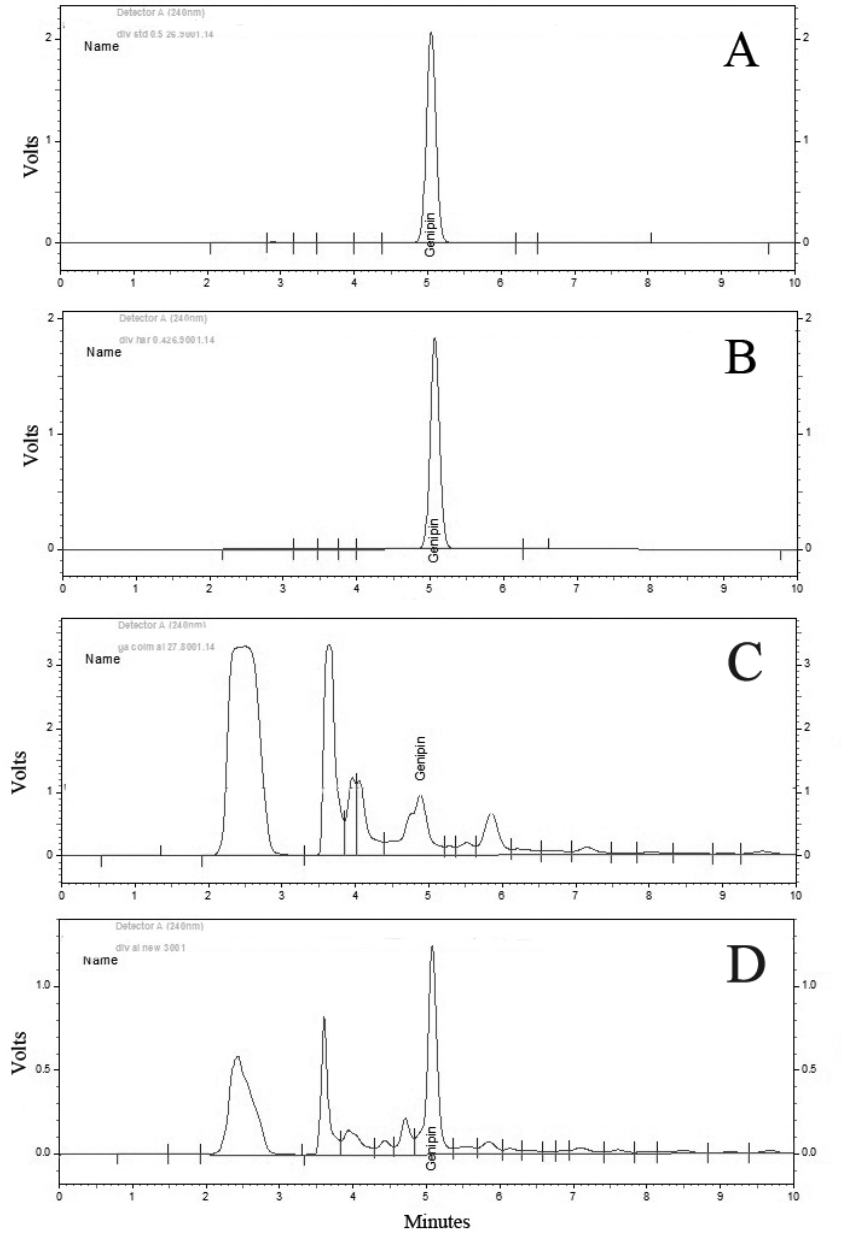

Figure 8.9 HPLC chromatogram of standard genipin (A) fraction B (B) Crude methanolic extract (C) and AMF (D) of *A. dimidiata*. Crude methanolic extract (40 mg/mL), AMF (30 mg/mL), standard genipin (0.5 mg/mL) and fraction B (0.4 mg/mL) were dissolved in methanol and injected

8.4.5 LC-MS profiling of AMF

LC-MS analysis of both standard genipin and fraction B gave *m/z* 227 (M) + as the prominent ion and matches with the molecular weight of genipin. The m/z spectrum also contains ions at m/z 248, some 23 Da higher than the expected molecular mass. These can be-identified as the sodium adduct ions, $(M + Na)^+$, and are quite common in electrospray ionisation. A fragmented ionic pattern of the fraction B was observed as; *m/z* 227, 209, 191, 177, 149 and 121 and a similar pattern was seen with the standard genipin (Figure 8.10). The percentage yield of active fraction B from the dried leaf powder of *Apodytes dimdiata* was calculated as 0.03128 % by Shimadzu Class VP offline processing software based on the peaks obtained from HPLC profile.

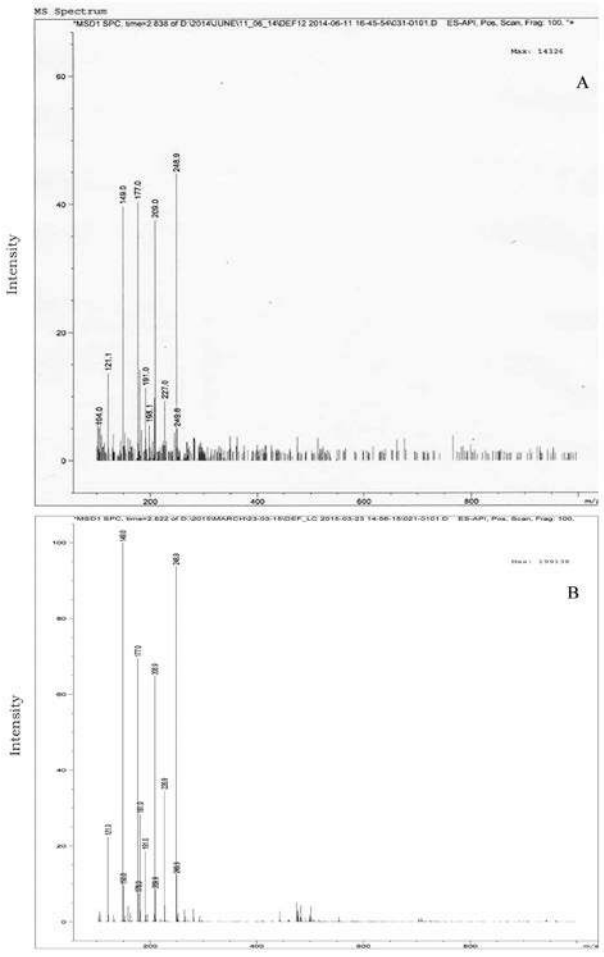

Figure 8.10 LC-MS spectra of (A) fraction B and (B) standard genipin using column: Eclipse Plus C18 4.6×250 mm, 5 μm, mobile phase: 5 mM ammonium formate: acetonitrile (50:50), flow rate 1 mL/min, wavelength 240 nm, column oven temperature at 25°C.

8.4.6 ^{1}H NMR spectrum analysis of AMF

^{1}H NMR spectrum of isolated fraction B and genipin was identical as evidenced from the chemical shifts and revealed a strong solvent ($CDCl_3$) peak at 7.26 ppm. The spectra gave peaks around δ 3.72 and 3.22 ppm due indicating –OCH3 group and cyclic OH group, respectively (Figure 8.11). Based on the proton-NMR data, the tentative structure of the fraction B is proposed (Figure 8.12) and this confirms that the isolated fraction B is genipin.

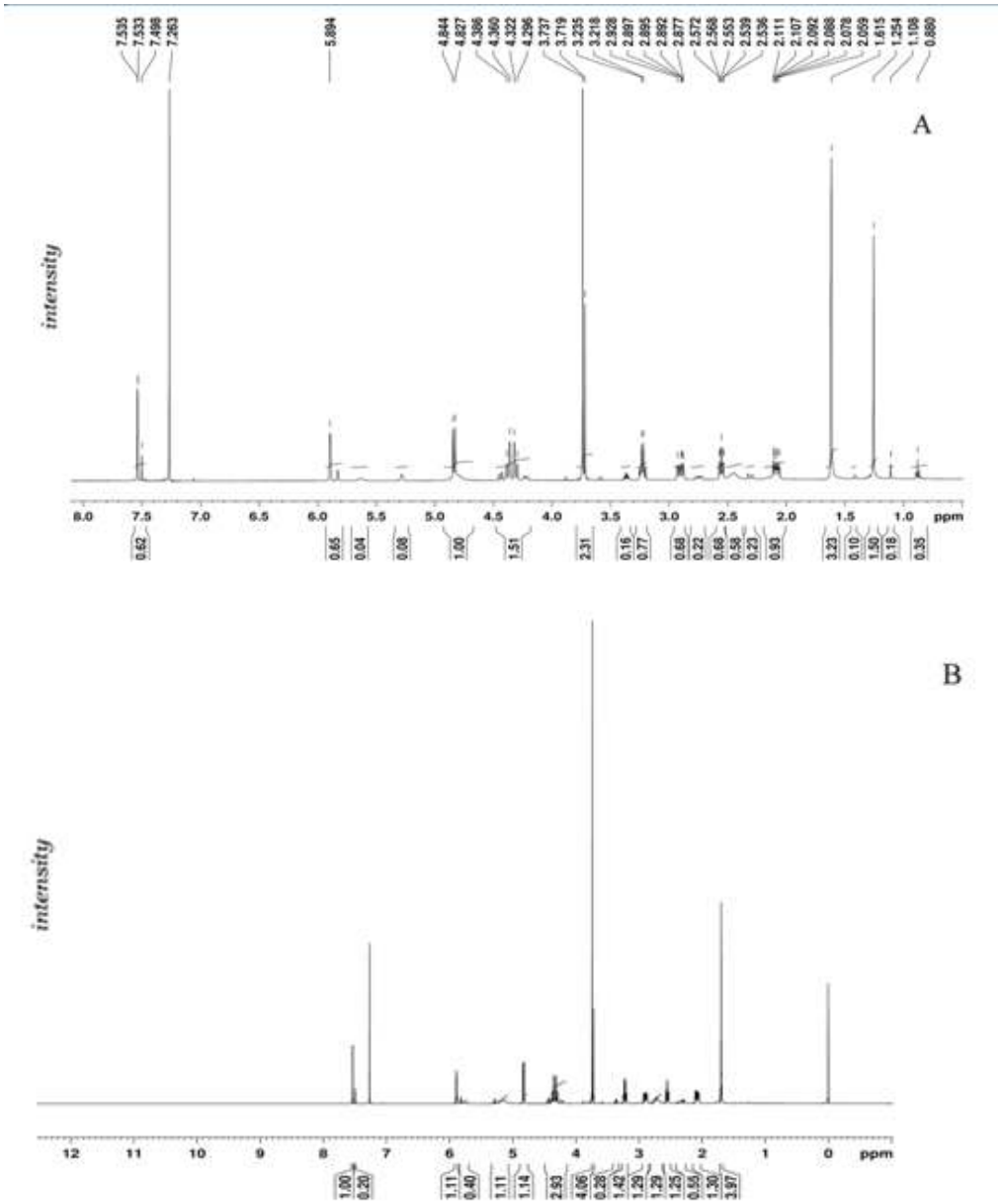

Figure 8.11 ^{1}H NMR (500 MHz, $CDCl_3$) analysis spectrum of fraction B and (B) standard genipin

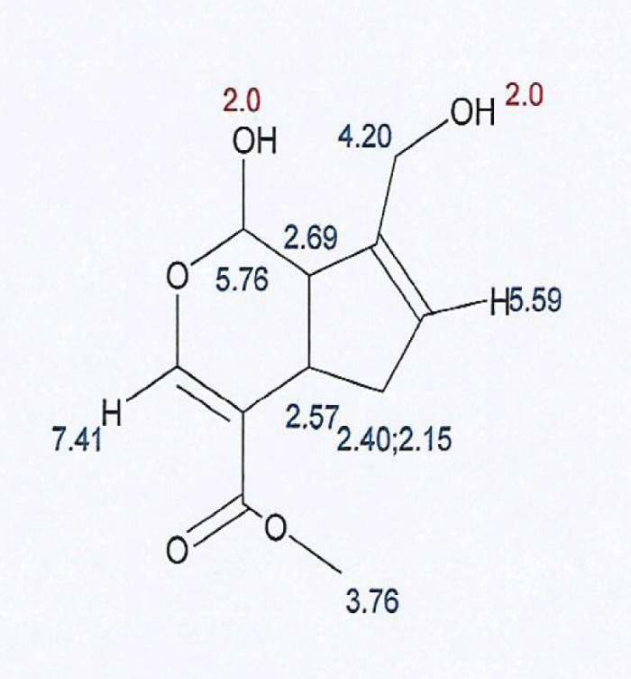

Figure 8.12- Structure of fraction B

8.5 Discussion

In the preliminary cytotoxic evaluation, the crude methanolic leaf extract of *A. dimidiata* showed significant cytotoxicity against cancer cell lines as shown in chap 3. Further purification of the extract by column chromatography yielded the active fraction in methanol (AMF). The cytotoxic effect of AMF obtained was comparable to that of the positive control, camptothecin used in the study and it was found to be more cytotoxic in cell lines like EAC and Jurkat. The American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation is less than 30 μg/mL (Abdel-Hameed *et al.*, 2012). However, crude extract having IC_{50} less than 20 μg/mL (Mahovarasirikul *et al.*, 2010) is considered highly cytotoxic. An IC_{50} value below this specified limit of NCI was observed for AMF against the entire cell lines studied, thereby proving its anti-cancer potential. The observed morphological alteration of the treated cells suggests apoptotic mode of cell death.

Considering the cytotoxic potential, the effect AMF on mouse tumour model was examined. A significant reduction in tumour development and extent of lifespan of tumour bearing mice was observed and the results were comparable to that of positive control,

cyclophosphamide. The reliable criteria on judging the value of any anti-cancer drug is the prolongation of lifespan of the animal (Clarkson and Burchneal., 1965). In sub-acute toxicity study, it was evidenced that AMF administration was non-toxic up to 500 mg/kg body weight dosage (discussed in chap 4). Since AMF was highly effective at the therapeutic dose (250 mg/kg b wt) in ascites as well as solid tumour model and this dosage being far below the expected LD_{50} value, it can be considered as a safe anti-tumour agent. The long-term use of the leaves and flower of *A. dimidiata* by the traditional healers (Hutchings, 1989) also hints its non-toxic nature. Moreover, there are reports on the plant being non-toxic based on mammal toxicity studies (Brackenbury *et al.*, 1997).

During the long-term cytotoxic analysis, a colour change of the culture medium to blue was noticed following the addition of fraction B. The color change observed was found irrespective of pH. It is reported that genipin is a natural cross linking agent which reacts with amino acids to generate blue pigment (Cho *et al.*, 2006) and the same principle has been well utilized in food industry to prepare a series of blue pigments (gardenia blue) (Hou *et al.*, 2008). Considering these indications, it is possible that the fraction B, like genipin could have interacted with the amino acids present in the RPMI medium which resulted in the colour change.

The λ max (240 nm) obtained for fraction B in absorption spectrum analysis was same as that of genipin. In HPTLC analysis, both genipin and the fraction B moved with the same relative mobility and in HPLC, both were eluted with the same retention time. In LCMS analysis, the ionic mass spectrum of fraction B was found similar to mass spectrum (m/z) of genipin, that is a prominent ion of *m/z* (M)+ 227 which is well in agreement with the molecular weight of genipin (Drewes *et al.*, 1996). The structure elucidation by H^1 NMR analysis also substantiated the fact that the fraction B purified from the leaf of *A. dimidiata* was genipin and the spectrum of genipin and fraction B showed identical chemical shifts. Even though, the alkaloid camptothecin is reported in the bark of *A. dimidiata*, the study shows that the leaves are devoid of the same (Ramesha *et al.*, 2013). In our studies also, we could not detect camptothecin in the leaf extract of *A. dimidiata*.

Genipin is an aglycone of geniposide isolated from the fruit of *Gardenia jasminoides* (Park *et al.*, 2002). It exhibits a wide range of biological properties, including anti-diabetic (Zhang *et al.*, 2006), anti-inflammatory (Hwa *et al.*, 2011), anti-bacterial (Lelono *et al.*, 2009), anti-

metastatic (Wang *et al*., 2012). Apoptotic induction associated with the activation of c-Jun NH_2-terminal kinase and p53 in HeLa cells (Cao *et al*., 2010) is also reported. It is suggested that genipin or its derivatives could be useful to inhibit uncoupling proteins (UCP2), the anti-apoptotic shield conferred to cancer cells (Zhang *et al*., 2006; Valle *et al*., 2010).

In view of the above discussed properties of genipin, the cytotoxic and anti-tumour efficacy exhibited by AMF could be largely due to genipin. Moreover other phytoconstituents having anti-tumour and chemopreventive properties identified in GC-MS analysis like squalene, hexadecanoic acid, oleic acid etc would have also contributed to the above discussed properties of AMF. Most of the natural drugs used for cancer are isolated from bark of stem or root of the specimen and the continuous extraction is a threat to the existence of plant species. In this context, the AMF can be used as a sustainable source as it is obtained from the leaf without much destruction to the plant. The present study thus provides an insight to the value added utility of *A. dimidiata* by exploring its possible anti-cancer potential.

Chapter 9

G1 arrest and induction of apoptosis by Apodytes dimidiata in human cervical cancer cell line, HeLa

9.1 Introduction

Chemotherapy and chemoprevention are the two important strategies implicated in the development of anticancer drugs. Even though the chemotherapeutic drugs follow many mechanisms of action like spindle arrest, topoisomerse inhibition, pore formation, obstruction in purine, pyrimidine synthesis etc, induction of cytotoxicity by inducing apoptosis seems to be the major or final event in most of the cases. Studies indicate that induction of apoptosis is the mechanism behind the anti-proliferative ability of many chemotherapeutics (Kelloff *et al*., 1994). But, toxicity remains the primary challenge for almost all the drugs used in cancer (Meyskens *et al*., 1994). Most of the chemotherapuetic drugs target not only cancer cells but also normal dividing cells (Kaelin, 2005) and the resistance shown by tumour cells towards chemotherapeutic drugs, better known as multiple drug resistance are the major concerns that of cancer therapy (Gottesman and Pastan, 1993).

The emergence of resistance to cancer chemotherapy has forced researchers to turn to natural products of plant and marine origin (Luqmani, 2005). Thus, developing chemo-preventive/-therapeutic agent with less toxicity and multiple drug resistance are the main criteria followed in cancer research. Since the plants or its products have lesser toxicity and greater structural diversity than synthetic compounds (Shah *et al*., 2006), extensive search for anti-cancer agents from the plant sources is under progress.

The word 'apoptosis' comes from the ancient Greek, meaning 'falling off ' (of petals from a flower) and refers to the formation of apoptotic bodies, a significant morphological change associated with the process (Kerr *et al*., 1972). There are various methods by which cell death can occur. Type I cell death or apoptosis involves characteristic changes like chromatin condensation and fragmentation, overall cell shrinkage, formation of apoptotic bodies enclosing nuclear or cytoplasmic material, blebbing of the plasma membrane and hydrolysis of nuclear DNA into internucleosomal fragments (Taylor *et al*., 2008). The type II cell death, also known as autophagic cell death is characterized by the formation of autophagosomes (massive accumulation of double-membrane containing vacuoles) that eventually fuses with lysosome vacuoles and Type III cell death is necrosis.

Apoptosis is an important cell death pathway employed for removing unwanted and harmful cells in a clean or silent manner during various growth stages and the alterations of apoptosis are umpired by the activation of intracellular proteases known as caspases

(Taylor *et al*., 2008). Caspases such as caspase 3, 6 and 7 cleave other cellular substrates, whereas the activation of the downstream effector caspases is regulated by the initiator caspases, 8, 9, and 10. The two main pathways by which the caspase activation occurs are the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. The extrinsic pathway mediated apoptosis plays significant role in the maintenance of tissue homeostasis, especially in the immune system, while the mitochondrial intrinsic pathway is activated in response to extracellular signals and DNA damages (Danial and Korsmeyer 2004; Debatin and Krammer, 2004).

Sometimes certain features of apoptosis seem to overlap with that of necrosis. It is therefore critical to confirm whether the cell death has occurred *via* or not. Since apoptosis is a well synchronised sequence of events, there are many ways to diagnose the process. When caspases become activated during apoptosis, they cleave specific substrates, either activating or inactivating them. The cleavages resulted in apoptosis by the activation of caspases produces distinct morphological changes and this one of the hallmarks of the process (Fischer *et al*., 2003).

Apoptotic drugs are usually chemical compounds that exhibit cytotoxicity (cell killing property). The cytotoxicity assays focuses on the alterations of plasma membrane permeability wherein the viable cells with intact plasma membrane and dead cells with damaged plasma membrane can be discriminated by differential staining. The Hoechst 33342 dye or acridine orange penetrates the plasma membrane and the highly condensed chromatin in the apoptotic nuclei is uniformly stained by Hoechst 33342, without permeabilization. In addition, the fluorescent dye, propidium iodide (PI) becomes highly fluorescent after binding to DNA and the stained and unstained cells are counted with flow cytometer. On the other hand, colorimetric assays like MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) measures cell viability based on the principle that dead cells are unable to metabolize various tetrazolium salts to a colored formazan product (Schulze-Osthoff, 2008).

In an organism, cell division is a highly controlled process and then cells do not undergo division without receiving appropriate signals. G0 phase (quiescence) is the resting stage of the cell cycle and various cascades of signals activate the cell division and the cell enters

into the active phases of the cell cycle. It is the G_1 to S phase transitory phase which decides whether a cell has to undergo division and many antitumor drugs seems to work by inducing cell cycle arrest.

In the previous study (chap 8), *Apodytes dimidiata* was found to exhibit significant cytotoxic and antitumor properties and an iridoid glycoside, genipin was isolated as the active component. Since we have done only a preliminary screening (Hoechst dye staining) for evaluating the apoptotic mode of action of the plant, the current study aimed to elucidate the apoptotic mechanism in detail using, morphological dual staining methods, quantification of caspase genes and detection of cell cycle arrest by flow cytometry.

9.2 Materials and methods

9.2.1 Preparation of AMF

The method for obtaining the active methanolic fraction of *A. dimidiata* (AMF) by partial purification is detailed in 2.2.1.1 of chap 2.

9.2.2 Cell lines

The cell line used for the present study was HeLa. The cells were maintained as per the conditions mentioned in 2.1.5. of chap 2.

9.2.3 Detection of cell viability by MTT assay

HeLa cells were seeded and incubated with various concentrations of AMF and the standard drug, camptothecin (2- 20 μg/ml), in RPMI media as described in 2.2.7.2 of Chap 2.

9.2.4 Morphological staining

The staining of HeLa cells by Hoechst dye and the dual staining, Acridine orange and ethidium bromide was done following the method outlined in 2.2.8.1 of Chap 2.

9.2.5 Gene expression study using RT-qPCR

The cDNA from the untreated HeLa cells and the cells treated with 2, 4 and 8 μg/ml of AMF was synthesised as per the protocol of Genei Bangalore. The gene expression of

caspases, 3, 8 and *9* was quantified using the CT values. The fold increase/decrease of the genes was also calculated. The details are given in 2.2.8.2.1, 2.2.8.2.2 and 2.2.8.2.3 of Chap 2.

9.2.6 Flow cytometric analysis

The role of AMF (2, 4 and 8 μg/ml) in the cell cycle arrest was analysed using flow cytometry in HeLa cell lines. The population of G0/G1, S, and G2/M were quantified using multi cycle Cell Cycle software. The procedure is described in 2.2.8.3. of Chap 2

9.3 Statistical analysis - As in 2.2.9. of chap 2.

9.4 Results

9.4.1 Effect of AMF on HeLa cells

9.4.1A Determination of cell viability by MTT assay

AMF induced cytotoxicty in HeLa cells in a dose dependent manner and the concentration at which 50% of the total cells were only viable was at 12.3 ± 1.4 μg/ml while the standard drug, camptothecin exhibited IC_{50} at 3.5 ± 1.3 μg/ml (Figure 9.1).

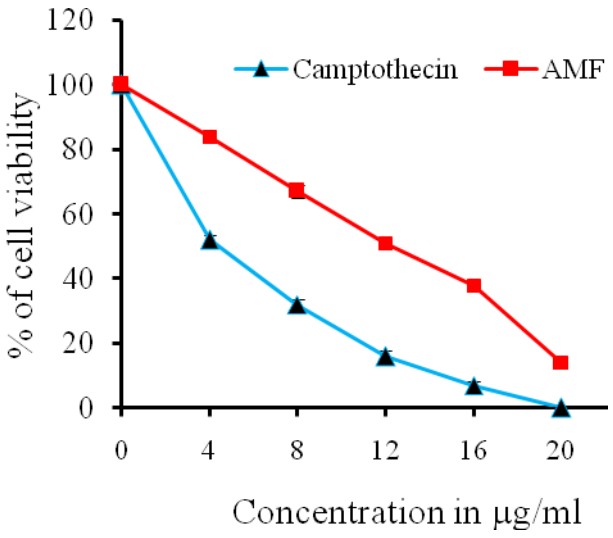

Figure 9.1 Effect of AMF on HeLa cells in MTT assay. All values are recorded as Mean ± SD based on three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment.

9.4.1B Detection of morphological alterations

It was seen that of the treated concentrations, at 2, 4 and 8 and 16 μg/ml concentrations of AMF, morphological features of apoptosis was very evident and clear. The morphological changes were observed by Hoechst staining and Ao/EtBr dual staining. Nuclear fragmentation, cell vacuolization and formation of apoptotic bodies were very apparent in the treated cells in staining with Hoechst dye (Figure 9.2). Compared to the control, the treated cells showed longer distances between cells.

On Ao/EtBr staining, the cells were differentially coloured based on the uptake of dyes. The control cells appeared green in colour while the treated dead cells were seen in orange colour, indicating the uptake of ethidium bromide into the nucleus. Some nuclear condensation and apoptotic bodies were also seen in treated cells (Figure 9.3).

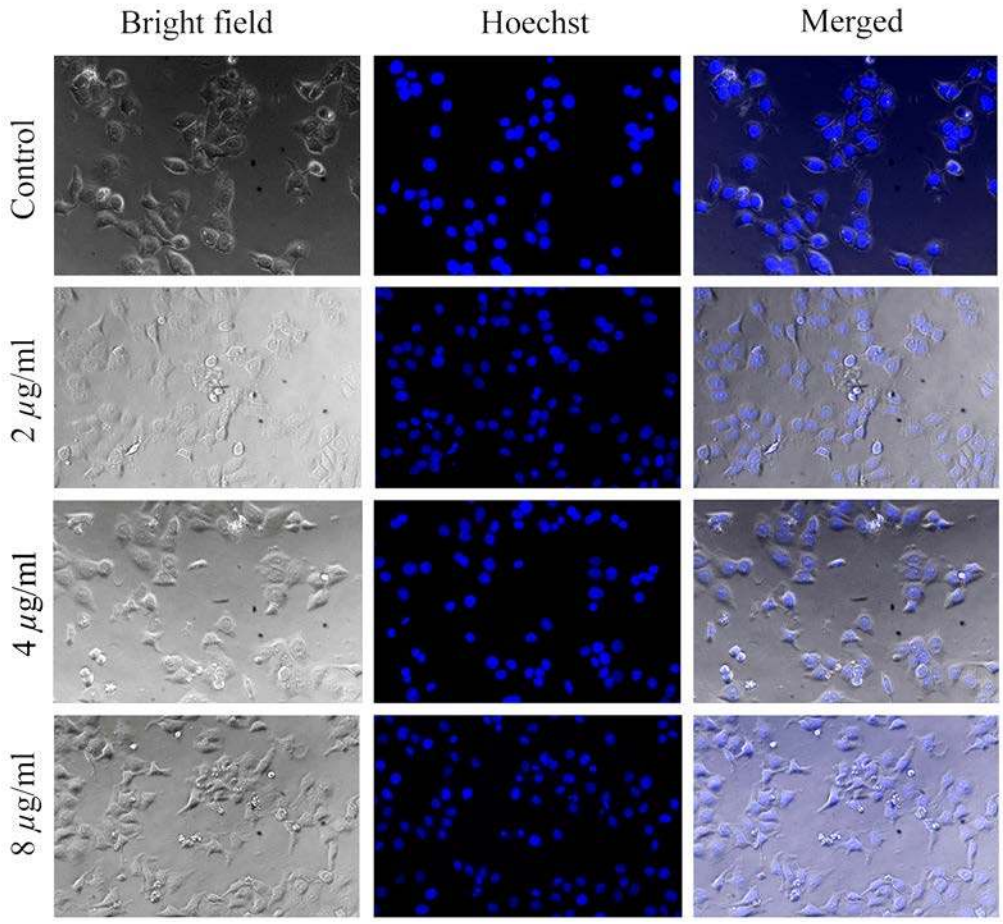

Figure 9.2 Effect of AMF on HeLa observed by the Hoechst dye staining method and viewed under fluroscent microscope. Control (A), Treated, 2 (B), 4 (C) and 8 μg/ml (D) of AMF

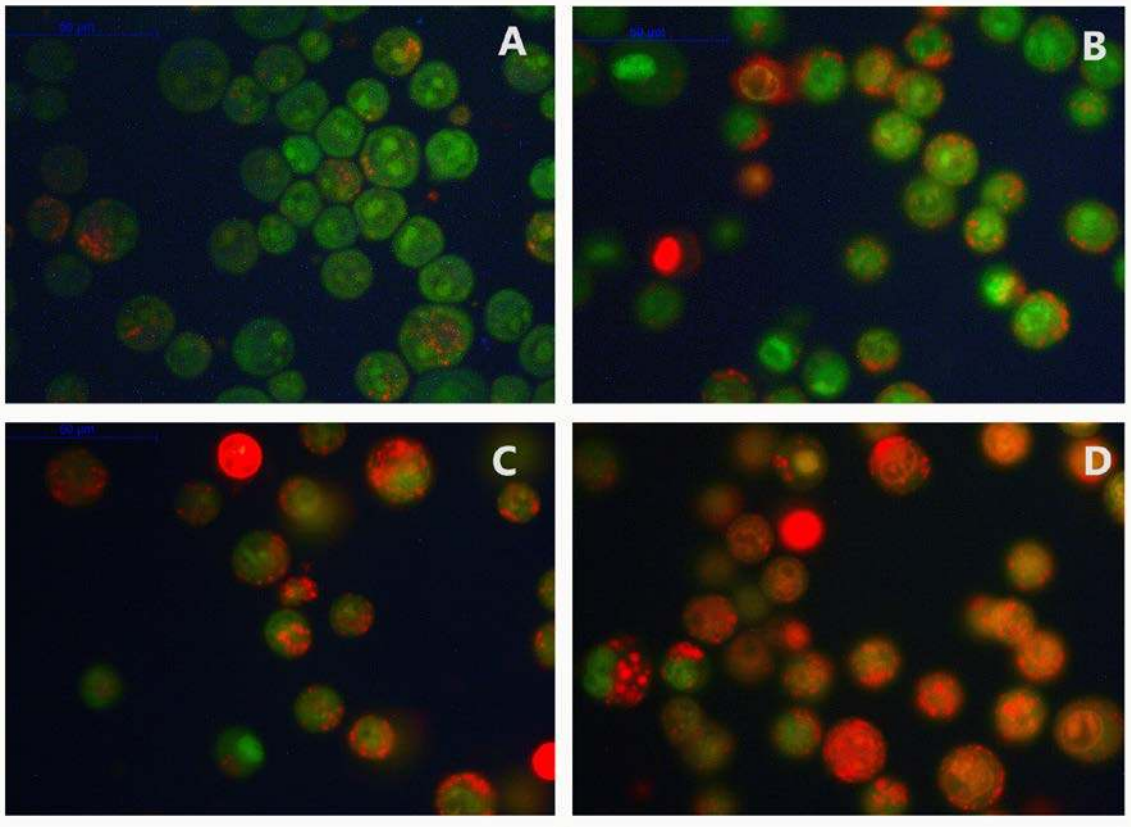

Figure 9.3 Effect of AMF on HeLa observed by the Ao/Etbr staining method and viewed under fluroscent microscope. Control (A), Treated, 2 (B), 4 (C) and 8 μg/ml (D) of AMF

9.4.1C Apoptotic gene expression profile

The gene expression was quantified using the ΔΔCT method and accordingly, a positive ΔΔCT indicates down-regulation and a negative ΔΔCT indicates upregulation. The ΔΔCT values obtained for *caspases 3, 8* and *9* were -4, 5 and -4 when HeLa cells were treated with 8 μg/ml of AMF (Figure 9.4). From the values obtained, it is evident that *caspase 9* and *3* were upregulated. Compared to the endogenous house-keeping gene, *β-actin*, the extent of target gene expression is showed as fold difference. Both, *caspase 9* and *3* showed a 16 fold increase of gene expression, while caspase showed a negligible 0.03 fold decrease in gene expression. The amplification plot from RT-qPCR substantiates the results obtained, as the expression of *caspase 9* and *3* were up-regulated in AMF treated cells, whereas *β-actin* which was taken as the control gene did not show any significant change in the amplification pattern in control and treated and cells (Figure 9.5).

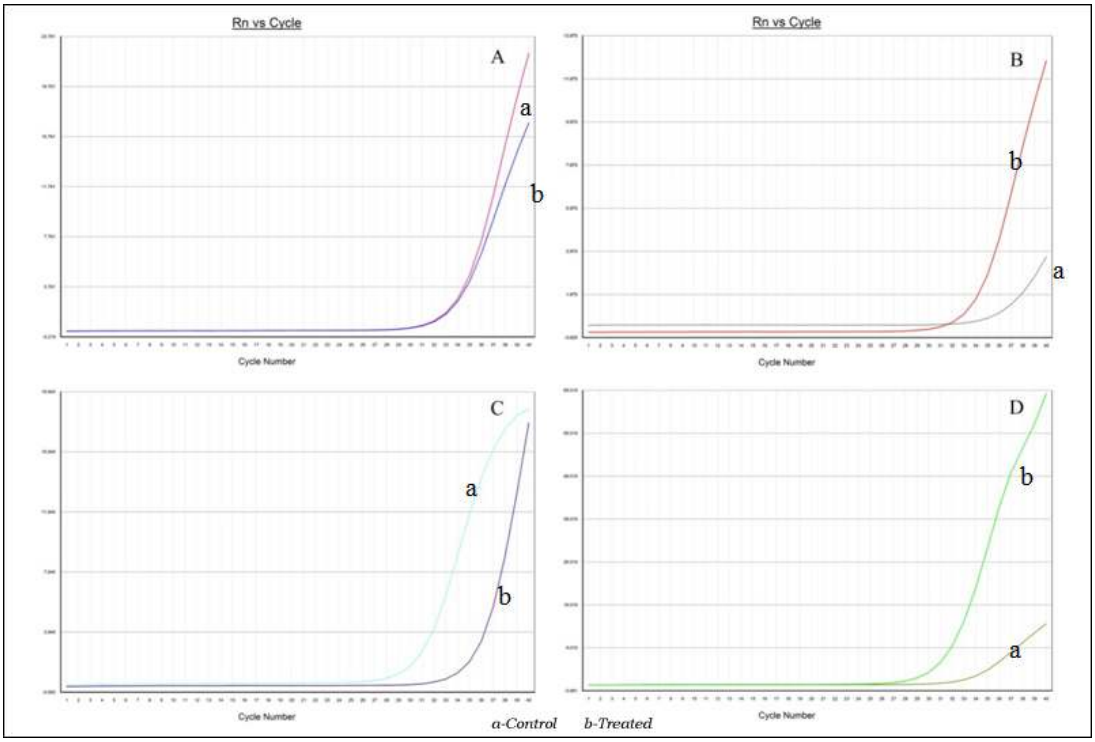

Figure 9.4 Expression profile of genes quantified in AMF (8 μg/ml) treated HeLa cells by RT-qPCR analysis. Housekeeping gene (A), *Caspase 3* (B), *Caspase 8* (C) and *Caspase 9* (D). "a" represents control cells and "b" refers to treated cells.

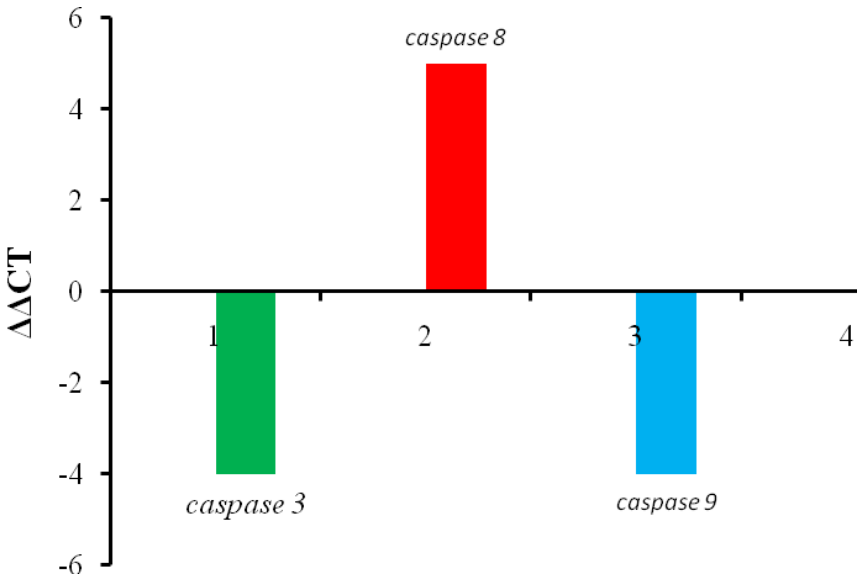

Figure 9.5 Bar graph indicating the regulation of apoptotic caspases by the treatment of HeLa cells by AMF (8 μg/ml). The gene expression is calculated using the Livak-DDCt method. A positive ΔΔCT indicates downregulation of genes, whereas a negative ΔΔCT indicates upregulation.

9.4.1D Cell cycle arrest of determined by flow cytometry

Results of the flow cytometric analysis indicate that that AMF inhibits the cell growth by inducing a block at G1/S transition phase of the cell cycle. It was evident from the histogram that there was an increase of cell population in the G_1 phase which was accompanied by a concomitant decrease of cell population in the S phase (Figure 9.6). Results indicate that 58.1 % of G_1 cells in the control population were increased to 77.3 % in 8 μg/ml AMF treated cells. The level of S phase was less in treated (8.4 %) than that of control (16 %) probably due to the gradual apoptotic elimination of cells. The cell population in G_2 phase was not affected much during the progression. The G_1 phase increase in HeLa treated with 2 and 4 μg/ml AMF was 64.2 and 65.3% respectively. The arrest was concentration dependent, and we can see the gradual increase in G_1 phase and the decrease in S phase population as the concentration of AMF increased (Figure 9.7).

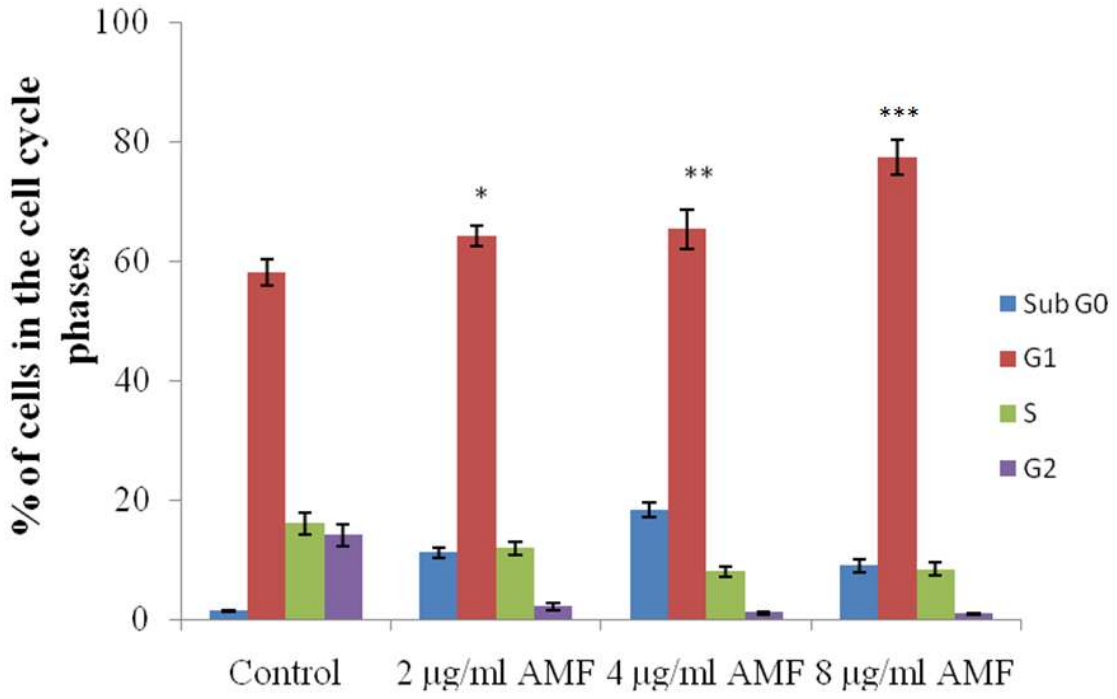

Figure 9.6 Percentage of HeLa cells in G1, S and G2 phases with and without AMF treatment. Each bar represents the mean ± SD of three independent experiments (The significance is calculated for the G1 phase cells as, $*P < 0.05$; $**P < 0.01$;$***P < 0.001$ compared to control).

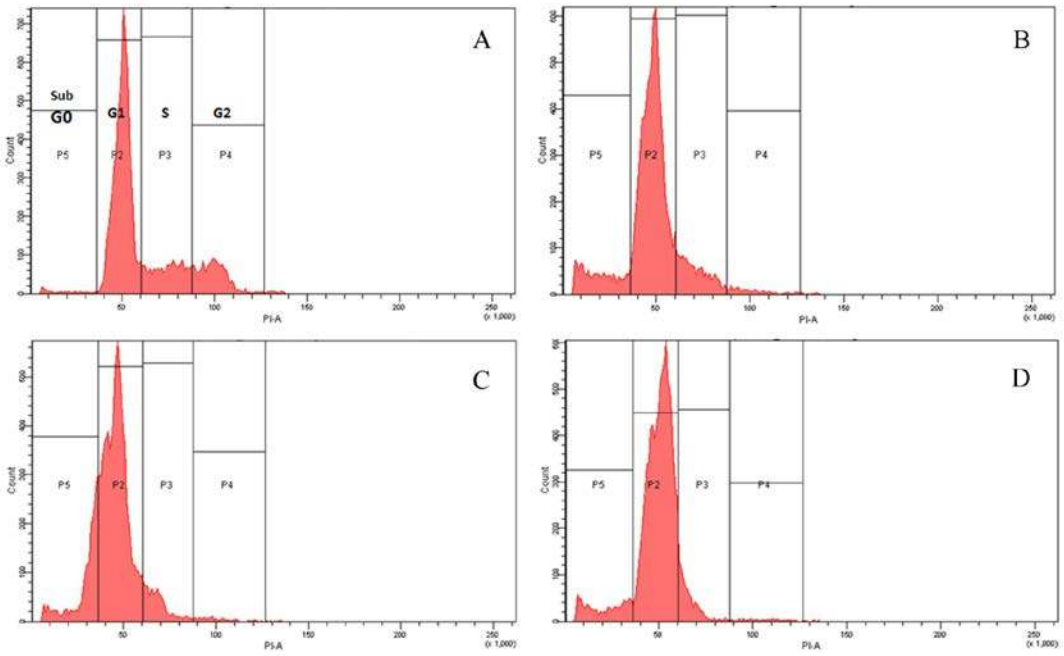

Figure 9.7 Effect of AMF in the cell cycle distribution of HeLa cells. Histograms showing cells untreated (A) and treated with 2 (B), 4 (C) and 8 (D) μg/ml of AMF

9.5 Discussion

Previous study has shown that AMF possess strong anti-tumor and cytotoxic properties and genipin was identified as one of the active molecules present in the extract. The present study is an illustration of the ability of AMF to cause apoptotic death, upregulation of the gene expression of *caspases* and cell cycle arrest at G1 phase in human cervical cancer cell line, HeLa.

The process of tumour development includes initiation, proliferation, invasion and metastasis (Dong *et al.*, 2011). Thus, drugs that can inhibit tumor cell proliferation and induce apoptosis are able to restrict tumor development effectively (Liu *et al.*, 2013). The morphological changes in AMF treated HeLa cells indicated apoptotic mode of cell death characterised by nuclear condensation and formation of apoptotic bodies. Moreover, AMF showed significant cytotoxicity towards HeLa cells in a dose dependent fashion, and the IC_{50} was determined at 12.3 ± 1.4 μg/ml. The results were comparable to that of the standard drug, camptothecin.

Caspases play the role of an executioner in apoptosis (Utz and Anderson, 2000; Saraste and Pulkki, 2000) and intrinsic or extrinsic pathways have been shown to trigger *caspase* activation (Solary *et al.*, 2002). In gene expression study, *caspase 9* and *caspase 3* was upregulated and both showed a 16 fold increase compared to the control gene. It is reported that *caspase 9* is associated with the activation of intrinsic mitochondrial pathway (Li *et al.*, 1997; Fulda *et al.*, 2001; Shi, 2001).

The cell cycle processes are properly coordinated by checkpoints to ensure the accurate replication of genome (Webster, 2000). This coordination takes place mainly at G1/S and G2/M phase transitions and allows the cells to respond appropriately to the proliferative signals (Elledge, 1996). AMF could induce cell cycle arrest at G1 phase in HeLa cells. Many anti-tumour agents derived from plant sources function by targeting G1 phase of cell cycle (Zeki *et al.*, 1999; Hsu *et al.*, 2004) and the G_1 phase is a vital checkpoint wherein the antigrowth signals exert their influence to block cell proliferation (Ringshausen *et al.*, 2006). The analysis of cell cycle regulation of AMF in HeLa cells showed a G1 specific cell cycle arrest. There was a shift of population from S to G1 phase in the treated cells. It is reported by Cao *et al.*, 2010 that genipin shows significant cytotoxicity in HeLa cells and inhibit its proliferation. Moreover, the ability of genipin to induce apoptosis was confirmed by DNA fragmentation assay, elevation of mitochondria associated bax protein and G1 cell cycle arrest. Similarly, here AMF also induced cell cycle arrest at G1 and the caspase activation was mediated by intrinsic pathway. It is thought that genipin which is identified as one of the active components in *A. dimidiata* would be playing a significant role in the cell cycle modulatory properties of AMF *via* apoptosis, apart from the other important phytoconstituents detected by GC-MS.

It is seen that certain compounds can exhibit both pro-oxidant and antioxidant effects, depending upon the concentrations and exposure time. Flavonoids are examples of substances with such dual behaviour (Perez-Trueba, 2003; Sakihama *et al.*, 2002) and baicalein is one such important flavonoid. It was reported that baicalein protects damage of rat liver mitochondria as an efficient free radical scavenger. But at high concentrations, it interferes with the mitochondrial bioenergetics by acting as a strong uncoupler, reducing ATP level and leads to cell death at high concentrations, in spite of showing excellent antioxidant capacity at all the tested concentrations (Pazina *et al.*, 2015). Eugenol (4-allyl-2-

methoxyphenol), a biologically active phenolic component of *Syzigium aromaticum* (cloves) is said to possess antioxidant, and anti-inflammatory activities (Pisano *et al.*, 2007; Ogata *et al.*, 2000) at low concentrations, whereas at higher concentration, it acts as a pro-oxidant and inducing apoptosis *via* the mitochondrial pathway (Manikandan *et al.*, 2011). Similarly, there is a possibility that AMF when administered directly onto the cancer cell lines; they are inducing cytotoxicity *via* apoptosis at higher concentrations. Earlier, we have seen that AMF (100 and 250 mg/kg b.wt) acts as an antioxidant agent against free radical stresses in the *in vivo* studies (Chap 5). This might be because at these concentrations, due to lower bioavaibility they are acting as antioxidants. AMF is also rich in various plant metabolites like flavonoids, polyphenols, saponins etc as evidenced from the preliminary phytochemical screening. Genipin, which was identified as one of the active principle in AMF is said to exhibit antioxidant, anti-inflammatory activities along with apoptosis inducing property via mitochondrial pathway in cancer cells. Thus, the synergistic action of all these components could be responsible for the cytotoxic and apoptotic properties of AMF.

Apoptosis is considered as the most appropriate suicidal pathway for the elimination of damaged or harmful cells (Singh *et al.*, 2002). Recently, the development of novel anticancer seems to orient upon apoptosis signalling systems (Hu and Kavanagh, 2003). It is reported that many of the plant-derived chemotherapuetic agents induces apoptosis (Ahmad *et al.*, 1997; Lee and Surh, 1998). The induction of apoptosis being one of the strong strategies of chemotherapuetic agents, AMF of *Apodytes dimidiata* could induce apoptosis by cell cycle arrest at G1. Moreover, the effector caspase 3 and the caspase 8 associated with intrinsic pathway was also found to be upregulated. Since, the restoration of altered regulatory checkpoints in cancer cells will been effective way of targeting them, further research focussing on the details of the apoptotic molecular machinery of AMF will help elevating *Apodytes dimidiata* as a promising anti-cancer agent.

Chapter 10
Summary and Conclusion

SUMMARY AND CONCLUSION

Regardless of the consistent efforts, cancer still remains an aggressive killer worldwide. By 2030, it is projected that there will be 26 million new cancer cases and 17 million cancer deaths per year (Jemal *et al.*, 2009). Moreover, during the last decade, novel synthetic chemotherapeutic agents currently in use clinically have not fully succeeded in spite of the considerable cost of their development (Solowey *et al.*, 2014). Therefore, there is an urgent need to develop new and affordable drugs for cancer treatment (Newman, 2008).

The use of compounds derived from plants to treat various ailments is a common practice in ancient time itself. Natural products have received an increasing attention over the past 30 years due to their potential as therapeutic agents (Gordaliza, 2007). In parallel, there is an increase in the use of plant-derived compounds as inhibitors of various stages of tumoriogenesis underlining their importance in cancer prevention (Solowey *et al.*, 2014). It is reported that the medicinal plants constitute a common alternative for cancer treatment in many developing and underdeveloped countries (Tascilar *et al.*, 2006). Around 10 to 40% of cancer patients use the plant-derived products for treatment and the rate seems to be increased up to 50% (Cassileth and Deng, 2004; Molassiotis *et al.*, 2006). Thus, the plants or plant derived products have always been the ultimate remedy for curing cancer. One of the best ways to advance in the field of drug discovery is to explore the plant biodiversity all over the world. Fortunately, India is blessed with a rich biodiversity and Western Ghats is considered to be one of the hot spots of diversity of flora and fauna. Keeping this in mind, we selected the allied taxa of *Nothapodytes nimmoniana*, a plant well known for anticancer alkaloid, camptothecin, which is not subjected to extensive pharmacological studies.

Ever since the discovery of the anti-cancer alkaloid, camptothecin from the plant *Nothapodytes nimmoniana*, the family Icacinaceae has received renowned significance. *N. nimmoniana* is one of the plants belonging to this family to have achieved world-wide attention for being a rich source of camptothecin. Later on, other species of the family like *Meriliodendron megacarpum* and *Pyrenacantha kleinii* was also found to contain camptothecin. Recently, 13 plants belonging to family Icacinaceae was reported as source of camptothecin (Ramesha *et al.*, 2012). Considering the importance of this family, the current study aimed to explore the pharmacological activities of the allied taxa of *Nothapodytes* found in Kerala emphasizing on anti-cancer properties.

The ability to scavenge free radicals, reduce inflammation and toxicity towards cancer cells are some of the crucial qualities of an anti-cancer agent. Thus, as part of preliminary screening, the *in vitro* anti-oxidant, anti-inflammatory and cytotoxic properties of *Apodytes dimidiata*, *Sarcostigma kleinii*, *Miquelia dentata* and *Gomphandra tetrandra* (allied taxa of *Nothapodytes*) were analysed. Out of the petroleum benzene, chloroform, acetone and methanolic fractions, the methanolic fraction was found to be the most active for all the plants. Of this, the crude extract of *Apodytes dimidiata* showed the highest and most profound cytotoxic and antioxidant activities. The methanolic fraction of *A.dimidiata* was partially purified through column chrmomatography. The fraction obtained in methanol through column showed better activities in all biological assays. Analysing the cytotoxicity in normal cell line Vero, the fraction was found very less toxic compared to cancer cells. Taking into account the overall results, *A. dimidiata* was selected out of the four plants and the partially purified fraction of *A. dimidiata* (AMF) was used for further studies.

Plants possess tremendous medicinal properties because of the presence of secondary metabolites produced in them (Savithramma *et al.*, 2011). Flavonoids and phenolic compounds constitute a group of secondary metabolites with significant anti-oxidant activity. In preliminary phytochemical screening of AMF, a number of phytochemicals were detected including tannins, terpenoids, saponins, sugar and flavonoids. These compounds are some of the most important bioactive compounds of plants (Edeoga, 2005). Moreover, appreciable amount of flavonoid and phenolic contents was detected in this extract. GC-MS analysis of AMF revealed the presence of 75 constituents. Of this, the known anti-oxidant, chemopreventive and anti-inflammatory compounds like squalene, dodecanoic acid, hexadecanoic acid and tetradecanoic acid were identified in AMF.

The safeness of the extract was confirmed by conducting sub-acute toxicity studies in mice. In the study, AMF did not produce any toxicity when administered orally up to the concentration of 500 mg/kg b wt. as shown in the results of haematological, biochemical and histopathological analysis. Toxicity and other side effects are the important challenges faced by the currently available chemotherapuetic drugs and free radicals generated by these drugs are implicated to be one of the main causes of the same (Plenderleith, 1990).

Even though Masoko and Nxumalo, 2013 has mentioned that *A. dimidiata* acetone extract possess free radical scavenging ability, a detailed study underlining this property was not done. Since nephrotoxicity induced by cisplatin is thought to be mediated by the free radicals generated, the study utilising the antioxidant property of *A. dimidiata* was done by evaluating the nephroprotective activity of the plant. Cisplatin is a widely used chemotherapeutic drug. But, the use in patients is limited due to nephrotoxicity caused by free radical stress. In the study, the nephrotoxicity associated with cisplatin administration was ameliorated by AMF in Wistar rats. The reduced SOD, catalase and GPx activities and GSH level was replenished by AMF. Moreover, the rise in lipid peroxidation was decreased by AMF administration. The study revealed the anti-oxidant potential of AMF and its possibility to be used conjointly with other drugs to reduce the free radical mediated side effects caused by chemotherapeutic agents.

It has been reported that *Apodytes dimidiata* is used in Zulu traditional medicine for treating inflammatory and gastric ailments. Presently, a scientific validation of these properties was ascertained, by examining the gastroprotective efficacy of AMF against ethanol induced gastric lesions in rats and by evaluating the *in vivo* anti-inflammatory property by carrageenan induced acute and formalin induced chronic models in mice. The ethanol induced gastric lesions and changes in biochemical parameters like SOD, GSH, GPx and catalase in the gastric mucosa was rectified by the oral administration of AMF. The paw oedema was significantly reduced and the results were comparable to the standard drug, diclophenac.

Cancer chemoprevention is currently regarded as one of the most promising platforms for cancer control (Tsao *et al.*, 2004). Considering the role of cellular oxidative stress in mutations and subsequent transformation, phytochemicals with anti-oxidant potential has become a primary choice as chemopreventive agents. Since AMF was found to contain many important phytoconstituents, the chemopreventive efficacy of AMF was studied. The results indicated that AMF could significantly revert the NPDA and NaN_3induced mutations in *Salmonella typhimurium* tester strains. The oxidative stress induced by NaF was also counteracted by AMF. In the DMBA/croton oil induced mouse skin papilloma study, a significant reduction of skin papilloma was observed. Since mutagenesis through oxidative DNA damage is hypothesised to be a frequent event in the cancer cells (Junod,

1996), it is possible that anti-oxidant and anti-mutagenic activities of AMF might have contributed to lessen the transformation of normal cells to a cancerous one, as evidenced by the reduction in papilloma formation in mice.

Since AMF of *A. dimidiata* showed significant activity against DLA and EAC cell lines, further studies were done to evaluate its cytotoxic and anti-tumour properties along with the characterisation of the bioactive component. In MTT assay, AMF showed profound cytotoxicity towards EAC, Jurkat and SK-BR-3 cell lines. The treated cells showed morphological alterations characteristic of apoptosis. Upon oral administration of AMF, the solid tumour volume in mice was significantly reduced and the life span of ascites tumour bearing mice increased significantly compared to untreated control. During the long-term cytotoxic analysis, a colour change of the culture medium to blue was noticed following the addition of fraction B. The colour change observed was found irrespective of pH. Further scrutiny on this regard lead to the finding of the component, genipin in AMF and through various phytochemical analysis, the presence of genipin was confirmed and it is thought to be one of the active component in AMF. Even though, the alkaloid camptothecin is reported in the bark of *A. dimidiata*, the study shows that the leaves are devoid of the same (Ramesha *et al*., 2013). In our studies also, we could not detect camptothecin in the leaf extract of *A. dimidiata*. Genipin is well known component and is reported as an anti-oxidant, anti-inflammatory and apoptosis inducing agent (Koo *et al*., 2004).

Further, the molecular mechanism of cytotoxic action of AMF was studied in human cervical cancer cell line (HeLa). The effect of AMF on HeLa cells were evaluated using MTT assay, the morphological changes were noticed by staining, the caspase genes expression were quantified using RT-qPCR and the modulation induced in cell cycle was studied by flow cytometry. AMF could induce cytotoxicity in HeLa cells significantly in a concentration dependent manner. Morphological features characteristic of apoptosis like nuclear condensation, formation of apoptotic bodies etc were noticed upon staining the HeLa cells treated with AMF. By RT-qPCR analysis, it was evidenced that the effector *caspase 3* and *caspase 9* related to the intrinsic mitochondrial pathway was up regulated in AMF treated HeLa cells. In flow cytometric analysis, AMF could specifically arrest the G_1 phase of the cell cycle. A significant decrease in the S phase cell population was also observed in the treated cells compared to the control.

The preventive mechanisms of tumour promotion by natural phytochemicals range from the inhibition of genotoxic effects, increased anti-oxidant and anti-inflammatory activity, inhibition of cell proliferation and modulate apoptosis and signal transduction pathways (Soobrattee, 2008). Here, AMF was found to be anti-oxidant, anti-inflammatory, anti-mutagenic and cytotoxic with the potential to induce apoptosis. The synergistic effects of plant metabolites offer higher efficacy during chemoprevention regimens (Guilford and Pezzuto, 2008). Presence of various classes of secondary metabolites like saponins, terpenes, flavonoids, phenols, squalene, hexadecanoic acid etc could have interplayed to produce the synergistic effects found in AMF.

Reducing oxidative stress is presumed to suppress the tumour cell proliferation and enhance apoptosis (Park, 2011; Sun, 2004). In the current study, AMF could reduce oxidative stress both in *in vitro* and *in vivo* experimental systems. Moreover, AMF could also induce apoptosis and modulate cell cycle during the crucial G1 phase. Reports show that apoptotic response is a promising approach for the treatment of various cancers (Nhan, 2006). Genipin was isolated as one of the active principle from *A. dimidiata*. Genipin is also said to induce apoptosis in HeLa cells during the G1 phase. Probably, the presence of genipin and other chemopreventive molecules in AMF would have influenced the apoptotic ability of AMF.

To revise, of the four plants, *Apodytes dimidiata* was found to be the most promising one and was chosen for further studies to explore possible chemotherapeutic and chemopreventive properties was well understood. The plant extract exhibited *in vitro* and *in vivo* antioxidant and anti-inflammatory properties, gastroprotective and nephroprotective potential, anti-mutagenic, anti-tumor and anti-carcinogenic ability, which highlights its ability to be considered for further development as a chemopreventive agent. Apart from these studies, *A. dimidiata* showed significant cytotoxicity against various cancer cell lines and the plant extract could arrest the G1 phase of cell cycle *via* apoptosis in HeLa cells, the results which indicated the possible chemotherapeutic potential of the plant. A less toxic and natural source of extract, AMF (active methanolic fraction of *A. dimidiata*) could be isolated and characterised from leaf of *A. dimidiata*. Its activity was well characterised by phytochemical studies and isolation of bioactive component, genipin. AMF showed very less toxicity to normal cells and was found non toxic to mice upon oral administration. It is

possible that presence of a well known anti-oxidant, anti-inflammatory and cytotoxic agent like genipin, and the other phytoconstituents identified by GC-MS analysis in AMF is mostly responsible for its anti-oxidant, anti-inflammatory, cytotoxic and apoptotic ability.

Since carcinogenesis is a multifactorial event, it is imperative to develop new drugs with multiple targeting actions and since the active fraction, AMF of *A. dimidiata* contain several components having different possible intracellular targets, this might provide an advantage. Since its non-toxic even at higher doses, it can be developed alone or in combination with other medications in preventing or reducing the incidence of cancer. AMF can also serve as a complementary therapy to ameliorate free radical induced damages associated with chemotherapeutic drugs. AMF can be obtained without any destruction to the plant because it is extracted from the leaf. Overall, the study provides a promising member, *Apodytes dimidiata* for the prevention as well as treatment of cancer from the Icacinaceae family.

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Appendix

Table i - Effect of AMF on antioxidant parameters in NaF treated mice

Groups	Antioxidant parameters			
	Catalase (K/g Hb)	SOD (U/gHb)	GSH (nmol/ml)	Lipid peroxidation (MDA nmol/mg protein)
Normal	35.6 8 ± 3.45	498.89 ± 73.45	42.64 ± 4.67	1.2 ± 0.12
NaF	26.74 ± 2.45	318.56 ± 53.46	38.67 ± 7.56	2.3 ± 0.23
Vehicle Control	28.56 ± 5.34	302.45 ± 45.98	35.23 ± 9.34	2.67 ± 0.34
NaF + Vit C (15 mg/kg)	33.21 ± 4.56 ^{ns}	502.41 ± 89.56*	53.24 ± 12.45**	1.34 ± 0.19**
NaF + AMF (100 mg/kg)	30.98 ± 4.23 ^{ns}	448.9 ± 98.53**	57.67 ± 8.89**	1.89 ±0.09**
NaF + AMF (250 mg/kg)	40.97 ± 6.56**	589.75 ± 87.32***	68.55 ± 10.32**	1.12 ± 0.18***

Values are mean ± S.D., n=6. *** P<0.001, ** P<0.01, * P<0.05, (Tukey test). Vehicle control group were compared with treated groups

Table ii - The percentage inhibition of papilloma by AMF on DMBA induced papilloma bearing mice

Groups	**No. of mice developed papilloma/ group**	**No: of papilloma/ mice**	**% inhibition in development of papilloma**
DMBA alone	0/7	0	0
Croton oil alone	0/7	0	0
DMBA + Croton oil	7/7	11.28 ± 1.25	0
DMBA + Croton oil + vehicle	7/7	10.42 ± 0.97	7.5
DMBA + Croton oil + 1% AMF	6/7	4.571 ± 0.78	59.49
DMBA + Croton oil + 3% AMF	4/7	3.75 ± 0.5	66.77
DMBA + Croton oil + 5% AMF	3/7	2.33 ± 0.57	79.32

List of publications

LIST OF PUBLICATIONS

1. Divya M K, Shalini S, Chubicka T, Raghavamenon A C and Babu T D (2015). Evaluation of cytotoxic and anti-tumor properties of *Apodytes dimidiata* and characterisation of the bioactive component. *Planta Medica* 81, 1705-1711.

2. Divya M K, Salini S, Meera N, Lincy L, Seema M, Raghavamenon A C and Babu T D (2015). Attenuation of DMBA/croton oil induced mouse skin papilloma by *Apodytes dimidiata* mediated by its antioxidant and antimutagenic potential. *Pharmacuetical Biology*, DOI-10.3109/13880209.2015.1107747. 3 (In press).

3. Divya M K, Lincy L, Raghavamenon A C and Babu T D (2016). Ameliorative effect of *Apodytes dimidiata* on cisplatin induced nephrotoxicity in Wistar rats. *Pharmacuetical Biology*, DOI- 10.3109/13880209.2016.1149494 (In press).

4. Divya Menon K, Sheema Dharmapal, Achuthan C R and Babu T D (2014). Cytotoxic and antitumor effects of *Tribulus terrestris* L fruit methanolic extract. *Journal of Pharmacognosy and Phytochemistry* 3, 1-4.

5. Salini S, Divya M K, Chubicka T, Meera N, Fulzele D P, Ragavamenon A C and Babu T D (2015). Protective effect of Scutellaria species on AAPH-induced oxidative damage in human erythrocyte. *Journal of Basic and Clinical Physiology and Pharmacology*. DOI 10.1515/jbcpp-2015-0081 (In press).

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Evaluation of Cytotoxic and Antitumour Properties of *Apodytes dimidiata* and Characterisation of the Bioactive Component

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Key words
- *Apodytes dimidiate*
- Icacinaceae
- cytotoxicity
- antitumour activity
- active principle
- genipin

Abstract

Apodytes dimidiata, belonging to the family Icacinaceae, is used for treating inflammation and various gastrointestinal ailments in Zulu traditional medicine. In the present study, significant cytotoxicity was exhibited by the methanolic extract of the *A. dimidiata* leaf against various cancer cell lines. The extract was purified partially through silica gel column by successive elution using various solvents of increasing polarity. Among these, the active methanolic fraction was found to be the most cytotoxic with IC_{50} values ranging from 0.92 to 3.95 µg/mL for Ehrlich's ascites carcinoma (a carcinoma cell line), Jurkat (human T lymphocyte cell line), and SK-BR-3 (mammary tumour cell line). The treated cells showed morphological alterations characteristic of apoptosis. Upon oral administration of active methanolic fraction at a dose of 250 mg/kg body weight, the solid tumour volume in mice was significantly reduced to 55.14% and the life span of the ascites tumour-bearing mice increased to 44.65% compared to untreated control. The active fraction with R_f value 0.56 was purified from the methanolic fraction by preparative thin-layer chromatography and was subjected to high-performance thin-layer chromatography, high-performance liquid chromatography, liquid chromatography-mass spectrometry, and nuclear magnetic resonance analysis. The iridoid glycoside genipin was identified as the active component.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

Considering the side effects of synthetic products even at the genomic level of healthy cells, natural molecules are currently being exploited as effective chemotherapeutic drugs. In modern medicine, approximately 50% of the prescriptions are phytochemicals or their analogues. Recently, various plant metabolites came out with exciting preclinical effects that could be a nontoxic alternative to the present day chemotherapies. Hence, the search for bioactive compounds from natural resources is more imperative [1,2].

Apodytes dimdiata E. Mey. Ex Arn., a small bushy tree belonging to the family Icacinaceae, is distributed in southern and eastern parts of Africa, the Western Ghats of India, and other Asian countries. The plant is commonly used in Zulu traditional medicine to treat gastrointestinal ailments, helminthes [3–5], and the leaves are used as a remedy for ear inflammation [6]. A well-known anticancer alkaloid, camptothecin, has been isolated from 13 members of the Icacinaceae family, and out of this a trace amount of camptothecin was reported in the stem bark of *A. dimidiata* [7]. Camptothecin content was also detected in a fungus, *Fusarium solani*, growing endophytically in *A. dimidiate* [8]. In addition, an iridoid glycoside genipin, a 10-monoacetate derivative of genipin, and an acetylated eudesmane glucoside were isolated from the bark and leaves, respectively, in association with the molluscicidal activity of *A. dimidiata* [9,10]. Genipin, a metabolite of geniposide isolated from *Gardenia jasminoides* J.Ellis (Rubiaceae), has been shown to possess various medicinal properties including anti-inflammatory [11] and anticancer [12]. Six new saponins, apodytines A–F, with antiprotozoal activity, were also isolated from the leaves of *A. dimidiata* [13]. Based on the toxicity assessment of mammals, the European Economic Community (EEC), the South Africa Bureau of Standards (SABS), and the World Health Organization (WHO) have classified the plant as nontoxic and nonirritating [14]. Apart

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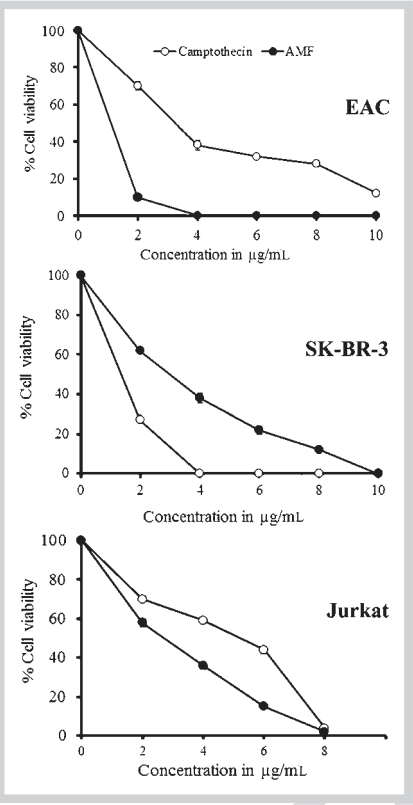

Fig. 1 Effect of AMF and camptothecin on various cancer cell lines in the MTT assay. All values are recorded as mean ± SD based on three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment.

from the screening of camptothecin from *A. dimidiata*, no biological studies emphasising its anticancer potential have been done so far. Hence, the present study investigated the cytotoxic and antitumour properties of *A. dimidiata* leaves with emphasis on the isolation of the active principle.

Results

In the preliminary screening, the crude methanolic extract of *A. dimidiata* leaves obtained by soxhlet extraction showed significant cytotoxicity with IC_{50} values of 9.30 ± 1.24 and 9.26 ± 0.73 µg/mL for Ehrlich's ascites carcinoma (EAC), and Dalton's lymphoma ascites (DLA) cells, respectively. A partially purified fraction of the extract obtained in methanol (active methanolic fraction, AMF) through column chromatography was found most cytotoxic with IC_{50} values of 3.0 ± 1.43 and 3.22 ± 0.84 µg/mL for EAC and DLA cells, respectively. Upon incubation with various cancer cell lines in a long-term MTT assay, AMF exhibited cytotoxicity in a dose-dependent manner and the IC_{50} values calculated were 0.92 ± 1.43, 2.48 ± 0.91, and 3.95 ± 1.57 µg/mL for EAC, Jurkat, and SK-BR-3 cells, respectively (**Fig. 1**). On statistical analysis, the cytotoxicity of AMF was found to be significant as much as the standard drug camptothecin (**Table 1**). On Hoechst staining, remarkable morphological changes like cell shrinkage and nuclear condensation, characteristic of apoptosis, were observed (**Fig. 2**).

On thin-layer chromatography (TLC) profiling, AMF separated into six bands (A–F) and the individual fractions obtained showed varying levels of cytotoxicity (**Table 2**). Among these, the band with an R_f value of 0.56 (fraction B) exhibited the highest level of cytotoxicity with IC_{50}s of 5.83 ± 1.92, 12.43 ± 3.35, and 14.82 ± 3.50 µg/mL for EAC, Jurkat, and SK-BR-3 cells, respectively (**Fig. 3**)

Table 1 Cytotoxicity of different fractions (R_f values of the fractions are also inserted) of AMF obtained after separation by TLC using the solvent system ethyl acetate:*n*-hexane (5 : 5 v/v) to DLA and EAC cell lines (trypan blue dye exclusion method). Values are mean ± SD of three separate determinations. The IC_{50} values were determined from the cytotoxicity curve at 50% of cell death after 3 h of treatment.

Fractions (bands with R_f values)	IC_{50} (µg/mL)	
	EAC	DLA
A (0.76)	48.79 ± 2.45	52.46 ± 0.61
B (0.56)	5.83 ± 1.92	5.25 ± 0.37
C (0.37)	22.61 ± 0.66	19.50 ± 1.64
D (0.31)	20.35 ± 1.40	19.33 ± 1.73
E (0.22)	28.60 ± 1.91	24.46 ± 2.28
F (0.10)	34.14 ± 2.30	32.50 ± 1.55

In *in vivo* antitumour studies, treatment with AMF increased the life span of tumour-bearing animals. The mean survival for the vehicle control group was 18.82 ± 2.27 days, whereas in the treated group, it was elevated to 24.24 ± 1.76 and 27.40 ± 3.85 days for 100 and 250 mg/kg b.wt. dose, respectively (**Fig. 4**). The percentage increase in life span of the drug-treated animals was calculated as 28.72 and 44.65% for 100 and 250 mg/kg b.wt., respectively. An elevated survival rate of 60.13% was exhibited in animals with the administration of cyclophosphamide, which was used as the standard.

The tumour volume was reduced in the drug-treated animals compared to the control in the solid tumour assay. On 32nd day of measurement, the volume of tumour was 3.25 ± 1.17 cm^3 in the case of control animals while it was significantly reduced to 1.42 ± 0.56 and 1.84 ± 0.38 cm^3 for 100 and 250 mg/kg b.wt., respectively, in the AMF-treated groups (**Fig. 5**). The animals that received cyclophosphamide showed a significant reduction in tumour volume to 1.13 ± 0.25 cm^3. The percentage of inhibition of tumour volume was calculated as 64.73 in the cyclophosphamide-treated group and 55.14 in the case of the AMF-treated group (250 mg/kg b.wt.).

The fraction B obtained from TLC was scanned in the wavelength range of 200–700 nm and showed the absorption maxima at 240 nm. The iridoid glycoside genipin also showed the maximum absorption at 240 nm (**Fig. 1 S**, Supporting Information). On high-performance thin-layer chromatography (HPTLC) analysis, AMF resolved into a total of six bands, named A–F, from the solvent front. The most active fraction B had the same R_f value of 0.56 as that of the standard, genipin (**Fig. 2 S**, Supporting Information). The R_f values of fractions A, C, D, E, and F are shown in **Table 1**. On high-performance liquid chromatography (HPLC) analysis, the crude methanolic extract and the AMF showed 21 and 14 peaks, respectively. Both the AMF and crude methanolic extract showed a peak corresponding to that of the standard genipin, at a similar retention time of 5.03. The retention time, 5.03 min detected for fraction B by HPLC analysis was same as that of the standard genipin (**Fig. 3 S**, Supporting Information). Liquid chromatography-MS (LC-MS) analysis of both genipin and fraction B gave *m/z* 227 $[M]^+$ as the prominent ion and matches the molecular weight of genipin. The *m/z* spectrum also contains ions at *m/z* 248, some 23 Da higher than the expected molecular mass. These can be identified as the sodium adduct ions, $[M + Na]^+$, and are quite common in electrospray ionisation. A fragmented

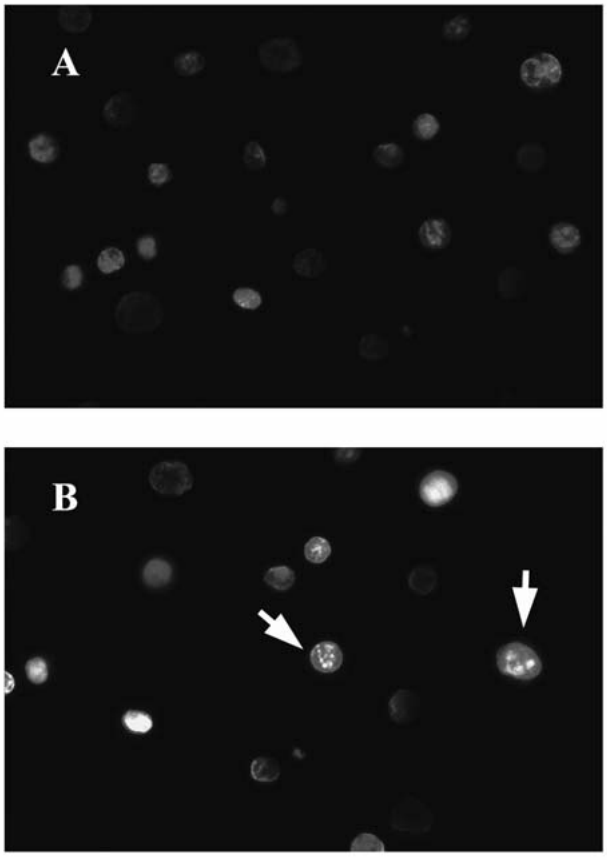

Fig. 2 Morphology of EAC cells stained with Hoechst dye and viewed under a fluorescence microscope (Leica Microsystem). **A** Control. **B** Cells treated with 5 µg/mL of AMF. The cells marked with the arrowhead indicate apoptotic bodies.

ionic pattern of fraction B was observed as *m/z* 227, 209, 191, 177, 149, and 121, and a similar pattern was seen with the standard genipin (**Fig. 4 S**, Supporting Information). The percentage yield of active fraction B from the dried leaf powder of *A. dimdiata* was calculated as 0.03128% by Shimadzu Class VP offline processing software based on the peaks obtained from the HPLC profile.

^{1}H NMR spectrum of isolated fraction B and genipin was identical as evidenced from the chemical shifts and revealed a strong solvent ($CDCl_3$) peak at 7.26 ppm. The spectra gave peaks around δ 3.72 and 3.22 ppm, indicating an $-OCH_3$ group and a cyclic OH group, respectively (**Fig. 5 S**, Supporting Information). Based on the proton-NMR data, the tentative structure of the fraction B is proposed (**Fig. 6 S**, Supporting Information) and this confirms that the isolated fraction B is genipin.

Discussion

The crude methanolic leaf extract of *A. dimidiata* showed significant cytotoxicity against cancer cell lines. Further purification of the extract by column chromatography yielded the AMF. The cytotoxic effect of the AMF obtained was comparable to that of the positive control, camptothecin, used in the study and it was found to be more cytotoxic in cell lines like EAC and Jurkat. The

Table 2 A comparison of the cytotoxicity results of camptothecin against AMF on various cell lines using the MTT assay. Values are mean ± SD of three separate determinations. The IC_{50} values were determined from the cell viability curve at 50% after 48 h of treatment. Results were considered statistically significant when $p < 0.05$ compared to the standard drug, camptothecin; ^{ns}: not significant.

Treatment	Cell lines (IC_{50} values in µg/mL)		
	EAC	Jurkat	SKBr3
Camptothecin	3.62 ± 2.18	4.46 ± 1.21	1.83 ± 1.67
AMF	0.92 ± 1.43^{ns}	2.48 ± 0.91^{ns}	3.95 ± 1.57^{ns}

American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation as less than 30 µg/mL [15]. However, a crude extract having an IC_{50} less than 20 µg/mL [16] is considered highly cytotoxic. An IC_{50} value below this specified limit of NCI was observed for the AMF against the entire cell lines studied, thereby proving its anticancer potential. The observed morphological alteration of the treated cells suggests an apoptotic mode of cell death.

Considering the cytotoxic potential of the AMF, an *in vivo* mouse tumour model was examined. The extent of life span and tumour reduction observed were comparable to that of the positive control, cyclophosphamide. The reliable criteria on judging the value of any anticancer drug is the prolongation of the life span of the animal [17]. In the subacute toxicity study, it was evidenced that AMF administration was nontoxic up to the 500 mg/kg body weight dosage (data not shown). Since the AMF was highly effective at the therapeutic dose (250 mg/kg body weight) in ascites as well as in the solid tumour model and this dosage was far below the expected LD_{50} value, it can be considered a safe antitumour agent. The long-term use of the leaves and flower of *A. dimidiata* by traditional healers [18] also hints at its nontoxic nature. Moreover, there are reports on the plant being nontoxic based on mammal toxicity studies [14].

During the long-term cytotoxic analysis, a colour change of the culture medium to blue was noticed, following the addition of fraction B. The colour change observed was found irrespective of pH. It is reported that genipin is a natural cross-linking agent that reacts with amino acids to generate a blue pigment [19] and the same principle has been well utilised in the food industry to prepare a series of blue pigments (gardenia blue) [20]. Considering these evidences, it is possible that fraction B, like genipin, could have interacted with the amino acids present in the Roswell Park Memorial Institute (RPMI) medium that resulted in the colour change.

The λ_{max} (240 nm) obtained for fraction B in the absorption spectrum analysis was the same as that of genipin. In HPTLC analysis, both genipin and fraction B moved with the same relative mobility and in HPLC, both were eluted with the same retention time. In LCMS analysis, the ionic mass spectrum of fraction B was found to be similar to the mass spectrum (*m/z*) of genipin, which is a prominent ion of *m/z* $[M]^+$ 227 and is well in agreement with the molecular weight of genipin [9]. The structure elucidation by H^1 NMR analysis also substantiated the fact that fraction B purified from the leaves of *A. dimidiata* was genipin and the spectrum of genipin and fraction B showed identical chemical shifts. Even though the alkaloid camptothecin is reported in the bark of *A. dimidiata*, the study shows that the leaves are devoid of the same [7]. In our studies also, we could not detect camptothecin in the leaf extract of *A. dimidiata*.

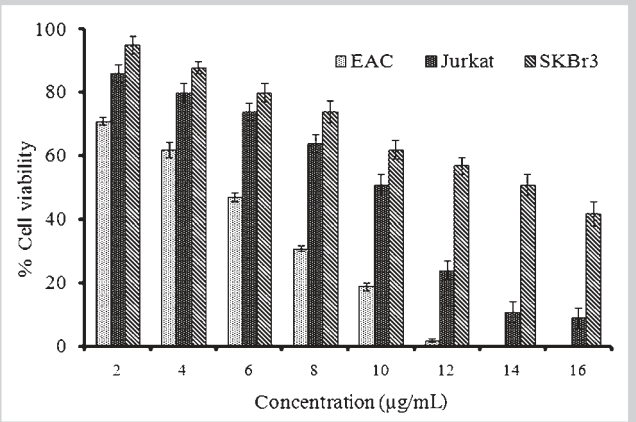

Fig. 3 Effect of fraction B on various cancer cell lines in the MTT assay. All values are recorded as mean ± SD based on three separate determinations. The IC_{50} values are determined from the cell viability curve at 50% after 48 h of treatment.

Genipin is an aglycone of geniposide isolated from the fruit of G. jasminoides [21]. It exhibits a wide range of biological properties, including antidiabetic [22], anti-inflammatory [23], antibacterial [24], and antimetastatic [25]. Apoptotic induction associated with the activation of c-Jun NH_2-terminal kinase and p53 in HeLa cells [26] is also reported. It has been suggested that genipin or its derivatives could be useful in inhibiting uncoupling proteins (UCP2), the antiapoptotic shield conferred to cancer cells [27,28]. In view of the above-discussed properties of genipin, the cytotoxic and antitumour efficacy exhibited by the AMF could be largely due to genipin. Most of the natural drugs used for cancer are isolated from the bark of the stem or root of the specimen, and the continuous extraction is a threat to the existence of plant species. In this context, the AMF can be used as a sustainable source as it is obtained from the leaf without much destruction to the plant. The present study thus provides an insight to the value added utility of *A. dimidiata* by exploring its possible anticancer potential.

Materials and Methods

Plant material

The leaves of *A. dimidiata* were collected from Periya, Wayanad district of Kerala, India (Altitude: 810 m, Geographical location: 11°51′03.19″N; 75°48′05.54″E), during the month of January 2014 and were identified by Dr. Sujanapal P, Taxonomist, Kerala Forest Research Institute (KFRI) Peechi, Kerala. A voucher specimen, No. KFRI 28 024, was lodged in the herbarium of KFRI.

Cell lines

DLA and EAC tumour cells, purchased from Adayar Cancer Institute, Chennai, India, were propagated in the peritoneal cavity of mice. Jurkat and SK-BR-3 cells obtained from NCCS, Pune, were maintained in RPMI-1640 medium as recommended by the supplier.

Animals

Swiss albino mice (Male, 25–30 g), purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University (KVASU), Thrissur, Kerala, were maintained under standard environmental conditions (22–28 °C, 60–70% relative humidity, 12-h dark/light cycle) and fed with standard rat feed (Sai Durga Feeds and Foods) and water *ad libitum*. The entire animal experiments (No. ACRC/IAEC/0006/2014) in the study were carried out with the prior approval from the Institutional Animal Ethics Committee by strictly following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

Chemicals and reagents

RPMI-1640 culture medium was purchased from Sigma-Aldrich and FBS from PAN Biotech. 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT), camptothecin (98% HPLC powder), and genipin (98% HPLC powder) were purchased from Sigma-Aldrich. Cyclophosphamide (95% pure) was obtained from Neon Laboratories Ltd. Acetonitrile, ammonium formate, methanol, and water were of HPLC grade and all other chemicals and reagents were of analytical grade (Merck).

Preparation of extracts

Leaves of *A. dimidiata* were dried under shade and powdered using a mixer grinder. Approximately, 30 g of the powder were extracted separately with 250 mL of petroleum benzene, chloro-

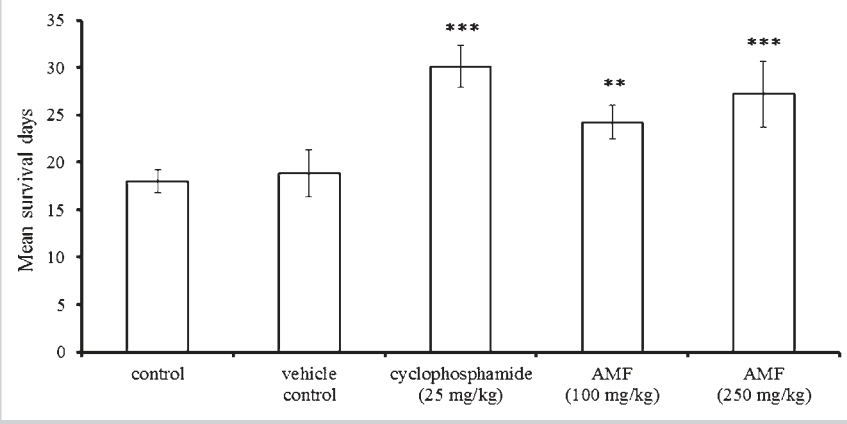

Fig. 4 Effect of AMF on ascites tumours induced by EAC. Values are mean ± SD (n = 6). $^{**}P < 0.01$, and $^{***}p < 0.001$ compared to the vehicle control.

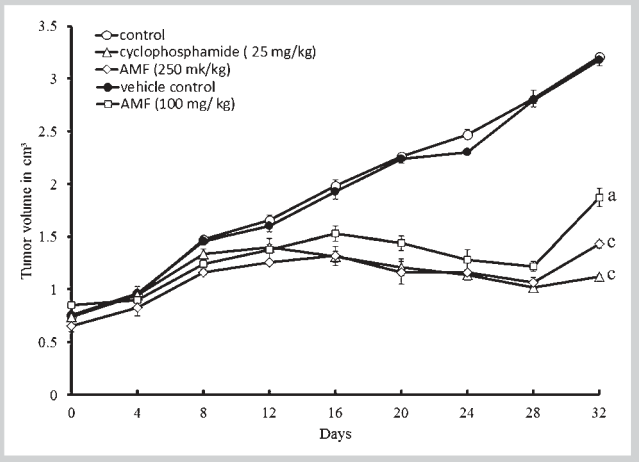

Fig. 5 Effect of AMF on solid tumours induced by DLA. Values are mean ± SD (n = 6); **a** ($p < 0.05$), **b** ($p < 0.01$), and **c** ($p < 0.001$) compared to the vehicle control.

form, acetone, and methanol using a soxhlet apparatus for 24 h. The extracts obtained were concentrated to dryness and the residue collected was dissolved in DMSO and resuspended in phosphate buffer saline (PBS). The most active crude methanolic extract (confirmed by short-term *in vitro* cytotoxicity assay) obtained from soxhlet (4 g) was loaded onto a column (600 × 30 mm) packed with 60–120 mesh silica gel and eluted successively by passing various solvents (150 mL each) of different polarities such as petroleum benzene, chloroform, acetone, and methanol for further purification. The individual fractions obtained were dissolved in DMSO and resuspended in PBS for cytotoxicity analysis. Further studies were carried out using the cytotoxically AMF obtained from column (0.92 g).

Cytotoxicity assays

Short-term cytotoxicity was assessed by the trypan blue dye exclusion method [29]. Briefly, 1×10^6 cells in 1 mL of PBS were incubated with different concentrations of samples for 3 h in an incubator at 37 °C. DMSO (0.1 % in PBS) was used as the vehicle control. After incubation, the cells were treated with trypan blue (100 µL of 0.1 % in normal saline) and the dead cells that appeared as blue were counted using a haemocytometer. The percentage of cell death was calculated using the following formula:

% of Cell death = number of dead cells/
number of total cells × 100 %

Long-term cytotoxicity was performed by the MTT assay. Approximately 1×10^6 cells of SK-BR-3 and Jurkat were seeded in 96-well plates and incubated to achieve 80 % confluency. The cells were then exposed to different concentrations of test materials (AMF, fraction B) and allowed to grow further at 37 °C for 48 h. EAC (1×10^6) cells were also seeded in RPMI medium and incubated with the samples for 48 h. MTT was added into the well plates (100 µL from the stock of 5 mg/mL PBS) and incubated for 4 h. DMSO (0.1 % in PBS) was kept as the vehicle control. After centrifugation, the supernatant was removed and the precipitate of the converted dye was solubilised in DMSO. The absorbance of the coloured product was measured at 570 nm and the percentage of viability was calculated. The average absorbance of the untreated negative control was taken as 100 % cell survival [30]. The results obtained were compared to the standard anticancer alkaloid camptothecin, which was kept as the positive control under same conditions. An aliquot (5 µL) of treated EAC cells was stained with 0.5 µg/mL of Hoechst dye for 10 min and viewed under a fluorescence microscope (Leica Microsystem) and photographed.

Ascites tumour analysis

Ascites tumour was developed by injecting 1×10^6 of EAC cells into the intraperitoneal cavity of mice. Male Swiss albino mice were divided into five groups of six each. Group 1 served as the untreated control and group 2 received a single intraperitoneal injection of the standard drug cyclophosphamide each day (25 mg/kg body weight) for 10 consecutive days. Group 3 was given 0.20 mL of propylene glycol orally (> 99 % pure) and served as the vehicle control. AMF 100 and 250 mg/kg body weight was given orally (0.20 mL) for groups 4 and 5, respectively. The drug treatment was started the next day following the injection of cells for 10 consecutive days. The animals were observed for death due to the tumour burden for 30 consecutive days. The life span of the animals was calculated using the following formula:

Percentage increase in life span (ILS) = (T-C)/C × 100

where T and C are the mean survival of treated and control mice.

Solid tumour analysis

Solid tumours were developed by injecting 1×10^6 DLA cells intramuscularly into the hind limb of the mice. The grouping of the animals and the administration of the drug was the same as that of the ascites tumour assay. The tumour development in each group of animals was determined by measuring the diameter of the tumour in two perpendicular planes using Vernier calipers every third day for 32 days. The tumour volume was calculated using the formula:

$V = 4/3\ \pi\ r_1^2 \times r_2$

where r_1 is the minor radius and r_2 is the major radius [31].

Thin-layer chromatography

AMF was subjected to TLC using the solvent system that gave the maximum separation of bands. Approximately 10 µg of the sample were spotted on a silica gel plate (20 × 20 cm^2; Merck, silica gel 60 F_{254}) and were developed using the solvent system ethyl acetate:*n*-hexane (5 : 5 v/v). The separation profile was visualised under UV at 366 nm and the six bands obtained were marked as A–F, carefully scrapped off, eluted with methanol, and centrifuged at 5000 rpm for 10 min. The supernatant was collected, filtered using a syringe filter (Millex GV filter unit 0.22 µm, Millipore), made silica free, and air-dried. The whole process was done under dim light conditions.

Absorption spectrum analysis

The λ_{max} of cytotoxically active fraction B (confirmed by trypan blue dye exclusion method) obtained after elution from TLC (1.20 mg/3 mL methanol) and the standard genipin (1 mg/3 mL methanol) was detected by scanning between wavelengths ranging from 200–900 nm using a UV-visible spectrophotometre (P G Instruments 80$^+$).

High-performance thin-layer chromatography analysis

HPTLC was performed using a CAMAG system. The samples (10 µL each) were spotted onto a silica gel plate (20 × 20 cm^2; Merck, silica gel 60 F_{254}) from the stocks of AMF (20 mg/mL), fraction B (1 mg/5 mL), and the standard genipin (0.50 mg/mL). The samples were dissolved in methanol and developed in the solvent system used for TLC. After separation, the bands were visualised under TL-600 UV at 366 and 254 nm and the R_f values of the individual bands were detected.

High-performance liquid chromatography analysis

The crude methanolic extract (40 mg/mL), AMF (30 mg/mL), the standard genipin (0.50 mg/mL), and fraction B (0.40 mg/mL) were dissolved in methanol and passed through the membrane filter (Nylon 6, 6 membrane 0.20 µm, Pall Life Sciences). The samples (20 µL) were injected to the end-capped, RPC-18, 5 µm size, 250 × 4.60 mm column and eluted by the solvent system, acetonitrile/ortho-phosphoric acid (0.01 M, pH 4) in the ratio of 30 : 70 with a flow rate of 1 mL/min using a 10 AT VP, HPLC system equipped with a UV-VIS detector. The column temperature was kept at 25 °C during running and the absorbance of the eluted samples was measured at 240 nm.

Liquid chromatography-mass spectrometry analysis

The active fraction B and standard genipin were analysed by LC-MS (LC-1200 infinity-MS-6120 Quadrapole LC/MS, Agilent). LC conditions of the analysis were as follows: Column: Eclipse Plus C18 4.60 × 250 mm, 5 µm; mobile phase: 5 mM ammonium formate: acetonitrile (50 : 50), flow rate 1 mL/min, wavelength 240 nm, diluent methanol, column oven temperature – 25 °C. A range varying from 100 to 700 *m/z* was scanned. An MSD detector was used and the analysis was performed by Chemstation Software (Agilent).

^{1}H nuclear magnetic resonance ($CDCl_3$) analysis

The concentrates of the isolated fraction B were crystallised in ether. A 500 MHz FT NMR spectrometer was used for obtaining the ^{1}H NMR spectrum of fraction B and genipin in deuterated chloroform ($CDCl_3$). The chemical shifts in the peak were recorded as peak shifts in ppm.

Statistical analysis

The values are expressed as mean ± SD. The statistical significance was compared between control and experimental groups by one-way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnet multiple comparison test) using Graphpad Instat 3 software.

Supporting information

Fig. 1 S to 6 S showing spectra and chromatography data as well as the structure of fraction B are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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