STUDIES ON ANTICANCER ACTIVITY OF SOME MEDICINAL PLANTS

By

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(Registration No. J-06-D-02-V)

Thesis submitted to
Faculty of Postgraduate Studies
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY
IN
VETERINARY PHARMACOLOGY & TOXICOLOGY

Division of Veterinary Pharmacology and Toxicology
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ABSTRACT

Title of the Thesis:	**Studies on anticancer activity of some medicinal plants**
Name of the student:	Dilip Manikrao Mondhe
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In the present investigation, leaves of *Azadirachta indica*, roots of *Asparagus racemosus*, flowers of *Butea monosperma,* and aerial parts of *Ipomoea nil* and *Costus speciosus* were studied for their anticancer activity. Alcoholic, hydro-alcoholic and aqueous extracts were prepared from the specified plant parts and studied for their *in vitro* cytotoxic potential against a panel of twelve cancer cell lines viz., Colo-205, 502713, HCT-15, SW-620, SK-N-SH , Du-145, A-549, MCF-7, OVCAR-5, HEP-2, IMR-32 and D17. Alcoholic extracts of *Azadirachta indica* (leaves), *Asparagus racemosus* (roots) and *Ipomoea nil* (aerial parts) were found to possess promising cytotoxic activity which were then fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and again investigated for their *in vitro* cytotoxic potential against the same panel of twelve cancer cell lines. The most promising fractions along with their respective extracts were further studied for their *in vivo* anticancer activity against seven models of murine tumors viz., Ehrlich ascites carcinoma (EAC), Sarcoma-180 (ascites), Methyl cholanthrene induced ascites, Ehrlich tumor (solid), Sarcoma-180 (solid), L1210 Lymphoid leukemia and P388 Lymphocytic leukemia and myelosuppressive effects in tumor bearing animals. Alcolholic extract of *Azadirachta indica* leaves (AILE) and its chloroform fraction (AILF2) showed poor to moderate *in vivo* anticancer activity whereas alcoholic extract of *Asparagus racemosus* roots (ARRE) and its chloroform (ARRF2) and n-butanol (ARRF3) fractions as well as alcolholic extract of *Ipomoea nil* aerial parts (INE) and its n-hexane fraction (INF1) showed excellent *in vivo* anticancer activity. ARRF3 and INF1 emerged to be enriched with therapeutic anticancer potential which on further investigation were found to arrest cell cycle at sub-G_1 phase and induce apoptosis through mitochondrial pathway. Light microscopic and scanning and transmission electron microscopic studies conducted on HL-60 cells, EAC cells and Sarcoma-180 (solid) tissue further confirmed the induction of apoptosis (as revealed by changes typical of apoptosis) with these isolates. However, the anticancer activity of INF1 was assosciated with severe myelosuppression whereas ARRF3 showed no significant myelosuppression on further investigation with respect to their effects on bone marrow cellularity. The study has identified ARRF3 and INF1 as isolates with definite potential of new anticancer therapeutics. It provides important leads for further studies to develop therapeutic molecules.

Keywords: Anticancer, *Azadirachta indica*, *Asparagus racemosus*, *Ipomoea nil*

Signature of Major Advisor **Signature of Student**

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ABBREVIATIONS AND SYMBOLS USED

α	Alpha
β	Beta
γ	Gamma
%	Per cent
&	And
μg	Microgram
β-HIVS	β-hydroxy isovaleryl shikonin
~	Approximately
μl	Microlitre
μM	Micromolar
5-HT	5-hydroxytryptamine
AIDS	Acquired Immunodeficiency Syndrome
AILE	Alcoholic extract of *Azadirachta indica* leaves
AILF2	Chloroform fraction from alcoholic extract of *Azadirachta indica* leaves
Apaf-1	Apoptotic proteases activating factor-1
ARRE	Alcoholic extract of *Asparagus racemosus* roots
ARRF2	Chloroform fraction from alcoholic extract of *Asparagus racemosus* roots
ARRF3	n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots
B(a)P	Benzo(a)pyrene
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
BW	Body weight
CA4	Combretastatin A-4 phosphate
CAD	Caspase activated DNase
CCl_4	Carbon tetrachloride
Cdk	Cyclin-dependent kinase
CHR	Complete hematologic remission
cm	Centimetre
CML	Chronic myelogenous leukemia
CPT-11	Camptothecin-11
DAL	Dalton's ascitic lymphoma
DDT	Dichlorodiphenyltrichloroethane
DEN	diethylnitrosamine
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EAC	Ehrlich Ascites Carcinoma
EDTA	Ethylene diamine tetra acetic acid
ET	Ehrlich Tumor (solid)
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf serum
FITC	Flurscein isothiocyanate
FSH	Follicle Stimulating Hormone
ft	Feet
GPx	Glutathione peroxidase
GSH	Glutathione
GST-P	Glutathion-S-transferase Pi
h	Hours
HBSS	Hank's Buffered Salt Solution
HHT	Harringtonine and homoharringtonine

HIV	Human Immunodeficiency virus
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
in	Inch
INE	Alcoholic extract of *Ipomoea nil* aerial parts
INF1	n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts
IU	International unit
KCl	potassium chloride
LDL	Low-density lipoprotein
LH	Lutenising Hormone
LPO	Lipid peroxidation
M	Molar
MBL-2	mannose-binding lectin 2
mg	Milligram
ml	Millilitre
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NCI	National Cancer Institute, USA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small-cell lung cancer
°C	Degrees Centigrade
OsO_4	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffer Solution
PCNA	Proliferating Cell Nuclear Antigen
PI	Propidium iodide
PLK-1	Polo-like kinase 1
RBC	Red Blood Cells
RH	Relative humidity
Rh-123	Rhodamine-123
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Rose Park Memorial Institute-1640
S-180	Sarcoma-180
SD	Standard deviation
SE	Estandard Error
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SRB	Sulphorhodamine B
SRBC	Sheep red blood cells
TCA	Trichloro acetic acid
TCF	Tissue Culture Flask
TNFR-1	Tumor necrosis factor receptor-1
TNF-α	Tumor necrosis factor-α
Tris	Tris-hydroxymethyl-aminomethane
USDA	United States Department of Agriculture
VDS	Vindesine
VEGF	Vascular endothelial growth factor
VRLB	Vinorelbine
WBC	White Blood Cells
WHO	World Health Organisation

Chapter I

Introduction

CHAPTER - I

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells (American Cancer Society). Defining feature of cancer is the rapid creation of abnormal cells that avoid natural process of cell death (apoptosis). The incidence of cancer rises dramatically with age. Cancer has been recognized as a major health problem in human beings globally, ranking second only to heart disease, both in developed and developing countries. Cancer and heart disease are reckoned to be the major modern diseases that constitute the main causes of death in old age. It was only really during the latter stages of the last century that large proportions of people began living to the seventh, eighth and ninth decades of life - decades at which chronic diseases are common. The large increase in life expectancy to current levels has been brought about by the cure or control of a large number of otherwise fatal diseases such as plague, cholera, diabetes, malnutrition, diseases of infancy and other infectious diseases such as tuberculosis. Scarlet fever, diphtheria, whooping cough, measles, smallpox, puerperal fever, syphilis, typhus and non-respiratory tuberculosis have now been virtually eliminated as a cause of death. Cancer is therefore a more common phenomenon nowadays than previously and to a large extent it is due to the growth of the world's population and relatively advanced age to which people now live, as it is a disease that is more common in elderly ages than in younger ages.

The global cancer burden (number of new cancer cases per annum) was estimated to be 5.9 million in 1975 (Parkin *et al.*, 1984) which rose to 10.1 million in 2000 (Parkin,

2001). In 2008, there were an estimated 12.4 million incident cases of cancer (6672000 in men and 5779000 in women) and 7.6 million deaths from cancer (4293000 in men and 3300000 in women). Over half of the incident cases occurred in residents of four WHO regions with a large proportion of countries of low- and middle-income. Globally, lung cancer was the commonest incident cancer and the cause of cancer-related mortality in men but, the most common incident cancer and the cause of cancer-related death in women was breast cancer (World Cancer Report, 2008). Presently, the main types of cancer leading to overall cancer mortality each year are: lung (1.31 million deaths/year), stomach (803000 deaths/year), liver (699000 deaths/year), colorectal (639000 deaths/year) and breast (519000 deaths/yar) (American Cancer Society, 2008).

In India, every year about 850000 new cancer cases are diagnosed resulting in about 580000 cancer related deaths each year. India has the highest number of the oral and throat cancer cases in the world. Every third oral cancer patient in the world is from India. Compared to developed nations overall there are less cancer cases in India but this could be due to under diagnosis and under reporting. Cancer is mainly a disease of old ages and the worldwide median age at diagnosis is about 60 yrs. The average life span in India is about 58 yrs compared to 75 yrs in the developed world. At the same time regional, ethnic, dietary and socio-economic factors might also results in difference in the cancer susceptibilities and the incidence. On an average cancers are diagnosed at a much later stages in India. Overall, cervical cancer is the number one cause of cancer deaths in India (Indian Cancer Society).

It is estimated that at present, about 26 million people are living with cancer worldwide and 13 per cent of all deaths are caused by cancer. That's more than the percentage of deaths caused by HIV/AIDS, tuberculosis and malaria put together. Cancer is thus a public health problem of serious concern, worldwide.

There are three clear scenarios under which the global cancer burden is set to increase over time. First of all, the increase in the world's population anticipated from 6.1 billion in 2000 through 6.7 billion in 2008 to attain 8.3 billion by 2030 (United Nations, 2007) will lead to an increase in the cancer burden even if the age-specific rates remain constant. Secondly, given the very large increases in cancer risk with age, if the population size and the age-specific rates remain constant, then the burden will increase

if the population ages. The third element that can lead to an increase in the cancer burden, even when the population size remains constant and the age distribution remains unchanged, is an underlying increase in the incidence rates. In France, cancer incidence rates increased by 1.3 per cent per annum between 1978 and 2000 (Boyle *et al.*, 2003; IARC, 2007). In the Indian cancer registries, between 1983 and 1997, the incidence rate increased at an annual rate of 0.5 per cent per annum. In China, between 1973 and 1997, the incidence rate increased at an annual 1.4 per cent per annum. In Latin American registries between 1985 and 1997 the incidence rate increased at an annual rate of 1.0 per cent per annum (Doll *et al.*, 1966; Doll *et al.*, 1970; Waterhouse *et al.*, 1976; Waterhouse *et al.*, 1982; Muir *et al.*, 1987; Parkin *et al.*, 1992; Parkin *et al.*, 1997; Parkin *et al.*, 2003).

Thus, the growth and ageing of the world's population and the continual increase in the underlying incidence rates in low- and middle-income countries will contribute to increases in the global cancer burden. Under the zero increase in cancer incidence scenario, International Agency for Research on Cancer (IARC) estimates that the global cancer burden will increase from 10.9 million in 2002 to nearly 20 million in 2030 and the cancer related mortality of 6.7 million in 2002 will increase to 12.9 million in 2030 (World Cancer Report, 2008).

By extrapolation of these data, taking into account demographic changes and factoring in a yearly increase in cancer incidence of 1 per cent, it could be expected that by 2030 there will be approximately 26.4 million incident cases of cancer and 17.0 million cancer deaths a year.

In domestic animals, varying incidence of cancer has been reported by various workers (Steiner and Bengston, 1951; Anderson and Jarrett, 1968; Brodey and Kelly, 1968; Priester, 1974; Hayes *et al.*, 1975; Hayes and Pendergrass, 1976; Priester, 1977; Moulton, 1990; Voigt, 2007). Among domestic animals, cancer is the leading cause of overall deaths in dogs, likely attributable to the lack of significant coronary disease in the species. Regardless of age, 1 in 4 deaths in dogs are due to cancer. Since cancer registries are not available in veterinary medicine, it is likely that this number is even higher. In a necropsy study of 2000 dogs, 45 per cent of dogs older than 10 years of age had died of cancer. Estimates of overall cancer incidence rates per 100000 pets at risk

range from 381 for dogs and 264 for cats (www.vetcancersociety.org). The most common cancers in dogs are mammary tumors, skin cancer, oral cancer, lymphoma and hemangiosarcoma. The most common cancers in cats are lymphoma, skin/connective tissue and mammary tumors.

In case of pancreatic carcinoma, Priester (1974) reported an annual incidence of 8.9 and 17.8 per 100000 human and canine populations, respectively. In other domestic animals, the incidence rates per 100000 populations were 3.9 for bovines, 1.6 for equines and 12.6 for felines. He found that the older age was strongly associated with increasing occurrence of pancreatic carcinoma in dogs and cats. From 11 North American veterinary university hospitals and clinics, 248 animals with a confirmed diagnosis of nervous-tissue tumor were identified by Hayes *et al.*, (1975). Out of the 248 cases, 7 tumors were found in cattle, 28 in horses, 14 in cats, 199 in dogs, and none in other species.

The therapeutic mainstays of cancer remain surgery, radiotherapy and chemotherapy, the relative contribution of each being mandated by the natural history of the specific tumor. However, the rising global cancer burden in people and animals makes the present day approaches to cure / control / manage cancer in patients quite far from adequate. In tumors where complete surgical resection is not possible, chemotherapy and radiation therapy are used to inhibit proliferating tumor cells. Unfortunately, both modalities inhibit normal proliferating cells as well. This general anti-proliferative effect results in the common and normal tissue toxicity observed in cancer patients, which limits the delivery of sufficient therapeutic doses to eradicate the tumor (Cornell, 2006). The world is therefore, in dire need of safer therapeutic agents that will control the growth of tumor without much harm to normal cells.

Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling (Geysen *et al.*, 2003; Lombardino and Lowe, 2004). Nevertheless, plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. The use of plants as medicines has involved isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th

century and subsequently led to the isolation of early drugs such as cocaine, codeine, digitoxin and quinine, of which some are still in use (Newman *et al.*, 2000; Butler, 2004).

The plant kingdom consists of about 400000 plant species and is a huge reservoir of bioactive molecules, many of which have been explored for various pharmaceutical applications (Badisa *et al.*, 2003). Plants can be regarded as chemical libraries of structurally diverse compounds that constitute a promising approach in drug discovery. Modern pharmacological studies on several medicinal plants resulted in number of highly valuable drugs (molecules) of unmatched therapeutic potential from their secondary metabolites and are in regular clinical practice. According to Newman *et al.*, (2003), 61 per cent of the 877 small-molecule New Chemical Entities (NCEs) introduced as drugs worldwide during 1981–2002 were inspired by natural products. These include: natural products (6 per cent), natural product derivatives (27 per cent), synthetic compounds with natural products-derived pharmacophore (5 per cent) and synthetic compounds designed from natural products (natural products mimics, 23 per cent). Thus, the natural products and particularly medicinal plants, remain an important source of new drugs, new drug leads and new chemical entities (NCEs) despite the recent interest of pharmaceutical companies in molecular modelling, combinatorial chemistry and other synthetic chemistry techniques as nature is the best combinatorial chemist and possibly has answers to all diseases of the mankind.

The best from plant / medicinal plant-based drug discovery is yet to come as only about one-tenth of the flowering species occurring globally have so far been investigated for their pharmaceutical potential. Therefore, the real treasure remains to be discovered. The diverse genetic resources and associated rich traditional knowledge available in India form the strong basis for bio-prospecting. Proper utilization of these resources and tools in bio-prospecting can certainly help in discovering novel lead molecules from plants by employing modern drug discovery techniques.

Plants have been used for over 3500 years in the treatment of cancer but, it was only in the late 1950s that the evaluation of crude plant extracts for their anti-proliferative potential was initiated in earnest. Since then, more than 120000 plant extracts from over 6000 genera have been tested, resulting in the development of a large number of

structurally divergent natural products as candidate anti-cancer agents. Some of these proved to be clinically useful and others served as tools to unravel the biochemical mechanisms involved in the growth and regulation of tumors.

The discovery and development of efficacious anticancer agents, such as vinblastine and vincristine isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don, provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents. These drugs include several anticancer agents such as podophyllotoxins, paclitaxel, camptothecin, or compounds based on these as lead structures. Each of these classes of compounds exhibit their anticancer activity by distinct mechanisms (Dev, 1999). The most important member of the clinically useful natural anticancer agents is paclitaxel (Taxol) (Suffness, 1995). Development of the anticancer drugs etoposide and teniposide as semi-synthetic derivatives of epipodophyllotoxin, isolated from *Podophyllum peltatum* L. and *P. emodii* Wall. (Cragg *et al.*, 1993a) have established the use of semi-synthetic drugs and synthesis of other newer drugs for the treatment of cancer.

Thus, the approach to anticancer drug discovery from plants, has a historical justification (as it has yielded many clinically useful newer anticancer pharmaceuticals), a biochemical rationale (the position of plants in the ecosystem demands that they produce defense substances, many of which have a novel phenotype), and a chemical rationale (natural products provide templates for drug design). Therefore, continuous search for new anticancer drugs from plants will be a fruitful frontier in cancer treatment and/or chemoprevention. Moreover, the fact that thus far only a relative handful of medicinal plants have been evaluated for their anti-cancer potential, holds promise for the identification of agents acting through even more sophisticated mechanisms.

With this view, the present studies were planned to investigate the anticancer potential of following medicinal plants which are traditionally used by the folklore for various ailments including cancer.

- *Azadirachta indica* A. Juss. (Common name: Neem; family: *Balsaminaceae*)
- *Asparagus racemosus* Willd. (Common name: Shatavari; family: *Asparagaceae*)

- *Ipomoea nil* L. Roth (Common name: Japanese Morning Glory, family: *Convolvulaceae*)
- *Costus speciosus* Koen ex. Retz. (Common name: Crape ginger, family: *Costaceae*)
- *Butea monosperma* (Lam.) Taub. (Common name: Palas, Palash, family: *Fabaceae*)

The studies on these plants were undertaken with the following objectives.

- To investigate *in vitro* cytotoxic potential of *Azadirachta indica* (leaves), *Butea monosperma* (flowers), *Asparagus racemosus* (roots), *Ipomoea nil* (aerial parts) and *Costus speciosus* (aerial parts) against ten cancer cell lines of human and/or animal origin and to identify the most promising plant extracts.

- To fractionate the promising plant extracts to identify active isolate(s) and to enrich the cytotoxic activity.

- To determine *in vivo* anticancer activity of the active isolate(s) in animal models of various cancers in order to evaluate their therapeutic potential.

- To evaluate the active isolate(s) for their myelosuppressive effects in tumor bearing animals.

- To determine the apoptotic potential of the active isolate(s) using light / electron microscopy and flow-cytometry techniques.

Chapter II

Review of Literature

CHAPTER - II

REVIEW OF LITERATURE

2.1 Drug Discovery from plants

For thousands of years medicine and natural products have been closely linked through the use of traditional medicines and natural poisons (Sneader, 1996; Grabley and Thiericke, 2000; Mann, 2000; Newman *et al.*, 2000; Buss *et al.*, 2003). Clinical, pharmacological and chemical studies of these traditional medicines, which were derived predominantly from plants, were the basis of most early medicines such as quinine (Sneader,1996), aspirin (Newman *et al.*, 2000), pilocarpine (Mann, 2000), morphine (Grabley and Thiericke, 2000) and digitoxin (Buss *et al.*, 2003). Similarly, plants have been the major source of drugs in Indian system of Medicine and other ancient systems throughout the world. Earliest description of curative properties of medicinal plants is found in Rigveda (2000-1800 BC). Charaka Samhita and Sushruta Samhita provide extensive description on various medicinal herbs. Plants still continue to provide mankind with newer remedies and serve as the basis of novel drug discovery. Therefore, classical sciences like taxonomy and ethnobotany have now become an integral part of drug discovery from plants. Around 250,000 flowering plant species are reported to occur globally and approximately half (125,000) of these are found in the tropical forests. They continue to provide the mankind with invaluable compounds for development of new drugs. The potential for finding new compounds is enormous as till date only about 1 per cent of tropical species have been studied for their pharmaceutical potential.

The success of drug discovery from plants resulted principally in the development of anti-bacterial and anti-cancer agents. The success of anti-cancer drug development can be illustrated from the efforts of the National Cancer Institute (NCI), USA. In this effort, field explorations are largely guided by the so-called biodiversity or 'random' collection approach, with ethnobotanical or ethnopharmacological information playing a minimal or no role. NCI launched its effort in 1955, and for the period between 1960 and 1982, about 114,000 extracts from an estimated 35,000 plant samples (representing 12,000–13,000 species) collected mostly from temperate regions of the world were screened against a number of tumor systems (Cragg and Boyd, 1996). A wide variety of compound classes were isolated and characterized. Clinically significant cancer chemotherapeutic agents that emerged from this programme included paclitaxel (*Taxus brevifolia* Nutt. and other *Taxus* sp.) and semi-synthetic derivatives of camptothecin (*Camptotheca acuminata* Decne., Nyssaceae) like hycamptamine (topotecan), CPT-11 and 9-aminocamptothecin (Cragg *et al.*, 1993). The programme was extended from 1986 to 2004, with an emphasis on global plant collections and screening against tumor cell cultures.

Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling (Geysen *et al.*, 2003; Lombardino and Lowe, 2004). According to Newman *et al.*, (2003), 61 per cent of the 877 small-molecule New chemical Entities (NCEs) introduced as drugs worldwide during 1981–2002 were inspired by natural products, out of which, 6 per cent were natural products, 27 per cent natural products derivatives, 5 per cent synthetic compounds with natural products-derived pharmacophore and 23 per cent synthetic compounds or mimics designed from natural products (Sneader, 1996; Newman *et al.*, 2003). Following are the few examples of successful drugs derived from plants.

Arteether is a potent anti-malarial drug and is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae), a plant used in traditional Chinese medicine (van Agtmael *et al.*, 1999; Graul, 2001).

Galanthamine, first isolated from *Galanthus woronowii* Losinsk. (Amaryllidaceae) in Russia is approved for the treatment of Alzheimer's disease. It slows down the process of

neurological degeneration by inhibiting acetylcholine esterase as well as binding to and modulating the nicotinic acetylcholine receptor (Heinrich and Teoh, 2004; Prittila *et al.*, 2004).

Tiotropium is an inhaled anticholinergic bronchodilator, based on ipratropium, a derivative of atropine, isolated from *Atropa belladonna* L. (Solanaceae) and other members of the *Solanaceae* family (Dewick, 2005). It has been released in the US for the treatment of chronic obstructive pulmonary disease (Frantz, 2005; Mundy and Kirkpatrick, 2004).

Morphine-6-glucuronide is reported as an alternative pain medication with fewer side effects than morphine (Lotsch and Geisslinger, 2001). It is a metabolite of morphine from *Papaver somniferum* L. (*Papaveraceae*).

Exatecan is an analogue of camptothecin isolated from *Camptotheca acuminata* Decne. (Nyssaceae) and is being developed as an anticancer agent (Butler, 2004; Cragg and Newman, 2004).

Vinflunine is a modification of vinblastine from *Catharanthus roseus* G. Don (Apocynaceae) for use as an anticancer agent with improved efficacy (Okouneva *et al.*, 2003).

Flavopiridol is totally synthetic, but the basis of its novel flavonoid structure is a natural product, rohitukine. Rohikutine, used for rheumatoid arthritis is isolated from *Dysoxylum binectariferum* Hook. f. (*Meliaceae*), which is phylogenetically related to the Ayurvedic plant, *Dysoxylum malabaricum* Bedd. Flavopiridol was one of the over 100 analogues synthesized during structure–activity studies and was found to possess tyrosine kinase inhibitory activity and potent growth inhibitory activity against a series of breast and lung carcinoma cell lines (Sausville *et al.*, 1999). It also showed broad-spectrum *in vivo* activity against human tumor xenografts in nude mice, which led to its selection for preclinical and clinical studies by the NCI in collaboration with Hoechst India Ltd. It is currently in phase I and phase II clinical trials, alone and in combination with other anticancer agents, against a broad range of tumors, including leukaemias, lymphomas and solid tumors (Cragg and Newman, 2005).

Over 60 per cent of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms. Plants have a long history of use in the treatment of cancer. Hartwell (1982) lists more than 3000 plant species that have reportedly been used in the treatment of cancer. The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960, focused mainly in temperate regions. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the taxanes and camptothecins. It took about 30 years for their development into clinically active agents. More recent semi-synthetic analogues of vinca alakaloids are vinorelbine (VRLB) and vindesine (VDS) which are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers. Vinblastine is used for the treatment of leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma, and Vincristine, in addition to the treatment of lymphomas, also shows efficacy against leukemias, particularly acute lymphocytic leukemia in childhood. VRLB has shown activity against non-small-cell lung cancer and advanced breast cancer (Cragg and Newman, 2005).

Podophyllum species (Podophyllaceae), *P. peltatum* Linnaeus (commonly known as the American mandrake or Mayapple), and *P. emodii* Wallich from the Indian subcontinent, have a long history of medicinal use, including the treatment of skin cancers and warts. *P. peltatum* was used by the Penobscot Native Americans of Maine for the treatment of cancer, and interest was promoted by the observation in the 1940s that venereal warts could be cured by topical application of an alcohol extract of the dried roots (called podophyllin). The major active constituent, podophyllotoxin, was first isolated in 1880, but its correct structure was only reported in the 1950s. Many closely related podophyllotoxin-like lignans were isolated during this period, and several of them were introduced into clinical trials, only to be dropped due to lack of efficacy and unacceptable toxicity. Extensive research at Sandoz Laboratories in Switzerland in the 1960s and 1970s led to the development of etoposide and teniposide as clinically effective agents which are used in the treatment of lymphomas and bronchial and testicular cancers (Cragg and Newman, 2005).

More recent addition to plant-derived chemotherapeutic agents is the class of molecules called taxanes. Paclitaxel (taxol) was initially isolated from the bark of *Taxus brevifolia* Nutt. (*Taxaceae*), collected in Washington State as part of a random collection program by the U.S. Department of Agriculture (USDA) for the National Cancer Institute (NCI). The use of various parts of *T. brevifolia* and other species of *Taxus* (e.g. *T. canadensis* Marshall, *T. baccata* L.) by several native American tribes for the treatment of certain non-cancerous conditions has been reported, while the leaves of *T. baccata* are used in the traditional Asiatic Indian (Ayurvedic) medicine system, including its use in the treatment of cancer. Paclitaxel and other key precursors (like baccatins) occur in the leaves of various *Taxus* species. The semi-synthetic conversion of the relatively abundant baccatins to paclitaxel, and to active paclitaxel analogs such as docetaxel (Taxotere), has provided a major renewable natural source of this important class of drugs. Paclitaxel is used in the treatment of breast, ovarian and non-small-cell lung cancer (NSCLC), and has also shown efficacy against Kaposi's sarcoma. Paclitaxel has also shown promise in the potential treatment of multiple sclerosis, psoriasis and rheumatoid arthritis. Docetaxel is primarily used in the treatment of breast cancer and NSCLC (Cragg and Newman, 2005).

Camptothecin, an active agent isolated from the Chinese ornamental tree, *Camptotheca acuminata* Decne (*Nyssaceae*), is another class of clinically-active agents. However, Camptothecin (salt) was dropped because of severe bladder toxicity but derivatives like Topotecan and Irinotecan are now in clinical use (Cragg and Newman, 2005).

Other plant-derived agents in clinical use are homoharringtonine, isolated from the Chinese tree, *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (Cephalotaxaceae). A racemic mixture of harringtonine and homoharringtonine (HHT) has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia. Purified HHT has shown efficacy against various leukemias, including some resistant to standard treatment, and has been reported to produce complete hematologic remission (CHR) in patients with late chronic phase chronic myelogenous leukemia (CML). Elliptinium is a derivative of ellipticine, isolated from species of several genera of the *Apocynaceae* family, including *Bleekeria vitensis* A. C. Sm., a Fijian medicinal plant with reputed anti-cancer properties, is licensed in France for the treatment of breast cancer.

2.2 Anticancer Agents of Plant origin under Clinical Development

Rohitukine, isolated by chemists at Hoechst India Ltd. in the early 1990s from *Dysoxylum binectariferum* Hook. f. (Meliaceae) is the basis for novel molecule flavopiridol. It is currently in Phase I and Phase II clinical trials, either alone or in combination with other anti-cancer agents, against a broad range of tumors, including leukemias, lymphomas and solid tumors. Flavopiridol alone probably is not a viable treatment, but use of this compound in conjunction with agents such as paclitaxel and cisplatin has led to partial and complete remissions in a number of Phase I patients, encouraging Phase II studies in patients with a variety of paclitaxel-resistant tumors (Cragg and Newman, 2005).

The combretastatins were isolated from the South African "bush willow", *Combretum caffrum* (Eckl. & Zeyh.) Kuntze (Combretaceae), collected in Southern Africa in the 1970s for the NCI. The combretastatins are a family of stilbenes which act as anti-angiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis. A water-soluble analogue, combretastatin A-4 phosphate (CA4), has shown promise in early clinical trials. Of interest is the number of combretastatin (CA4) mimics being developed. Three are in clinical trials, while 11 are in preclinical development.

An interesting agent in early clinical development is roscovitine which is derived from olomucine, originally isolated from the cotyledons of the radish, *Raphanus sativus* L. (*Brassicaceae*), but now produced synthetically. Olomucine stimulated interest as a result of its inhibition of cyclin-dependent kinases (Cdk) - proteins which play a major role in cell cycle progression. Chemical modification of olomucine resulted in the more potent inhibitor, roscovitine, which is under clinical development in Scotland and currently is in Phase II clinical trials in Europe. Further development of this series, following synthesis of a focused library via combinatorial chemistry techniques, has led to the purvalanols which are even more potent and are in preclinical development (Cragg and Newman, 2005).

2.3 Studies on anticancer activity of plants

Withaferin A (WA), a major chemical constituent of *Withania somnifera*, reportedly shows cytotoxicity in a variety of tumor cell lines. Withaferin A primarily induces oxidative stress in human leukemia HL-60 cells and in several other cancer cell lines. Malik *et al.* (2007) reported that the withanolide induced early ROS generation and mitochondrial membrane potential loss, which preceded release of cytochrome C, translocation of Bax to mitochondria and apoptosis inducing factor to cell nuclei. These events paralleled activation of caspases –9, –3 and PARP cleavage. Withaferin A activated extrinsic pathway significantly as evidenced by time dependent increase in caspase- 8 activity with respect to over expression of TNFR-1. It mediated decreased expression of Bid, which probably is an important event for cross talk between intrinsic and extrinsic signaling. Furthermore, withaferin A inhibited DNA binding of NF-kB and caused nuclear cleavage of p65/Rel by activated caspase- 3. N-acetyl-cysteine rescued all these events suggesting thereby a pro-oxidant effect of withaferin A.

Tinospora cordifolia (Guduchi) has been used for centuries for treating various ailments including cancer in Ayurvedic system of medicine. *Tinospora cordifolia* has antitumor activity (Singh *et al.,* 2004; 2005). Thippeswamy and Salimath (2007) reported that treatment of Ehrlich ascites tumor (EAT) bearing animals with hexane fraction of *T. cordifolia* (TcHf) resulted in growth inhibition and induction of apoptosis in a dose-dependent manner. TcHf induced the formation of apoptotic bodies, nuclear condensation, typical DNA ladder, activation of caspase-3, decreased cell number and ascites volume. It inhibited the proliferation of EAT cells by blocking cell cycle progression in the G_1 phase. Apoptosis in the EAT cells was associated with the constitutive expression of caspase activated DNase (CAD) in both nucleus and cytoplasm after TcHf treatment. Furthermore, the expression of pro-apoptotic gene, Bax was up-regulated and the expression of anti-apoptotic gene, Bcl-2 was down-regulated in a time dependent manner by TcHf treatment which indicated the apoptosis inducing capability of hexane fraction of the plant *in vivo*. Dahanukar and Thatte (1997) reported inhibition of proliferation of bone marrow cells and also the induction of apoptosis in malignant cells by the *Tinospora cordifolia* aqueous extract.

Boswellia serrata triterpenediol (TPD) mixture comprising of isomeric mixture of 3α, 24-dihydroxyurs-12-ene and 3α, 24-dihydroxyolean-12-ene induces apoptosis in cancer

cells (Bhushan *et al.*, 2007). It inhibited cell proliferation with IC_{50} ~ 12μg/ml and produced apoptosis as measured by various biological end points, e.g., increased sub-G_0 DNA fraction, DNA ladder formation, enhanced AnnexinV-FITC binding of the cells etc.

Bhushan *et al.* (2006) reported that AP9-cd, a standardized lignan composition from *Cedrus deodara* consisting of (-)-wikstromal, (-)-matairesinol, and dibenzyl butyrolactol, showed cytotoxicity in several human cancer cell lines. It inhibited Molt-4 cell proliferation with IC_{50} of approximately 15 μg/ml, increased sub-G_0 cell fraction without any mitotic block, produced apoptotic bodies and induced DNA ladder formation. AnnexinV-FITC/PI-stained cells showed time-related increase in apoptosis and post-apoptotic necrosis, which indicated cell death by apoptosis. Singh *et al.* (2007) studied the *in vivo* anticancer activity of the same lignin composition from *Cedrus deodara* (AP9cd) against Ehrlich ascites carcinoma and colon carcinoma (CA-51) models in mice. The tumor growth inhibition observed with Ehrlich ascites carcinoma and CA-51 was 53 and 54 per cent respectively, when AP9cd was administered @ 300 mg/kg, i.p. for nine days in the Ehrlich ascites carcinoma bearing mice and 400 mg/kg, i.p. for the same period in the CA-51 model. The activity was comparable with 5-fluorouracil administered @ 22 mg/kg and 20 mg/kg, respectively.

Sharma *et al.* (2009) studied the *in vitro* cytotoxicity and *in vivo* anticancer activity of the essential oil from a variety of lemon grass, *Cymbopogon flexuosus.* They also studied the morphological changes in tumor cells treated with *Cymbopogon flexuosus* oil to ascertain the mechanism of cell death. The *in vitro* cytotoxicity studies showed dose-dependent effects against various human cancer cell lines. Intra-peritoneal administration of the oil significantly inhibited both the ascitic and solid forms of Ehrlich and Sarcoma-180 tumors in a dose-dependent manner. Morphological studies of the oil treated HL-60 cells revealed loss of surface projections, chromatin condensation and apoptosis. Morphological studies of ascites cells from animals treated with the oil too revealed the changes typical of apoptosis.

The Chinese herbal medicine, *Radix sophorae* is widely applied as an anti-carcinogenic/ anti-metastatic agent against liver cancer. Cheung *et al.* (2007) showed that Leachianone A, isolated from *Radix Sophorae,* possessed a profound cytotoxic activity against human

hepatoma cell line HepG2 *in vitro*, with an IC_{50} value of 3.4 mg/ml post-48 h treatment. Its mechanism of action involved both extrinsic and intrinsic pathways of apoptosis. Its anti-tumor effect was further demonstrated *in vivo* by 17–54 per cent reduction of tumor size in HepG2-bearing nude mice, in which no toxicity to the heart and liver tissues was observed.

Punica granatum, especially its fruit, possesses a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal value. The tree/fruit can be divided into several anatomical compartments: seed, juice, peel, leaf, flower, bark and roots, each of which has interesting pharmacologic activity. Juice and peels, for example, possess potent antioxidant properties, while juice, peel and oil are weakly estrogenic and heuristically of interest for the treatment of menopausal symptoms. Lansky and Newmana (2007) showed the juice, peel and oil of pomegranate tree, *Punica granatum*, to possess anticancer activities, including interference with tumor cell proliferation, cell cycle invasion and angiogenesis.

Feijoa acetonic extract has been reported to exert selective anti-cancer activities on solid and hematological cancer cells by Bontempo *et al.* (2007) as the extract did not show toxic effects on normal myeloid progenitors. Fractionation and subsequent purification of *Feijoa acetonic* extract identified flavone as the active component. Flavone induced apoptosis was accompanied by caspase activation and p16, p21 and TRAIL over-expression in human myeloid leukemia cells.

William and Cho (2007) observed *Astragalus membranaceus*, a commonly used Chinese medicinal plant, to be capable of restoring the impaired T cell functions in cancer patients. *In vitro* and *in vivo* anti-tumor effects of *Astragalus membranaceus* were investigated. It could effectively suppress the *in vivo* growth of syngeneic tumor in mice. Results showed that murine macrophages pre-treated with *Astragalus membranaceus* had increased *in vitro* and *in vivo* cytostatic activities towards MBL-2 tumor. It could also act as a priming agent for tumor necrosis factor production in tumor-bearing mice.

Selaginella tamariscina induces apoptosis mediated by the caspase activation pathway in HL-60 cells (Ahn *et al.*, 2006) and modified gene expression and cytokine production (Kuo *et al.*, 1998). Lee *et al.* (1999) reported that the aqueous extract of the plant

efficiently increased p*53* gene expression and induced G_1 arrest and suggested that this might contribute to cytotoxic effects by causing apoptotic DNA fragmentation in human leukaemia cell line U-937 and human ovarian cancer cell line A-2780.

The hot water extract of the bark of *Acer nikoense* (Nikko maple) showed concentration-dependent inhibitory effects on the growth of three cell lines (Nitta *et al.,* 1999). DNA fragmentation and morphological changes, accompanied by condensed and fragmented nuclei, were observed in the leukemia cell lines cultured with hot water extract of the bark of Nikko maple.

Uncaria tomantosa (Willd.) DC, also known as 'Cat's claw', has been used in South American traditional medicine (Taraphdar *et al.*, 2001). The water extract of *Uncaria tomantosa* has been shown to enhance DNA repair, mitogenic response and leukocyte recovery after chemotherapy-induced DNA damage, *in vivo* (Sheng *et al.*, 2001). He also reported that the water extract of the plant induced apoptosis mediated cytotoxicity in human leukaemic cell lines HL-60, K-562 and human EBV-transformed B-lymphoma cell line.

Seeds from *Acalypha wilkesiana* (Euphorbiaceae) are essential components of a complex plant mixture used empirically by traditional healers in south-west Nigeria to treat breast tumours and inflammation. Bussing *et al.* (1999) observed induction of apoptosis and generation of reactive oxygen intermediates in granulocytes by an aqueous extract of the seeds. The extract induced the release of the pro-inflammatory cytokines TNF-α and interleukin-6 (IL-6) and also T-cell associated cytokines, interleukin-5 (IL-5) and interferon-gamma.

Kavimani and Manisenthilkumar (2000) evaluated antitumour activity of methanolic extract of *Enicostemma littorale* (MEL) against Dalton's ascitic lymphoma (DAL) in swiss albino mice. Significant enhancement of mean survival time of MEL treated tumour bearing mice was found with respect to the control group.

Andrographolide, the major diterpenoid of the *Andrographis paniculata* extract has shown cytotoxic activity against KB (human epidermoid carcinoma) and P-388 (lymphocytic leukemia) cells. The methanol extract of aerial parts of *Andrographis*

paniculata and some of the isolated compounds showed growth inhibitory and differentiating activity on Ml (mouse myeloid leukemia) cells (Kumar *et al.*, 2004).

Devi *et al.* (1994) studied the effect of alcoholic root extract of *Plumbago rosea* on experimental mouse tumors, Sarcoma-180 (solid) and Ehrlich ascites carcinoma, *in vivo*. Intraperitoneal injection of 50 mg/kg of Plumbago extract (PE) for 10 days starting from 24 h after intradermal inoculation of Sarcoma-180 cells in BALB/c mice produced about 16 per cent complete response (CR), which increased to 50 per cent with increase in drug dose to 100 mg/kg for 10 days.

Curcuma longa, commonly known as Turmeric has been shown to possess variety of pharmacological properties like anti-inflammatory, anti-carcinogenic and anti-oxidant by different workers. Chakravarty and Yasmin (2008) have reported that turmeric also activates the lymphocytes and induces apoptosis of tumor cells. Kavimani *et al.* (1999) evaluated the antitumor activity of the methanolic extract of *Glinus lotoides* against Dalton's ascitic lymphoma (DAL) in Swiss albino mice. A significant enhancement of mean survival time of tumor bearing mice was observed with respect to the control group. Aqueous extract of *Rhodiola imbricata* rhizome has very potent anti-cancer activities (Mishra *et al.*, 2009). Its rhizome inhibits proliferation of an erythroleukemic cell line K-562 by inducing apoptosis and cell cycle arrest at G_2/M phase. Chakraborty *et al.* (2004) found the alkaloid fraction from *Tiliacora racemosa* to be cytotoxic against four cell lines but *Semecarpus* oil only in leukaemic cells.

Hu and Kavanagh (2003) studied the effect of water-soluble macromolecular components of *Artemisia capillaris* on human hepatoma cell line (SMMC-7721) and reported inhibition of cell proliferation and induction of apoptosis. Aqueous extract of *Viscum album* L. (Loranthaceae), European mistletoe, which is one of the most widely used alternative cancer therapies in Europe, is reported to possess cytotoxic and immunomodulatory properties that support its usefulness in cancer therapy (Sooryanarayana *et al.*, 2001). β-Hydroxy isovaleryl shikonin isolated from the plant *Lithospermum radix* (roots of *Lithospermum erythrorhizon*) induces apoptosis in various cell lines of human tumor cells (Masuda *et al.*, 2003). They postulated that the suppression of the activity of PLK-1 (polo-like kinase 1) via inhibition of tyrosine kinase

activity by β-hydroxy isovaleryl shikonin (β-HIVS) might play an important role in the induction of apoptosis.

Sun Chang-Ming *et al.* (2003) performed bioassay directed fractionation of *Saussurea lappa* which led to the isolation of a novel lappadilactone and seven sesquiterpene lactones as cytotoxic principles against selected human cancer cell lines. Lappadilactone, dehydrocostuslactone and costunolide exhibited the most potent cytotoxicity against Hep-G2, OVCAR-3 and HeLa cell lines.

Alcoholic extract of *Ganoderma lucidum* induced apoptosis in MCF-7 human breast cancer cells, which might be mediated through up-regulation of a pro-apoptotic Bax protein and not by the immune system (Hounbo *et al.*, 2002).

Alpinia oxyphylla Miquel (Zingiberaceae) has also been shown to induce apoptosis-mediated cytotoxicity in HL-60 cells in culture by exhibiting internucleosomal DNA fragmentation in time- and concentration-dependent manners (Lee, 1999).

Liu *et al.* (2000) reported that *Salvia miltiorrhiza,* a traditional Chinese herbal medicine exerted cytotoxic effects and strongly inhibited the proliferation of human hepatoma cell line HepG2 through apoptosis.

Betulinic acid (BA), a pentacyclic triterpene isolated from the root bark of *Morus australis* or *Clerodendrum mandarinorum* and many other medicinal plants (Zhu *et al.*, 1996; Ko *et al.*, 1997), has been reported to have cytotoxic activity against several tumor cell lines (Pisha *et al.*, 1995; Fulda *et al.*, 1997, 1998; Wick *et al.*, 1999; Kwon *et al.*, 2002; Wachsberger *et al.*, 2002). Betulinic acid is a novel experimental antineoplastic agent for human melanoma cells *in vitro* (Pisha *et al.*, 1995; Wachsberger *et al.*, 2002). Betulinic acid also induces apoptosis in neuroectodermal tumour and glioma cells (Fulda *et al.*, 1997, 1998; Wick *et al.*, 1999). Furthermore, BA has been shown to inhibit growth factor-induced angiogenesis *in vitro* (Kwon *et al.*, 2002).

Ren and Tang (1999) reported that *Solanum muricatum* plant extract induced DNA ladder formation and PARP cleavage. Water extract of *Paeoniae radix* is also found to induce apoptosis in HEpG2 and HEp3B hepatoma cells (Lee *et al.*, 2002).

Although number of plants deserve to be explored for their therapeutic potential against cancer, in the present study five plants viz., *Azadirachta indica* (leaves), *Butea monosperma* (flowers), *Asparagus racemosus* (roots), *Ipomoea nil* (aerial parts) and *Costus speciosus* (aerial parts) have been identified to be studied for their anticancer activity as they are relatively unexplored for this activity.

2.4 *Azadirachta indica* A. Juss

2.4 .1 Principal Constituents:

The alcoholic extract of the fresh stem and bark yields the bitter principles nimbin, nimbinin and nimbidin. The alcoholic extract of the air-dried root bark yields nimbin and nimbidin. All parts of the plant yield β-sitosterol (Indian patent No. 13343, 1927; Bhattacharji *et al.*, 1953; Mitra *et al.*, 1953; Narsimhan, 1957).

The leaves contain nimbin, nimbinene, 6-desacetylnimbinene, nimbadiol, nimbolide and quercetin. The presence of β-sitosterol, n-hexacosanol and nonacosane is also reported (Dakshinamurti, 1954; Basak and Chakraborty, 1968; Awasthi and Mitra, 1971). The diterpenoids, margolone, nimbogone, nimbonolone and mimbolinin have been isolated from the plant (Hanson, 1991).

2.4.2 Pharmacological activities:

Roy *et al.* (2006) found that Nimbolide, a natural triterpenoid present in the edible parts of the neem tree was growth-inhibitory in human colon carcinoma HT-29 cells at 2.5 – 10 μM. Flow cytometric analysis of HT-29 cells showed that nimbolide treatment (2.5 μM, 12 h) caused a 6.5-fold increase in the number of cells (55.6 per cent) in the G_2/M phase, which decreased to decreased to 18 per cent at 48 h, but the cells in G_0/G_1 phase increased to 52.3 per cent. Western blot analysis revealed that nimbolide-mediated G_2/M arrest was accompanied by the up-regulation of p21, cyclin D2, Chk2; and down-regulation of cyclin A, cyclin E, Cdk2, Rad17. Roy *et al.* (2007) also reported that Nimbolide, extracted from the flowers of the neem tree exerted moderate to strong growth inhibition at 0.5-5.0 μM concentrations against U937, HL-60, THP1 and B16 cancer cell lines. Flow cytometric analysis of U937 cells revealed that nimbolide produced cell cycle disruption by decreasing the number of cells in G_0/G_1 phase, with initial increases in S and G_2/M phases. Cells exposed to a higher dose of nimbolide for a

longer period displayed a severely damaged DNA profile, resulting in a remarkable increase in the number of cells in the sub-G_1 fraction, with a reciprocal decrease of cells in all phases. Quantification of the expression of phosphatidylserine in the outer cell membrane showed that doses of nimbolide higher than 0.4 µM exerted remarkable lethality, with over 60 per cent of cells exhibiting apoptotic features after exposure to 1.2 µM nimbolide.

Harish *et al.* (2009) reported dose- and time-dependent growth inhibition of human choriocarcinoma (BeWo) cells with nimbolide with IC_{50} values of 2.01 and 1.19 µM for 7 and 24 h respectively, accompanied by down-regulation of proliferating cell nuclear antigen. Examination of nuclear morphology revealed fragmentation and condensation indicating apoptosis. Increase in the generation of reactive oxygen species (ROS) that was reversed by addition of reduced glutathione suggested ROS involvement in the cytotoxicity of nimbolide. A decrease in Bcl-2/Bax ratio with increased expression of Apaf-1 and caspase-3, and cleavage of poly (ADP-ribose) polymerase provided compelling evidence that nimbolide-induced apoptosis was mediated by the mitochondrial pathway.

Similar results have been reported by Kumar *et al.* (2006) with ethanolic extract of neem leaves in prostate cancer cells (PC-3) which induced apoptosis as evidenced by increase in DNA fragmentation and a decrease in cell viability. Western blot studies indicated a decreased level of Bcl-2 and increased level of Bax protein. Ricci *et al.* (2008) also evaluated a component of the whole Neem oil obtained by methanolic extraction and defined as MEX for cytotoxicity against two different cell populations: a stabilized murine fibroblast line (3T6) and a tumor cell line (HeLa). The data obtained suggested a differential sensitivity of these two populations, the tumor line exhibiting a significantly higher sensitivity to MEX.

Baral and Chattopadhyay (2004) inoculated Swiss and C57BL/6 mice with Ehrlich Cacinoma and B16 Melanoma tumors on day 0 and after 7 days treated them with NLP (Neem Leaf Preparation) (1 unit/mice/week) for 4 weeks. Treatment of mice with NLP after tumor inoculation, did not result in any significant difference in tumor growth as compared to that in PBS treated control groups during the period of 35 days tumor growth. No difference in survivability between NLP and PBS treated tumor bearing mice

was also observed. However, they observed significant reduction ($p<0.01$) of tumor growth in mice prophylactically treated with NLP (1 unit/mice) for 4 weeks and after 7 days inoculated with Ehrlich Carcinoma and B16 Melanoma tumors, respectively. Further, they cultured Ehrlich Carcinoma and B16 Melanoma cells with different concentrations of NLP for 24 and 72 h, respectively and found that there was no significant difference in cell viability as compared to that of untreated control, suggesting that the NLP had no direct cytotoxic effect on Ehrlich Carcinoma and B16 Melanoma cells.

Haque and Baral (2006) observed that neem leaf preparation (NLP)-mediated activation of mononuclear cells from blood and spleen in Swiss and C57BL/6 mice enhanced cytotoxicity to murine Ehrlich Carcinoma cells *in vitro*. NLP-stimulated spleen cells showed greater secretion of TNF-α and IFN-γ.

Subapriya *et al.* (2006) reported that the administration of ethanolic neem leaf extract during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis significantly decreased the expression of proliferating cell nuclear antigen (PCNA), mutant p53 and Bcl-2 and overexpression of cytokeratin in the buccal pouch tissues.

Koul *et al.* (2006) observed that DMBA induced skin tumor in male BALB/c mice caused hepatic damage characterized by a decreased hepatosomatic index and significantly increased activities of the hepatic tissue injury marker enzymes, viz., alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase. However, upon treatment with aqueous *Azadirachta indica* leaf extract (AAILE), the above-mentioned alterations, including the increased activities of hepatic tissue injury marker enzymes, were significantly reversed. Increased oxidative stress was also observed in the hepatic tissue of skin tumor bearing mice as revealed by a significant increase in lipid peroxidation levels and a decrease in reduced glutathione contents and activities of various antioxidant enzymes, namely glutathione-S-transferase, glutathione peroxidase and glutathione reductase. Gangar and Koul (2007) observed that oral administration of AAILE (100 mg/kg body wt for 2 weeks) reduced the aryl hydrocarbon hydroxylase (AHH) activity and enhanced the UDP-glucuronosyltransferase activity in murine forestomach and liver in Benzo(a)pyrene (B(a)P) biotransformation, suggesting its

potential in decreasing the activation and increasing the detoxification of carcinogens. The LPO levels decreased upon AAILE treatment in the hepatic tissue, suggesting its antioxidative and thereby enhancing its anti-carcinogenic effects.

Gangar and Koul (2008a) further reported enhanced apoptotic index in the forestomach tumors of mice receiving AAILE along with benzo(a)pyrene (B(a)P) which was confirmed by presence of classical morphological features of apoptosis including chromatin condensation/marginalization, nuclear fragmentation, and formation of apoptotic bodies as revealed in transmission electron microscopy. No mitotic cells were found to be present; however, certain cells showing shrinkage and blebbings (characteristics of apoptosis) were observed. DNA fragmentation was observed to increase exclusively in the tumors of mice that received AAILE along with B(a)P.

Gangar and Koul (2008b) reported the activities of phase I biotransformation enzymes to increase, whereas a decrease in GSH content as well as glutathione-S-transferase in mice receiving only B(a)P during and after 2 weeks of B(a)P instillations. In mice that received AAILE along with B(a)P instillations, a decrease in phase I enzymes was accompanied by an increase in phase II enzymes as well as GSH contents.

Manikandan *et al.* (2008a) observed that the administration of neem leaf fractions reduced the incidence of DMBA-induced Hamster Buccal Pouch (HBP) carcinomas at a lower concentration compared to the crude extract. Chemoprevention by neem leaf fractions was associated with modulation of phase I and phase II xenobiotic-metabolising enzymes, lipid and protein oxidation, upregulation of antioxidant defences, inhibition of cell proliferation and angiogenesis, and induction of apoptosis. However, ethyl acetate fraction (EAF) was more effective than methanolic fraction (MF) for its antiproliferative and antiangiogenic effects and expression of Cytochrome-P450 isoforms. The greater efficacy of EAF was postulated to be due to higher content of constituent phytochemicals as revealed by HPLC analysis. Manikandan *et al.* (2008b) also studied the combinatorial chemopreventive efficacy of *Azadirachta indica* (AI) and *Ocimum sanctum* (OS) against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis, based on changes in oxidant-antioxidant status, cell proliferation, apoptosis and angiogenesis. Rats administered MNNG developed forestomach carcinomas that displayed low lipid and protein oxidation coupled to enhanced antioxidant activities, and over-expression of

PCNA, GST-P, CK, VEGF and Bcl-2 with downregulation of Bax, cytochrome C and caspase-3. Co-administration of AI and OS extracts suppressed MNNG-induced gastric carcinomas accompanied by modulation of the oxidant-antioxidant status, inhibition of cell proliferation and angiogenesis, and induction of apoptosis.

Vinothini *et al.* (2009) studied the chemopreventive potential of the ethyl acetate fraction (EAF) and methanolic fraction (MF) of *Azadirachta indica* (neem) leaf on 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary carcinogenesis. Estradiol and estrogen receptor status, xenobiotic-metabolizing enzyme activities, redox status, DNA and protein modifications, and the expression of cell proliferation, and apoptosis related proteins in the mammary gland and liver were used as biomarkers of chemoprevention. The authors reported that administration of both EAF and MF at a dose of 10 mg/kg BW effectively suppressed tumour incidence and that the chemoprevention by neem leaf fractions was associated with modulation of hormone and receptor status, xenobiotic-metabolising enzymes and lipid and protein oxidation with upregulation of antioxidants, inhibition of oxidative DNA damage, protein modification and cell proliferation and induction of apoptosis. The EAF, rich in constituent phytochemicals was more effective than MF in modulating multiple molecular targets.

Two water-soluble polysaccharides GIa and GIb isolated from the bark of *Melia azadirachta*, demonstrated strong anti-tumor effect with complete regression of the tumours, when administered in mice at a daily dose of 50 mg/kg for four days from 24 h after subcutaneous inoculation of Sarcoma-180 cells (Fujiwara *et al.*, 1982).

Haque *et al.* (2006) observed significant restriction of growth of Ehrlich's carcinoma following prophylactic treatment in Swiss albino mice with neem leaf preparation @ 1 unit once weekly for four weeks. Toxic effects of different doses like 1 unit, 0.5 unit and 2 units were evaluated on different murine physiological systems. All the mice could tolerate 4 injections of 0.5 and 1 unit NLP doses and these stimulated hematological systems as evidenced by the increase in total count of RBC, WBC and platelets and hemoglobin percentage. Histological changes as well as elevation in serum alkaline phosphatase, SGOT, SGPT were not observed in mice treated with three different doses of NLP. However, NLP dose of 2 units could not exhibit such immunostimulatory

changes; NLP mediated immunostimulation was correlated well with the growth restriction of murine carcinoma.

Ghosh *et al.* (2006) observed that pretreatment of mice with neem leaf preparation (NLP) reduced the extent of leucopenia and neutropenia in normal and tumor bearing cyclophosphamide (CYP) treated mice. NLP pretreatment enhanced *in vitro* tumor cell cytotoxicity by peripheral blood mononuclear cells (PBMC) from CYP treated mice in either normal or tumor bearing conditions. Similarly, NLP pretreatment of mice enhanced the CYP mediated *in vivo* tumor growth inhibition and survivability of the host. They further investigated the ability of NLP to protect against apoptosis of circulating blood cells induced by cisplatin and 5-fluorouracil (cis + 5-FU) in carcinoma-bearing mice and found it to be comparable to granulocyte colony stimulating factor (GCSF) (Ghosh *et al.*, 2009).

Bose *et al.* (2009) have demonstrated augmentation of the CD3-CD56+ natural killer (NK) and CD8+CD56-T-cell-mediated tumor cell cytotoxicity by neem leaf glycoprotein (NLGP). NLGP induced TCRalphabeta-associated cytotoxic T lymphocyte (CTL) reaction to kill oral cancer (KB) cells. This CTL reaction was assisted by NLGP-mediated up-regulation of CD28 on T cells and HLA-ABC, CD80/86 on monocytes. CTL-mediated killing of KB cells and NK-cell-mediated killing of K562 (erythroleukemic) cells was associated with activation of these cells by NLGP.

Nair *et al.* (2007) assessed antibacterial activity of *Azadirachta indica* against six bacterial strains--*Pseudomonas testosteroni, Staphylococcus epidermidis, Klebsiella pneumoniae, Bacillus subtilis, Proteus morganii, Micrococcus flavus. A. indica* showed strong activity against tested bacterial strains. Koul *et al.* (2009) observed that aqueous and petroleum ether extracts of *A. indica* leaf reduces the inflammation caused by *S. typhimurium* as assessed by paw flicking response. Significantly lower levels of monokines (IL-6 and TNF-alpha) were also observed in the presence of petroleum ether extract of *A. indica* leaf than aqueous extract.

Chandra *et al.* (2008) reported that oral treatment of diabetic rats with *Azadirachta indica* extract (500 mg/kg of body weight) not only lowered the blood glucose level but also inhibited the formation of lipid peroxides, reactivated the antioxidant enzymes, and restored levels of GSH and metals. The herbal extract (50-500 μg) inhibited the

generation of superoxide anions (O_2^{2-}) in both enzymatic and nonenzymatic *in vitro* systems.

Owolabi *et al.* (2008) investigated the effect of methanolic extract of neem leaves on the histology of the ovary and also on serum levels of FSH and LH in female Wistar rats. Histological sections of the experimental groups revealed no histopathological features. However, serum levels of LH were significantly reduced when compared with the control.

2.5 *Asparagus racemosus* Willd.

2.5.1 Principal constituents:

The plant contains saponins, alkaloids, proteins, starch, tannin, mucilage and disogenin. The type of saponin varies with the geographical distribution of the species. Plants found in south India have saponin-A4 fraction but not in north Indian samples (Kanitkar *et al.*, 1969b; Sankarasubramanian and Nair, 1969). The plant contains triterpene saponins – Shatavarin I – IV, which are phytoestrogen compounds. Steroid saponin, Shatavarin I is the major glycoside with 3 glucose and rhamnose moieties attached to sarsasapogenin, whereas shatavarin-IV has 2 glucose and one rhamnose moieties with sarsasapogenin. Vanillin, coniferin and sarsasaponin were also identified from roots.

2.5.2 Ethnopharmacological uses:

The tubers of *Asparagus racemosus* and *A. sarmentosus* are eaten as a sweetmeat. The fresh juice of the root is given with honey as a demulcent in bilious dyspepsia or diarrhoea. The roots have oleaginous, cooling, antispasmodic, appetizer, stomach tonic, aphrodisiac, galactogogue, astringent, antidiarrhoeal, antidysenteric and laxative properties and are useful in tumors, inflammations, diseases of blood and eye, throat complaints, tuberculosis, leprosy, epilepsy, night blindness and kidney troubles (Chopra *et al.*, 1994). *Asparagus racemosus* (Shatavari) is recommended in Ayurvedic texts for prevention and treatment of gastric ulcers, dyspepsia and as a galactogogue. *A. racemosus* has also been used successfully by some Ayurvedic practitioners for nervous disorders, inflammation, liver diseases and certain infectious diseases (Goyal *et al.*, 2003). In Ayurveda, Shatvari is considered to be the best general tonic for women.

2.5.3 Pharmacological activities:

Alcoholic extract has anti-oxytocic activity. Saponin-glycoside (A4) produced a specific and competitive block of the pitocin-induced contraction of rat, guina pig and rabbit uteri *in vitro* and *in situ*. It also blocked the spontaneous uterine motility (Joshi and Dev, 1988). Hypotensive action of syntocinin in cat was unaffected by previous administration of saponin A4 (Gaitonde and Jetmalani, 1969).

Various extracts from the roots of *A. racemosus* have been shown to cause contraction of smooth muscles of rabbit duodenum, guinea pig ileum and rat fundal strip without affecting peristaltic movement. These actions were found to be similar to that of acetylcholine and were blocked by atropine, suggesting a cholinergic mechanism of action (Jetmalani *et al.,* 1967). However, no effect was observed on isolated rectus abdominis.

Root extracts increase the weight of mammary glands in post-partum and estrogen-primed rats and uterine weight in estrogen-primed rats (Sabnis *et al.,* 1968). It also has galactogogue action in buffaloes (Patel and Kanitkar, 1969). Systemic administration of the alcoholic extract of *A. racemosus* in estrogen-primed rats showed well developed lobulo-alveolar tissue and lactation. Increase in mammary gland weight and growth of the lobulo-alveolar tissue was suggested to be due to the action of released corticoids and prolactin (Meites, 1962). However, Sharma *et al.* (1996) did not observe any increase in prolactin levels in females complaining of secondary lactational failure with *A. racemosus* suggesting that it has no lactogenic effect.

Pandey *et al.* (2005) observed prominence of the mammary glands, a dilated vaginal opening and a transversely situated uterine horn in pregnant female albino rats administered alcoholic extract of *A. racemosus* rhizome daily for 15 days (days 1-15 of gestation). However, the ovary revealed no effect of the drug. Goel *et al.* (2006) reported that methanolic extract of *A. racemosus* roots (ARM; 100 mg/kg/day for 60 days) showed teratological disorders in terms of increased resorption of fetuses, gross malformations e.g. swelling in legs and intrauterine growth retardation with a small placental size in Charles Foster rats.

Both, aerial parts and roots have amylase and lipase activities (Dange *et al.*, 1969). Aerial parts are reported to have anticancer activity in human epidermal carcinoma of the nasopharynx (Dhar *et al.*, 1968).

Rao (1981) reported that virgin female rats, normal or primed with 17-β-estradiol treatment, put on diets containing 0.25, 0.5, 1 or 2 per cent *Asparagus* root extract powder prior to their exposure to DMBA, displayed a sharp decline in mammary tumor incidence thereby indicating the inhibitory effect of *Asparagus racemosus root extract* on DMBA-induced mammary tumorigenesis.

Agrawal *et al.* (2008) observed that the hepatic tissues of Wistar rats treated with diethylnitrosamine (DEN) (200 mg/kg bw, i.p.) once a week for 2 weeks, followed by treatment with DDT (0.05 per cent in diet) for 2 weeks and kept under observation for another 18 weeks, demonstrated the development of malignancy. Pretreatment of Wistar rats with the aqueous extract of the roots of *Asparagus racemosus* prevented the incidence of hepatocarcinogenesis.

Saponins are the major constituents and suggested to be the active principles responsible for the biological activities of asparagus (Goryanu *et al.*, 1976). Shao *et al.* (1996) further reported that crude saponins were found in n-butanol fraction of methanolic extract of the shoots (edible part) of asparagus (asparagus crude saponins; ACS) and have antitumor activity. The ACS inhibited the growth of human leukemia HL-60 cells in culture and macromolecular synthesis in a dose and time dependent manner. At 75-100 µg/ml range it was cytostatic but the concentrations greater than 200 µg/ml were cytocidal to HL-60 cells. ACS at 6 and 50 µg/ml inhibited the synthesis of DNA, RNA and protein in HL,-60 cells by 84, 68 and 59 per cent, respectively and the inhibitory effect of ACS on DNA synthesis was irreversible.

Rege *et al.* (1989) observed a significant decrease in the intraperitoneal adhesions induced by caecal rubbing in animals receiving *Asparagus racemosus*. This was associated with significant increase in the activity of macrophages.

The powdered dried root of *A. racemosus* is used in Ayurveda for dyspepsia. Oral administration of powdered dried root of *A. racemosus* has been found to promote gastric

emptying in healthy volunteers. Its action is reported to be comparable with that of the synthetic dopamine antagonist metoclopromide (Dalvi *et al.*, 1990).

In Ayurveda, *A. racemosus* has also been mentioned for the treatment of ulcerative disorders of stomach and duodenal ulcers. The juice of fresh roots of *A. racemosus* has been shown to have definite curative effect in patients of duodenal ulcers (Kishore *et al.*, 1980). It has been suggested to heal the ulcers by potentiating defensive factors and many hypotheses have been put forward for its possible mechanism (Singh and Singh, 1986).

Treatment of rats with *Asparagus racemosus* (Shatavari) crude extract (100 mg/kg/day orally) for 15 days significantly reduced ulcer index when compared with control group (Bhatnagar and Sisodia, 2006). The reduction in gastric lesions was comparable to a standard antiulcer drug Ranitidine (30 mg/kg/day orally). Crude extract also significantly reduced volume of gastric secretion, free acidity and total acidity. A significant increase in total carbohydrate (TC) and TC/total protein (TP) ratio of gastric juice was also observed.

Immunomodulatory property of *A. racemosus* has been shown to protect rat and mice against experimentally induced abdominal sepsis (Dahanukar *et al.*, 1986; Thatte *et al.*, 1987). Oral administration of decoction of powdered root of *A. racemosus* has been reported to produce leucocytosis and predominant neutrophilia along with enhanced phagocytic activity of the macrophages and polymorphs. Percentage mortality of *Asparagus racemosus* treated animals was found to be significantly reduced while survival rate was comparable to that of the group treated with a combination of metronidazole and gentamicin (Dahanukar *et al.*, 1986; Thatte *et al.*, 1987). This protection offered by *Asparagus racemosus* against sepsis was not due to its antibacterial activity but due to altered function of macrophages, which indicates its possible immunomodulatory property (Dahanukar *et al.*, 1986). Dhuley (1997) has reported the revival of macrophage chemotaxis and interleukin-I (IL-I) and tumor necrosis factor-α (TNF-α) production by the oral treatment of *Asparagus racemosus* root extract in Ochratoxin-A treated mice. Alcoholic extract has been found to enhance both, humoral and cell mediated immunity of albino mice injected with sheep red blood cells as particulate antigen (Muruganadan *et al.*, 2000). Muruganadan *et al.* (2000) have also shown the alcoholic extract of root of *A. racemosus* to significantly reduce the

enhanced levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in CCl_4-induced hepatic damage in rats, indicating antihepatotoxic potential of *A. racemosus*.

Alcoholic extract of roots of *A. racemosus* was also found to have slight diuretic effect in rats and hypoglycemic effect in rabbits, but, no anti-convulsant and anti-fertility effect was observed in rats and rabbits respectively. However, it did show some anti-amoebic effect in rats (Roy *et al.*, 1971).

Thatte and Dahanukar (1988) reported the protective effects of *A. racemosus* against myelosuppression induced by single injection of Cyclophosphamide (CYP). Leukopenia produced by CYP was prevented to a varying degree, suggesting immunostimulatory activity.

Mandal *et al.*, (2000) found that different concentrations (50, 100, 150 µg/ml) of the methanol extract of the roots of *Asparagus racemosus* Willd. showed considerable *in vitro* antibacterial efficacy against *Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella flexneri, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium, Pseudomonas putida, Bacillus subtilis and Staphylococcus aureus.*

Parihar and Hemnani (2004) investigated the potential of extract of *Asparagus racemosus* (AR) against kainic acid (KA)-induced hippocampal and striatal neuronal damage. The excitotoxic lesion in brain was produced by intra-hippocampal and intra-striatal injections of kainic acid (KA; 0.25 µg in a volume of 0.5 µl) to ketamine and xylazine (200 and 2 mg/kg b.w. respectively) anesthetized mice. The results showed impairment of hippocampus and striatal regions of brain after KA injection marked by an increase in lipid peroxidation and protein carbonyl content and decline in glutathione peroxidase (GPx) activity and reduced glutathione (GSH) content. The AR extract supplemented mice displayed an improvement in GPx activity and GSH content and reduction in membranal lipid peroxidation and protein carbonyl.

Ethanolic extract of *Asparagus racemosus* Willd. was evaluated for its inhibitory potential on urolithiasis induced by oral administration of 0.75 per cent ethylene glycolated water to adult male albino Wistar rats for 28 days (Christina *et al.*, 2005). The ionic chemistry of urine was altered by ethylene glycol, which elevated the urinary

concentration of crucial ions viz., calcium, oxalate, and phosphate, thereby contributing to renal stone formation. The ethanolic extract, however, significantly reduced the elevated level of these ions in urine. Also, it elevated the urinary concentration of magnesium, which is considered as one of the inhibitors of crystallization. The high serum creatinine level observed in ethylene glycol-treated rats was also reduced following treatment with the extract.

Hannan *et al.* (2007) evaluated the effects of the ethanol extract and five partition fractions of the roots of *A. racemosus* on insulin secretion. The ethanol extract and each of the hexane, chloroform and ethyl acetate partition fractions concentration-dependently stimulated insulin secretion in isolated perfused rat pancreas, isolated rat islet cells and clonal beta-cells. The stimulatory effects of the ethanol extract, hexane, chloroform and ethyl acetate partition fractions were potentiated by glucose, 3-isobutyl-1-methyl xanthine (IBMX), tolbutamide and depolarizing concentration of KCl.

Dutta *et al.* (2007) reported racemoside-A, a water-soluble steroidal saponin purified from the fruits of *Asparagus racemosus*, to be a potent anti-leishmanial molecule effective against antimonial-sensitive (strain AG83) and -unresponsive (strain GE1F8R) Leishmania donovani promastigotes, with IC_{50} values of 1.15 and 1.31 µg/ml, respectively. Incubation of promastigotes with racemoside-A caused morphological alterations including cell shrinkage, an aflagellated ovoid shape and chromatin condensation. This compound exerted its leishmanicidal effect through the induction of programmed cell death mediated by the loss of plasma membrane integrity as detected by binding of annexin V and propidium iodide, loss of mitochondrial membrane potential culminating in cell-cycle arrest at the sub-G_0/G_1 phase, and DNA nicking shown by deoxynucleotidyltransferase-mediated dUTP end labelling (TUNEL). Racemoside-A also showed significant activity against intracellular amastigotes of AG83 and GE1F8R at a 7-8-fold lower dose, with IC_{50} values of 0.17 and 0.16 µg/ml, respectively, and was non-toxic to murine peritoneal macrophages up to a concentration of 10 µg/ml.

Antidepressant effect of methanolic extract of roots of *Asparagus racemosus* (MAR) standardized to saponins (62.2 per cent w/w) was evaluated in rats by Singh *et al.* (2009). Rats were given MAR in the doses of 100, 200 and 400 mg/kg daily for 7 days

and then subjected to forced swim test (FST) and learned helplessness test (LH). The results showed that MAR decreases immobility in FST and increases avoidance response in LH indicating antidepressant activity. In behavioral experiments, MAR increased the number of head twitches produced by 5-HT and increased clonidine-induced aggressive behavior indicating facilitatory effect on both serotonergic and adrenergic systems respectively.

Visavadiya *et al.*, (2009) evaluated antioxidant activity of the aqueous (ARA) and ethanolic extracts (ARE) of *Asparagus racemosus* (AR) roots in a series of *in vitro* assays including ROS generation in chemical and biological model systems. The ARA and ARE extracts showed dose-dependent scavenging activity against DPPH (IC_{50} = 60.7 and 52.5 µg/ml), nitric oxide (IC_{50} = 141.9 and 63.4 µg/ml), superoxide (IC_{50} = 221 and 89.4 µg/ml), hydroxyl (IC_{50} = 318.7 and 208.8 µg/ml) and ABTS+ (IC_{50} = 134.5 and 71.9 µg/ml) radicals. ARA and ARE extracts were also found to be effective at suppressing lipid peroxidation induced by Fe^{2+}/ascorbate system in rat liver mitochondrial preparation (IC_{50} = 511.7 and 309.2 µg/ml, respectively). Further, ARA and ARE root extracts significantly decreased copper-mediated human LDL oxidation by prolongation of lag phase time with decline in oxidation rate, maximal yield of conjugated dienes, lipid hydroperoxides and malondialdehyde concentrations.

Treatment of experimental mice with aqueous root extract of *A. racemosus* (100 mg/kg bw p.o.) resulted in significant increase of CD3(+) and CD4/CD8(+) percentages suggesting its effect on T cell activation. ARE treated animals showed significant up-regulation of Th1 (IL-2, IFN-γ) and Th2 (IL-4) cytokines suggesting its mixed Th1/Th2 adjuvant activity. Consistent to this, ARE also showed higher antibody titres and DTH responses. ARE, in combination with LPS, ConA or SRBC, produced a significant proliferation suggesting effect on activated lymphocytes (Gautam *et al.*, 2009).

2.6 *Ipomoea nil* L. Roth

2.6.1 Principal constituents:

The seeds contain a resin from which a resin glucoside, pharbitin has been isolated. The plant has unnamed alkaloid in leaves and flowers (Kapoor, 1990). Phytochemical studies on the seeds and flowers of this plant have resulted in the isolation of resin glycosides

(Kawasaki *et al.*, 1971; Okabe *et al.*, 1971; Ono *et al.*, 1990), gibberellins (Yokota *et al.*, 1969; Murofushi *et al.*, 1968; Yokota *et al.*, 1971), flavonoids, chlorogenic acid derivatives (Saito *et al.*, 1994a) and anthocyanins (Saito *et al.*, 1993; 1994b; 1996).

Schimming *et al.* (2005) reported the isolation of a novel spermidine alkaloid, N1,N10-ditigloylspermidine, from the seeds of *Ipomoea nil* (L.) Roth. Structural elucidation was achieved by EIMS, HRMS, 1H NMR, and 13C NMR spectroscopy.

Jung da *et al.* (2008) isolated two new oleanene-type triterpene glycosides, pharbitosides A and B, together with beta-sitosterol, beta-sitosterol glucoside (daucosterol), caffeic acid, and methyl caffeate from the seeds of *Pharbitis nil*. The structure of pharbitoside A was elucidated to be queretaroic acid 3-O-alpha-L-rhamnopyranosyl-(1-->2)-O-beta-D-glucopyranosyl-(1-->2)-beta-D-glucopyranoside. Pharbitoside B is a 21alpha-hydroxyoleanolic acid saponin carrying the same sugar moiety as that of pharbitoside A .

Kim *et al.* (2009) isolated six new ent-kaurane diterpene glycosides, pharbosides A-F, and a new ent-gibbane diterpene glycoside, pharboside G, together with three known ent-kaurane diterpenoids, 7beta,16beta,17-trihydroxy-ent-kauran-6alpha,19-olide, 6beta,7beta,16alpha,17-tetrahydroxy-ent-kauranoic acid, and 6beta,7beta,16beta,17-tetrahydroxy-ent-kauranoic acid, from an ethanolic extract of the seeds of *Pharbitis nil*.

2.6.2 Pharmacological activities:

The herb is reported to be a good purgative, anthelmintic, blood purifier, acrid, light and hot and useful in vitiated conditions of *kapha* and *vata*. Tribal people in Brazil use it as laxative (Albuquerque *et al.*, 2007). Seed is used as a purgative in cases of gastric disorders, flatulence, oedema, fever, headache and worms. It is also beneficial in rheumatism and paralytic affections. Seed paste is a good application to cure skin diseases (Kapoor, 1990). Seeds of this plant are reported to show antitumor and antifungal activities (Saito *et al.*, 1996; Koo *et al.*, 1998).

Matsui *et al.* (2001a,b) examined the alpha-glucosidase (AGH) inhibitory effect of natural anthocyanin extracts. They used a free AGH assay system and found that *Pharbitis nil* extract showed the strongest maltase inhibitory activity, with an IC_{50} value

of 0.35 mg/ml, as great as that of *Ipomoea batatas* extract (IC_{50}=0.36 mg/ml). However, the extract did not inhibit sucrase activity at all.

Ko *et al.* (2004) analyzed the effects of 80 per cent ethanol extract of roots of *Pharbitis nil* on proliferation and on expression of cell growth/apoptosis related molecules, using an AGS gastric cancer cell line. The treatment of *Pharbitis nil* dramatically reduced cell viability in a dose and time-dependent manner. FACS analysis and Annexin V staining assay also showed that *Pharbitis nil* induces apoptotic cell death of AGS. *Pharbitis nil* increased expression of apoptosis related Bax and cleavage of active caspase-3 protein. They also confirmed the translocation of Bax to mitochondria.

Kim *et al.* (2009) evaluated the compounds isolated from seeds for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) using the SRB assay *in vitro*. Compounds Pharboside D and Pharboside E exhibited moderate cytotoxic activity against A549, SK-OV-3, SKMEL-2, and HCT15 cells (IC_{50} (Pharboside D): 25.15, 28.73, 14.29, and 17.23 μM, and IC_{50} (Pharboside E): 23.60, 13.77, 15.22, and 22.12 μM, respectively).

2.7 *Costus speciosus* Koen ex. Retz.

2.7.1 Principal constituents:

The rhizomes of *Costus speciosus* are well known for their diosgenin content and also for several saponins (Dasgupta and Pandey, 1970; Tschesche and Pandey, 1978). Singh *et al.* (1980) reported the seeds of this plant as an additional source of diosgenin. Rhizomes, roots, stems and leaves contain Diosgenin 396.0, 70.0, 83.0 and 70.0 µg/g, respectively. Diosgenin and sitosterol are isolated from seeds, stem and roots. Singh and Thakur (1982) isolated ten steroidal saponins from the seeds of *Costus speciosus* and elucidated their structures as β-sitosterol-β-D-glucopyranoside, prosapogenin-B of dioscin, prosapogenin-A of dioscin, dioscin, gracillin, 3-O-[α-L-rhamnopyranosyl (1-2)-β-D-glucopyranosyl], 26-O-[β-D-gluconopyranosyl]-22α-methoxy-(25R)-furost-5-en-3β,26-diol,methyl protodioscin and protodioscin. Recently, Eliza *et al.* (2009a) isolated Costunolide from the hexane extract of *C. speciosus* roots following bioassay guided fractionation. The structure was elucidated using X-ray crystallography. Very recently,

Eliza *et al.* (2009b) isolated Eremanthin from *C. speciosus* roots. The structure was identified using gas chromatography–mass spectrometry (GC–MS) analysis.

2.7.2 Ethnopharmacological uses:

In ayurveda, the rhizomes are ascribed to be bitter, astringent, acrid, cooling, aphrodisiac, purgative, antihelminthic, depurative, febrifuge, expectorant and tonic (Mandal *et al.*, 2007). Rhizome paste is applied externally on tumors and the decoction of powdered rhizome is given internally in constipation and stomachache (Mali and Bhadane, 2008). Root is bitter, astringent, stimulant, digestive, anthelmintic, depurative and aphrodisiac (Kirtikar and Basu, 1934; Nadkarni, 1954). Root is useful in catarrhal fevers, coughs, dyspepsia, worms, skin diseases and snake-bites. Tuber is cooked and made into a syrup or preserve which is very wholesome. The Korku tribe of Amravati district of Maharashtra, India, takes half-cup fresh juice of rhizomes twice with a gap of 3 h in a day to cure stomachache. They also apply rhizome paste of tuber externally to cure tonsils (Jagtap *et al.*, 2006). It is also useful in curing ailments like burning sensation, constipation, fever, inflammation, worm infection and diseases like leprosy, asthma, bronchitis and anemia. The tribes inhabiting in Bay Islands generally consume cooked rhizomes. The roots are used as tonic and are antihelminthic. The sap from the crushed stem is used to treat diarrhoea and eye infections. An infusion or a decoction from leaves is utilized as subdorific or in bath for relieving patients from high fever. The juice of fresh rhizome is considered to be a purgative. It is also applied on the head to have a cooling effect and to get relief from headache and earpain. Rhizomes are eaten with sugar for deworming purpose (Mandal *et al.*, 2007).

2.7.3 Pharmacological activities:

Total alkaloids (comprising of four alkaloids, Rf. 0.80, 0.52, 0.24 and 0.09), isolated from the rhizomes of *Costus speciosus* (Koen) Sm. possess anticholinesterase activity (Bhaitacharya *et al.*, 1972).

Vijayalakshmi and Sarada (2008) investigated different parts of *Costus speciosus* for their polyphenol content and antioxidant activity. Polyphenol content of methanolic extracts was high in root and peel of the stem of *C. speciosus* when compared to leaves.

The methanolic extracts showed more hydroxyl radical scavenging activity and free radical quenching ability.

Antihyperglycemic, antihyperlipemic and antioxidant potency of an ethanol extract of *Costus speciosus* root in alloxan-induced diabetic male (Charles Foster) rats was studied by Bavarva and Narasimhacharya (2008). Administration of 150 mg/kg BW dose neither improved glucose nor lipid metabolism and antioxidant levels. Administration of 300 and 450 mg/kg BW doses, however, resulted in a reversal of diabetes and its complications. Both doses significantly brought down blood glucose concentration (26.76 and 34.68 per cent, respectively), increased glycogenesis and decreased glyconeogenesis bringing the glucose metabolism towards normalcy. These doses also reversed the hyperlipidemia by reducing plasma total lipid (12.87 and 178.24 per cent), cholesterol (21.92 and 30.77 per cent) and triglyceride (25.32 and 33.99 per cent) and improved hepatic antioxidant enzyme activities. The high dose (450 mg/kg BW) was found to have more potential antioxidant activities compared with glibenclamide.

Eliza *et al.* (2009a) administered Eremanthin, isolated from *Costus speciosus* roots to streptozotocin (STZ) (50 mg/kg bw) induced diabetic male Wistar rats at different doses (5, 10 and 20 mg/kg BW) for 60 days. They found that the plasma glucose level was significantly reduced in a dose dependent manner when compared with the control. In addition, oral administration of eremanthin (20 mg/kg BW) significantly decreased glycosylated hemoglobin (HbA1c), serum total cholesterol, triglyceride, LDL-cholesterol and at the same time markedly increased plasma insulin, tissue glycogen, HDL-cholesterol and serum protein. Eremanthin also restored the altered plasma enzyme (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase and acid phosphatase) levels to near normal.

Very recently, Eliza *et al.* (2009b) administered Costunolide isolated from the hexane extract of *C. speciosus* root to streptozotocin (50 mg/kg BW)-induced diabetic male Wistar rats at different doses (5, 10 and 20 mg/kg BW) for 30 days to assess its effect on fasting plasma glucose and cholesterol levels. Plasma glucose was significantly reduced in a dose-dependent manner. In addition, oral administration of costunolide (20 mg/kg BW) significantly decreased glycosylated hemoglobin (HbA1c), serum total cholesterol, triglyceride, LDL cholesterol and at the same time markedly increased plasma insulin, tissue glycogen, HDL cholesterol and serum protein. Also, costunolide restored the

altered plasma enzyme (aspartate aminotransferase, alanine aminotrasferase, lactate dehydrogenase, alkaline phosphatase and acid phosphatase) levels to near normal. They postulate that the costunolide might have stimulated beta islets to secrete insulin by inhibiting the expression of nitric oxide synthase.

Keller *et al.*, (2009) investigated *Insulina* (*Costus spicatus*) which is in popular use by Dominicans in the Dominican Republic and the United States for the treatment of diabetes. They tested the ability of a tea made from the leaves of *Costus spicatus* to alter glucose homeostasis in C57BLKS/J (KS) *db/db* mice, a model of obesity-induced hyperglycemia with progressive beta cell depletion. Their data however suggest that at the dose given, tea made from *Costus spicatus* leaves had no efficacy in the treatment of obesity-induced hyperglycemia.

2.8 *Butea monosperma* (Lam.) Taub.

2.8.1 Principal constituents:

The main constituent of the flower is butrin (1.5 per cent) besides butein (0.37 per cent) and butin (0.04 per cent). Flowers also contain flavonoids and steroids (Murti and Seshadri, 1940; Rao and Seshadri, 1941). Later studies prove that isobutrin slowly changes to butrin on drying (Puri and Seshadri, 1953). Other than these, coreopsin, isocoreopsin, sulphurein (glycoside) and other two monospermoside and isomonospermoside structures are also identified in flowers (Gupta *et al.*, 1970). The bright colour of the flower is attributed to the presence of chakones and aurones. Several nitrogenous constituents have also been reported which include palasonin (Raj and Karup, 1967), monospermin (Mehta and Bokadia, 1981), allophonic acid derivatives (Porwal *et al.*, 1988) and palasimide (Guha *et al.*, 1990).

Seeds have been reported to contain α–amyrin, β-sitosterol, β-sitosterol glucoside and hexeicosanoic acid δ-lactone (Bishnoi and Gupta, 1979). Palasonin, isolated from seeds showed anthelmintic activity (Raj and Karup, 1968). From resin fraction fraction of seed-lac, isolation of four acid esters designated as jalaric ester I, jalaric ester II, laccijalaric ester I and laccijalaric ester II has been reported (Singh *et al.*, 1974). From the stem, isolation of two new compounds 3α-hydroxyyeuph-25-ene and 2,14-dihydroxy-11,12-dimethyl-8-oxo-octadec-11-enylcyclohexane has been reported (Mishra

et al., 2000). Roots contain glucose, glycine, glucosides and aromatic compounds (Tandon *et al.*, 1969). Tetramers of leucocynidin are isolated from gum and stem bark (Seshadri and Trikha, 1971). Seed contains oil.

Shah *et al.* (1990) reported the presence of at least seven flavones and flavonoid constituents including butrin and isobutrin and also four free amino acids in the dried flowers of *Butea frondosa* Roxb.

2.8.2 Ethnopharmacological uses:

The plant is known for number of activities in Indian System of Medicine. The fresh juice is applied to ulcers and for congested and septic sore throats. The gum is a powerful astringent given internally for diarrhoea and dysentery, phthisis and hemorrhage from stomach and the bladder, in leucorrhoa, ringworm and as a substitute for gum Kino. The bark is reported to possess astringent, bitter, pungent, alliterative, aphrodisiac and anthelmintic properties. It is useful in tumors, bleeding piles and ulcers. The decoction is useful in cold, cough, fever and menstrual disorders.

Roots are useful in elephantiasis and in curing night blindness and other eyesight defects. Roots also cause temporary sterility in women. Root extract is applied in sprue, piles, ulcers, tumors and dropsy. Leaves have astringent, tonic, diuretic and aphrodisiac properties. They are also used to cure boils, pimples, tumors, hemorrhoids and piles. Flowers are reported to possess astringent, diuretic, depurative, aphrodisiac and tonic properties. They are used as emmenagogue and to reduce swellings. Flowers are also reported effective in leprosy, leucorrhoea and gout (Chopra *et al.*, 1956; Wealth of India, 1988).

2.8.3 Pharmacological activities:

A fraction containing sodium salt of phenolic constituent isolated from the bark has shown potential as an anti-asthmatic agent and estrogenic activity in mice. Savitri and Samaranayake (1989) reported antifungal constituents from petroleum and ethyl acetate extracts of stem bark. Extract exhibited significant antifungal activity against *C.*

cladosporiodes. Ratnayake *et al.* (1989) further isolated (-)-Medicarpin from stem bark of *Butea monosperma* and identified it as active principle for antifungal activity.
Sharma *et al.* (2005) reported anti-diarrhoeal potential of ethanolic extract of stem bark of *Butea monosperma* in castor oil induced diarrhoea model and PGE_2-induced enteropooling in rats. Extract also reduced gastrointestinal motility after charcoal meal administration.

The effect of alcoholic bark extract on cutaneous wound healing in rats was investigated by Suguna *et al.* (2005). Excision wounds were made on the back of rat and extract was applied topically. The granulation tissue formed on days 4, 8, 12 and 16 (post-wound) was used to estimate total collagen hexosaamine, protein, DNA and uronic acid. Further epithelialization and wound contraction was confirmed by histopathalogical examination.

Hot alcoholic extract of the seeds showed significant anti-implantation and anti-ovulatory activities in rats and rabbits respectively. It also showed abortive effect in mice (Khanna and Choudhury, 1968; Garg *et al.*, 1978; Kamboj and Dhawan, 1982). Aqueous extract of the flowers also shows significant anti-implantation activity (Laumas and Uniyal, 1966; Khanna and Chaudhury, 1968). Purified alcoholic extract at lower dose level and ethereal and water extracts at higher dose level have been found to exhibit significant anti-estrogenic activity in immature mice, while ethyl acetate extract containing butrin and isobutrin exhibited poor activity. Significant inhibition of uterus weight gain, vaginal epithelium cornification and characteristic histological changes have been observed (Shah *et al.*, 1990).

Isobutrin and butrin have been identified as anti-hepatotoxic principles from flowers of *Butea monosperma* (Wagner *et al.*, 1986). Activity was monitored by means of CCl_4 and GaIN-induced liver lesion *in vitro*. The anti-hepatotoxic principles isolated consisted of two known flavonoids, isobutrin (3, 4, 2', 4'-tetrahydroxychalcone-3, 4'-diglucoside), and the less active butrin (7, 3', 4'-trihydroxyflavanone-7, 3'-diglucoside). These compounds from flowers (butrin and isobutrin) have also been reported to possess hepatoprotective activity by Sehrawat *et al.* (2006). Hepatoprotective action of the extract containing these compounds has been studied against thioacetamide induced hepatotoxicity. Further, authors studied DOC and H3 thymidine incorporation and demonstrated its inhibition suggesting that the extract may inhibit tumor formation.

Mishra *et al.* (2000) evaluated free radical scavenging activity of various extracts of flowers by using different *in vitro* models like reducing power assay, scavenging of 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide radical, super oxide anion radical, hydroxyl radical and inhibition of erythrocytes hemolysis using 2,2' azo-bis (amidinopropane) dihydrochloride (AAPH). Methanolic extract along with its ethyl acetate and butanol fractions showed potent free radical scavenging activity. They attributed the observed activity to higher phenolic contents in the extracts.

A triterpene present in the n-hexane-ethyl acetate (1:1) fraction of the petroleum ether extract of *Butea monosperma* flowers has been reported as active principle for anticonvulsive activity in laboratory animals (Kasture *et al.,* 2002). The triterpene exhibited anticonvulsant activity against seizures induced by Maximum Electro Shock (MES). It also inhibited seizures induced by pentylene tetrazole, electrical kindling and the combination of lithium sulphate and pilocarpine nitrate.

Bhatwadekar *et al.* (1999) reported *Butea monosperma* flowers to possess anti-stress activity which they attributed to the decrease in brain 5-HT and suppression of hypothalamo-hypophyseal-adrenocortical axis.

The seed oil of *Butea monosperma* has been reported to possess bactericidal and fungicidal activities (Mehta *et al.,* 1983). The methanol extract of *Butea monosperma* seeds, tested *in vitro*, showed significant anthelmintic activity (Prashant *et al.,* 2001). An Ayurvedic rasayana (herbal medicine) containing *Butea monosperma* as one of the constituent has been reported for the management of giardiasis perhaps by immunomodulation as the rasayana had no killing effect on the parasite *in vitro* (Agarwal *et al.,* 1994).

Wongkham *et al.* (1996) studied the oligosaccharide specificity of *Butea monosperma* agglutinin and its characteristics were shown to be useful in identifying malignancy-associated alteration of AFP sugar chains.

2.9 Programmed cell death (Apoptosis)

An exciting and very promising new area of anticancer drug development is the identification of molecular targeted therapies. Evidence has emerged from various studies that suggests that the plant products useful in the treatment of cancer comprise a diverse group of compounds with different mechanisms of action, but their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of action. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both, normal and cancer cells is significantly affected by the rate of apoptosis. Thus, modulating apoptosis may be useful in the management and treatment or prevention of cancer. Certain plant products are known to induce apoptosis in neoplastic cells but not in normal cells (Chiao *et al.,* 1995; Kaufman and Earnshaw, 2000). It has become increasingly evident that apoptosis is an important mode of action for many anti-tumor agents, including ionizing radiation, alkylating agents such as cisplatin and 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), topoisomerase inhibitors e.g., etoposide, cytokines e.g., tumour necrosis factor (TNF) (Shih and Stutman, 1996), taxol (Gibb *et al.,* 1997), and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (Pero, 1997). Induction of apoptosis is a new target for innovative mechanism-based drug discovery (Workman, 1996). It is thus considered important to screen apoptosis inducers from plants, either in the form of crude extracts or bioactive components isolated from extracts for their anticancer activity.

Apoptosis, which is a major way of programmed cell death, plays an important role in the regulation of tissue development and homeostasis in eukaryotes (Hengartner, 2000). A balance between cell death and cell proliferation is required to maintain homeostasis. Disruption of this balance leads to many diseases including cancer (Thompson, 1995). It has been demonstrated that apoptosis helps to establish a natural balance between cell death and cell renewal in mature animals by destroying excess, damaged, or abnormal cells (Okada *et al.,* 2004). Apoptosis requires stringent regulation and any deviation in such control can have pathological implications.

Kerr *et al.,* (1972) described first time a little known and curious form of cell death known as *apoptosis,* which today is one of the most intensively studied topics in modern

biology. Apoptosis represents a universal and exquisitely efficient cellular suicide pathway. It is a type of cell death that is clean and quick and involves a predictable sequence of structural changes that cause a cell to shrink and can be rapidly digested by the neighbouring cells (McConkey, 1998). Dramatic changes occur within the nucleus of cells undergoing apoptotic death. It is commonly thought that the nuclear changes are due to activation of endogenous nuclease(s) which cleaves DNA into oligonucleosomal fragments. Apoptosis can be measured by number of parameters such as Annexin V, Cell cycle, DNA laddering, Mitichondrial membrane potential, etc.

Morphological characteristics of apoptosis include plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation and formation of apoptotic bodies (Wyllie *et al.*, 1980; Wyllie *et al.*, 1997). Mitochondria are currently regarded to play a central role in mediating 'intrinsic death signals' and thus could serve as a novel target for chemotherapy (Grad *et al.*, 2001). Cytochrome C, a mitochondrial protein can activate caspases but, the release of cytochrome C is regulated by the anti- and pro-apoptotic members of the Bcl-2 family. Once cytochrome C is in the cytoplasm, it binds Apaf-1 to pro-caspase-9, leading to the activation of caspase-9 and to the initiation of caspase cascade. Then caspase-9 cleaves and activates caspase-3, the executioner caspase, which cleaves poly (ADP-ribose) polymerase (PARP) and activates caspase activated DNase to fragment DNA (Green and Reed, 1998).

The mechanism of apoptosis is highly complex and sophisticated. Multiple death promoting molecules, amplifying cascade effects and positive feedback loops all ensure that the process, once initiated, is quick, fatal and irreversible. Cells undergoing apoptosis usually exhibit a characteristic morphology including fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of the DNA into small oligonucleosomal fragments (Steller, 1995). The cells or fragments are then phagocytosed by macrophages. Recent studies have documented two major pathways involved in the regulation of apoptosis (Kaufmann and Earnshaw, 2000). One pathway is mediated via cell surface death receptors, such as Fas/CD95 and TNFR1, which upon activation recruit cytoplasmic tails of the receptors and down stream associated signaling complex leading to the activation of caspase-8. The second pathway is mitochondrial-dependent, which is regulated by signaling cascade-involving members of Bcl-2 family. A loss of mitochondrial membrane potential

brings about translocation of pro-apoptotic Bax to mitochondria and cytochrome C from mitochondria to cytosol resulting in caspase-9 activation.

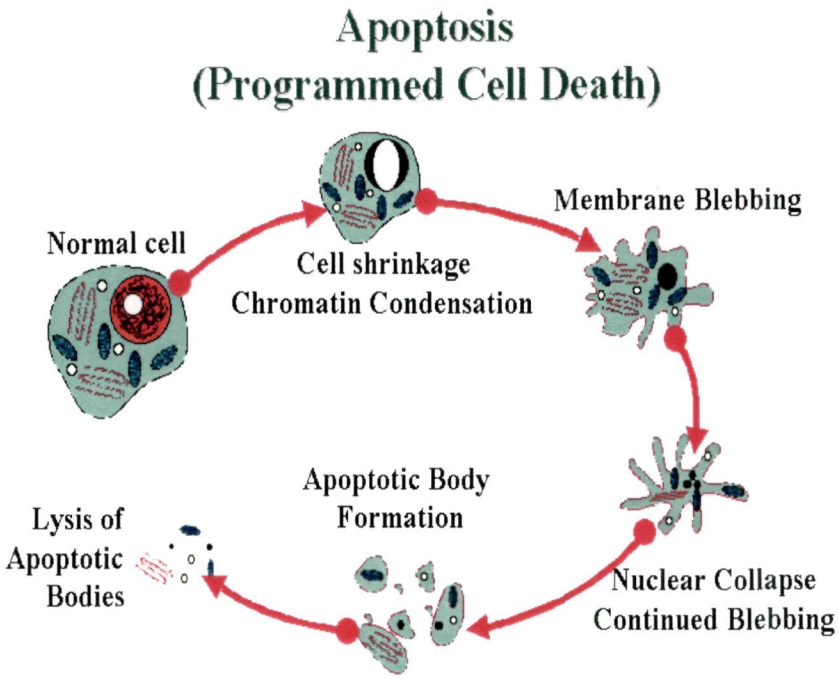


Fig 2.1: Stages of apotosis

2.10 Toxicity: Myelosuppression

In the treatment of cancer, dose escalation of chemotherapy and/or radiation usually results in better tumor control but with an increase in unacceptable adverse effects. The full benefit of dose escalation for cancer treatment with chemotherapy or radiation is not realized due to dose limiting toxicity to normal tissues (Frei and Canellos, 1980; Hryniuk and Levine, 1986; Crown, 1997; Savarese *et al.*, 1997; Honkoop *et al.*, 1998). Treatment regimens involving dose escalation have a devastating effect on bone marrow (BM) cells. Myelosuppression is the most common dose-limiting toxicity of anti-tumor agents as they inhibit stem and progenitor cell proliferation. Progenitor cells have shorter duplication times than stem cells and are more sensitive to chemotherapy-induced damage. Since neutrophil leukocytes have the shortest half-life in circulation, their continuous renewal is essential to prevent the sequel of chemotherapeutic agent-induced neutropenia, infection and death (Benko *et al.*, 2003).

Oredipe *et al.* (2003) reported the number of bone marrow cells in the femurs and tibia of normal C57BL/6, BALB/c, C3H-HEN and DBA-2 strains of mice to be 1.84 ± 0.07 $x10^7$, 1.93 ± 0.08 $x10^7$, 1.87 ± 0.05 $x10^7$ and 1.94 ± 0.07 $x10^7$, respectively. Benko *et al.* (2003) reported 41 per cent reduction in bone marrow cellularity as compared to vehicle treated BDF1 female mice following a single intraperitoneal administration of 5-Fluorouracil (100 mg/kg).

Pawar *et al.* (2006) observed the number of cells per femur to be $5x10^6$ in group treated with Cyclophosphamide (25 mg/kg body wt) for 10 days in swiss albino mice as against $14.4x10^6$ in vehicle treated control group on day 15. Kumar and Kuttan (2005) treated adult BALB/c mice with Cyclophosphamide at a dose of 25 mg/kg b.wt (i.p.) daily for 14 days and recorded femoral bone marrow cellularity on days 3, 9, 12 and 30. On day 3, the bone marrow cellularity was found to be 6.2 $x10^6$ in treated group as against 15.8 $x10^6$ in normal control animals. On day 30, the values recorded for the treated and control groups were $8.8x10^6$ and $16.2x10^6$, respectively. The similar dose of Cyclophosphamide has been earlier shown to produce severe myelosuppression in mice (Praveenkumar *et al.*, 1995).

Valadares *et al.* (2006) investigated the effect of Ehrlich ascites tumor on the femoral bone marrow cellularity in male BALB/c mice. Mice were inoculated intraperitoneally (i.p.) on day 0 with 6×10^6 viable tumor cells per mouse in a volume of 0.1 ml. They reported femoral marrow cell counts as 11.0 ± 1.1 $x10^6$ and 9.0 ± 1.5 $x10^6$, for control and tumor bearing animals respectively, on day 5. Earlier, they had found femoral marrow cell counts to be 15.4 ± 1.5 $x10^6$ and 10.4 ± 1.2 $x10^6$ for control and tumor bearing animals on day 5 after intraperitoneal inoculation of 6×10^6 viable Ehrlich tumor cells per mouse (Valadares and Queiroz, 2002).

Ghosh *et al.* (1999) studied the effect of Ehrlich ascites carcinoma (EAC), Sarcoma-180 (S-180) (ascites) and Dalton's ascitic lymphoma (DAL) on femoral bone marrow cellularity in male Swiss albino mice after i.p. inoculation with 1.0×10^6 respective viable cells/mouse at an injection volume of 0.2 ml. On day 9 after inoculation, the femoral marrow cell counts were found to be 12 $x10^6$ per µl in normal control animals. In EAC, S-180 and DAL bearing animals the femoral marrow cell counts were 0.36 ± 0.75 $x10^6$, 0.60 ± 0.85 $x10^6$ and 0.12 ± 1.05 $x10^6$ per µl, respectively. Overall, in tumor bearing

animals, a decrease (20–25 per cent) in marrow cellularity was observed on day 9 ($P<0.01$) which further decreased to 30–40 per cent on day 21.

Page *et al.* (2003) reported that the treatment of C57BL/6 mice with 50 mg/kg of DMBA for 48 h resulted in approximately 50 per cent decrease in the total number of bone marrow cells. They further investigated this effect in knock-out mice deficient in p55, p75, or both (p55/75) isoforms of the TNF-α receptor. Wild-type (wt), TNFR p55 null, p75 null, and p55/75 null mice were injected (i.p.) with 50 mg/kg of DMBA (or vehicle as a control) and euthanized 48 h later. The bone marrow cells were isolated and counted. Significant decrease in the total number of bone marrow cells in the wt without any significant decrease in the bone marrow cellularity of the TNFR p55, p75, or p55/75 null mice was observed. A slight decrease in the TNFR p75 null mice observed was not found to be statistically significant (Page *et al.*, 2004).

Chapter III

Materials and Methods

CHAPTER - III

MATERIALS AND MEHODS

3.1 Plants selected for the study

Azadirachta indica (leaves), *Asparagus racemosus* (roots), *Ipomoea nil* (aerial parts), *Costus speciosus* (aerial parts) and *Butea monosperma* (flowers) were selected for studying their anticancer activity. These plants are briefly described below.

3.1.1 *Azadirachta indica* A. Juss

3.1.1.1 Taxonomical classification:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Sapindales
Family: Balsaminaceae
Genus: Azadirachta
Species: *Azadirachta indica*

3.1.1.2 Nomenclature:

Common Names: Indian-lilac, margosa, margosier, neem, nim tree.
Synonyms(s): Antelaea azadirachta (L.) Adelb., Antelaea javanica Gaertn., Melia azadirachta L., Melia indica (A. Juss.) Brandis
Sanskrit: Nimba, Nimbac, Nimbak.

Bengali:	Bim, Nim, Nimgach.
Hindi:	Balnimb, Neem, Nim, Nind.
Chinese:	Lian shu, Ku lian, Lian zao zi, Yin du lian shu (Taiwan).
English:	Bead tree, Burmese neem tree, Chinaberry, Indian cedar, Indian-lilac, Margosa, Margosa tree, Neem, Neem tree, Nimtree.

3.1.1.3 Habitat:

It is commonly found throughout the greater part of India. Though not a forest-tree, it generally grows wild.

3.1.1.4 Morphology:

It is a large evergreen tree with long, spreading branches forming a broad crown. The bark is grey and rough, the leaves are alternate, the leaflets 8-19, glossy and bluntly serrate. The flowers are white or pale-yellow, small, scented, numerous and found in long, axillary panicles. The drupes are yellow on ripening, aromatic, oblong and smooth with a single exalbuminous seed.

3.1.2 *Asparagus racemosus* Willd.

3.1.2.1 Taxonomical classification:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Order:	Asparagales
Family:	Asparagaceae
Genus:	Asparagus
Species:	*Asparagus racemosus*

3.1.2.2 Nomenclature:

Common Names:	Sahansarmuli, Sahanspal, Satavar, Satawari, Satmuli, Shataavar, Shatamuli, Shatavar, Shatavari, shatavir, Shatavri, Sootmooli
Sanskrit:	Challagadda, hiranyasringi, Satavari, Shataavarii, Shatavari
Hindi:	Sahansarmuli, Sahanspal, Satavar, satawari, Satmuli,

	Shataavar, Shatamuli, Shatavar, Shatavari, shatavir, Shatavri, Sootmooli
Chinese:	Chang Ci Tian Men Dong
English:	Indian Asparagus, Sataver White, Sataver Yellow, Wild Asparagus

3.1.2.3 Habitat:

It is found throughout tropical Africa, Java, Australia, India, Sri Lanka and southern parts of China. In India, it is found in plains to 4000 ft high, in tropical, sub-tropical dry and deciduous forests and in the Himalayas.

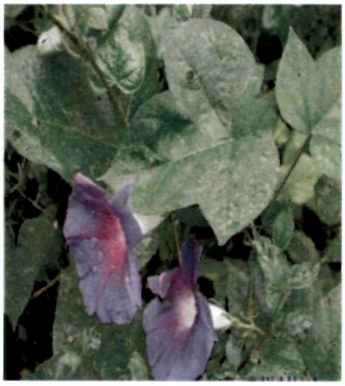
3.1.2.4 Morphology:

It is an under-shrub, climbs up to 1-3 m high, with stout and creeping root stock. The root occurs in clusters or fascicle at the base of the stem with succulent and tuberous rootlets. The stem is scandent, woody, triquetrous, striate, terete and climbing. The young stem is delicate, brittle and smooth. The spines are long, sub-recurved or straight. Cladodes are in tufts of 2-6 in a node, slender, finely acuminate, falcate divaricate. The flowers are solitary or fascicles, simple or branched racemes of 3 cm long. The pedicel is slender and jointed in the middle. Perianth lobes are white, fragrant and 3 mm in length. The anthers are minute and purple. The berry is globular or obscurely 3 lobbed, purple-reddish and the seeds are hard with brittle testa.

3.1.3 *Ipomoea nil* L. Roth

3.1.3.1 Taxonomical classification:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Solanales
Family:	Convolvulaceae
Genus:	Ipomoea
Species:	*Ipomoea nil*



3.1.3.2 Nomenclature:

Common Names:	campainha, corda-de-viola, corriola, Japanese morning-glory, qian niu, white- edge morning-glory
Synonym(s):	Convolvulus hederaceus L., Convolvulus nil L., Ipomoea hederacea auct. non Jacq., Pharbitis nil (L.) Choisy
Sanskrit:	Kalanjani, Krishnabijah
Hindi:	Kalmua, Kalmi, Kalmisag, Patuasag.
Chinese:	Weng cai (Cantonese Ngung choi, Ung tsoi), Kong xin cai (Cantonese Hung sam choi, Ong tung tsoi), Tong cai, Ong choi, Ong choy, Ohng choy.
English:	Chinese water spinach, Water convolvulus, Water spinach, Swamp cabbage, Swamp morning glory, Tropical spinach.

3.1.3.3 Habitat:

It is native of America. It was introduced in India by Portuguese by about 1500 A.D. This is now naturalized in all parts of India. The species is polymorphic.

3.1.3.4 Morphology:

It is a large, glabrous twinning shrub. Leaves are simple, alternate, ovate-cordate, acuminate, entire or 3-lobed, 11 x 9 cm, adpressed hairy above and puberulous below. Flowers are large, showy, solitary or in axillary flowered cymes. Calyx has 5 lobes. Corolla is funnel-shaped with 5 cm long tube, white, limb purple. There are 5 unequal stamens attached to the base of the corolla tube. Ovary is conical, filiform style and capitate stigma. Seeds are black and glabrous.

3.1.4 *Costus speciosus* Koen ex. Retz.

3.1.4.1 Taxonomical classification:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Liliopsida
Order:	Zingiberales
Family:	Costaceae
Genus:	Costus
Species:	*Costus speciosus*

3.1.4.2 Nomenclature:

Common Names:	Cane-reed, crepe-ginger, wild ginger
Synonym(s):	Banksea speciosa
Sanskrit:	Kemuka, Kustha
Hindi:	Keu, Keukand
Chinese:	bi qiao jiang
English:	Cane-Reed, Canereed, Crepe Ginger, Crepe-Ginger, Spiral Ginger, Wild Ginger

3.1.4.3 Habitat:

It is native of southeast Asia, especially the Indonesia. It is cultivated in India for its medicinal uses and elsewhere as an ornamental plant.

3.1.4.4 Morphology:

Costus speciosus Koen ex. Retz. is a succulent perennial herb, growing up to 2.7 m high and having an erect stem. The plant possesses horizontal rhizomatous rootstock. It has leafy stem, 6-9 ft in height. Leaves are 1/2 - 1 ft. or more, oblong, acute and thinly silky beneath. Spik is very dense flowered, 2-4 in., bracts ovate, bright red, 1-1 1/2 in. Calyx is 1 inch, with 3 segments, ovate and cuspidate. Corolla is white, oblong, 1-1 1/2 inch, lip white, sub-orbicular, the margins incurved and meeting. Filament is 1/2-2 inch, crowned with the persistent calyx. Fruit is an ellipsoidal, many-seeded capsule (Whistler, 2000). It generally grows luxuriantly on clayey loamy soil near inland forest under moderate shade. The plant propagates vegetatively through rhizomes or via seeds dispersed by birds. Rhizome is the plant part used as medicine.

3.1.5 *Butea monosperma* (Lam.) Taub.

3.1.5.1 Taxonomical classification:

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Fabales
Family:	Fabaceae
Genus:	Butea
Species:	*Butea monosperma*

3.1.5.2 Nomenclature:

Common Names:	arbre à laque, bastard-teak, Bengal kino, dhak, flame-of-the-forest, Kinobaum, Lackbaum, palas, palash
Synonym(s):	Butea frondosa, Erythrina monosperma
Sanskrit:	Palas
Hindi:	Dhak, Palas, Palash
German:	Kinobaum, Lackbaum
English:	Bastard Teak, Bastard-Teak, Bengal Kino, Dhak, Flame of the Forest, Flame-Of-The-Forest, Nourouc, Palas, Parrot Tree

3.1.5.3 Habitat:

It is found in greater parts of India, Burma and Sri Lanka. It is capable of growing in water logged situations, black cotton soils, saline, alkaline, swampy badly drained soils and on barren lands except in arid regions.

3.1.5.4 Morphology:

It is an erect, medium sized tree of 12-15m height, with a crooked trunk and irregular branches. The shoots are clothed with gray or brown silky pubescence. The bark is ash coloured. The leaves are 3 foliate, large and stipulate. Petiole is 10-15 cm long. Leaflets are obtuse, glabrous above, finely silky and conspicuously reticulately veined beneath with cunnate or deltoid base. From January to March the plant is bald. Flowers are rigid racemes of 15 cm long, densely brown velvety on bare branches. Calyx is dark, olive green to brown in colour and densely velvety outside. The corolla is long and bright orange red with silky silvery hairs outside. Stamens are diadelphes and anthers uniform. Ovary is composed of 2 ovules, style is filiform, curved and stigma is capitate. Pods are argenteo-canesent, narrowed, thickened at the sutures, splitting round the single apical seed. The seeds are flat, reniform and curved (Jhade *et al.*, 2009).

3.2 Collection and authentication of plant materials

The leaves of *Azadirachta indica,* roots of *Asparagus racemosus,* flowers of *Butea monosperma* and aerial parts *of Ipomoea nil* and *Costus speciosus* were collected from Jammu and its surrounding areas after authentication by the taxonomist at the site of collection.

Fig. 3.1: *Azadirachta indica* (plant, leaves, fruits and flowers)

Fig. 3.2: *Asparagus racemosus* (aerial parts and roots)

Fig. 3.3: *Ipomoea nil* (aerial parts)

Fig. 3.4: (A)-*Costus speciosus* plants; (B)-Closer view of *Costus speciosus* flower

Fig. 3.5: *Butea monosperma* flowers (A); *Butea monosperma* leaves (B)


A


B


A


B

3.3 Preparation of extracts from the plant materials

Freshly collected plant parts were chopped and dried under shade. Each of the dried coarse plant material was divided into three parts for the preparation of three extracts as described below.

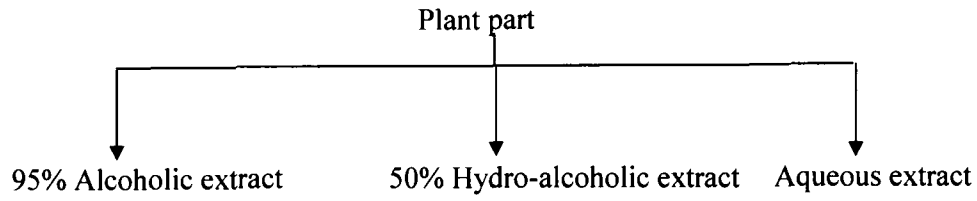


Fig. 3.6: Flow chart for the preparation of extracts from the plant parts

3.3.1 Preparation of 95 per cent alcoholic extract

Dried and chopped plant material was placed in a conical glass percolator and kept submerged with 95 per cent ethanol for 16 h. The percolator was occasionally shaken during extraction. The solvent was removed and stored. The process was repeated four times and all the four extracts were pooled together. The pooled extracts were evaporated to dryness under reduced pressure at 50°C. The final drying was done initially in vacuum desiccators and finally in lyophilizer. The dried extracts were scrapped off and transferred to glass containers. Nitrogen was blown in the container before capping. The extracts were stored in refrigerator under desiccation.

3.3.2 Preparation of 50 per cent hydro-alcoholic extract

Another lot of shade-dried and chopped plant parts was placed in conical glass percolators and kept submerged in 50 per cent ethanol for 16 h. The percolator was occasionally shaken during extraction. Solvent was removed and stored and the process was repeated four times. All the four extracts were pooled together and evaporated to dryness under reduced pressure at 50°C. The final drying was done initially in a vacuum desiccator and then in lyophilizer. The dried extracts were scrapped off and transferred to a glass container. Nitrogen was blown in the container before capping and the material was in refrigerator under desiccation.

3.3.3 Preparation of aqueous extract

The third lot of shade-dried and chopped plant parts was heated with distilled water on steam bath in a round bottom flask for 4 h. The extracts were drained and filtered. Using additional amounts of distilled water, the process was repeated four times with the same material. The pooled solvent was filtered and centrifuged at 1000 rpm for 20 min. Finally, drying was done using freeze dryer. The dried extracts were scrapped off and transferred to a glass container. Nitrogen was blown in the container before capping and the material was stored in refrigerator under desiccation.

3.4 Fractionation of extracts

95 pr cent alcoholic extracts of specified parts of all the plants mentioned above were further fractionated into n-hexane, chloroform, n-butanol and water soluble fractions.

3.4.1 Preparation of n-hexane fractions from 95 per cent alcoholic extracts

Dried alcoholic extracts of the specified plant parts were taken in stoppard conical flasks and shaken vigorously with n-hexane. The flasks were kept standing for 30 min. and the supernatants were decanted. The procedure was repeated thrice using fresh n-hexane every time. All the three supernatants were pooled together and evaporated to dryness under reduced pressure at 50°C. The dried isolates were scrapped off and transferred to air tight containers. Nitrogen was blown in the container before capping and the material was stored in refrigerator under desiccation.

3.4.2 Preparation of chloroform fractions from 95 per cent alcoholic extracts

The residue left after removing n-hexane soluble part was further macerated with chloroform four times as described for preparing n-hexane fraction. All the four supernatants were pooled together and evaporated to dryness under reduced pressure below 50°C. The dried isolates were scrapped off and transferred to air tight containers. Nitrogen was blown in the container before capping and the material was stored in refrigerator under desiccation.

3.4.3 Preparation of n-butanol and aqueous fractions from 95 per cent alcoholic extracts

The residue left after removing n-hexane and chloroform soluble parts was further suspended in water. The suspensions were taken to separating funnels and extracted with n-butanol four times. All the four n-butanol extracts were pooled together and evaporated to dryness under reduced pressure below 50°C. The dried isolates were scrapped off and transferred to air tight containers. Nitrogen was blown in the container before capping and the material was stored in refrigerator under desiccation. Water percolate fraction was filtered, centrifuged at 1000 rpm for 20 minutes and finally dried using freeze dryer. The complete extraction with each solvent was ensured in all the above cases by placing few drops of fresh extracts on watch glass and allowing it to evaporate. If no residue was noticed on the watch glass, extraction was considered complete. In cases where residues were noticed on the watch glass, the extraction process with the required solvent was repeated. The fractionation process is illustrated in the following flow chart.

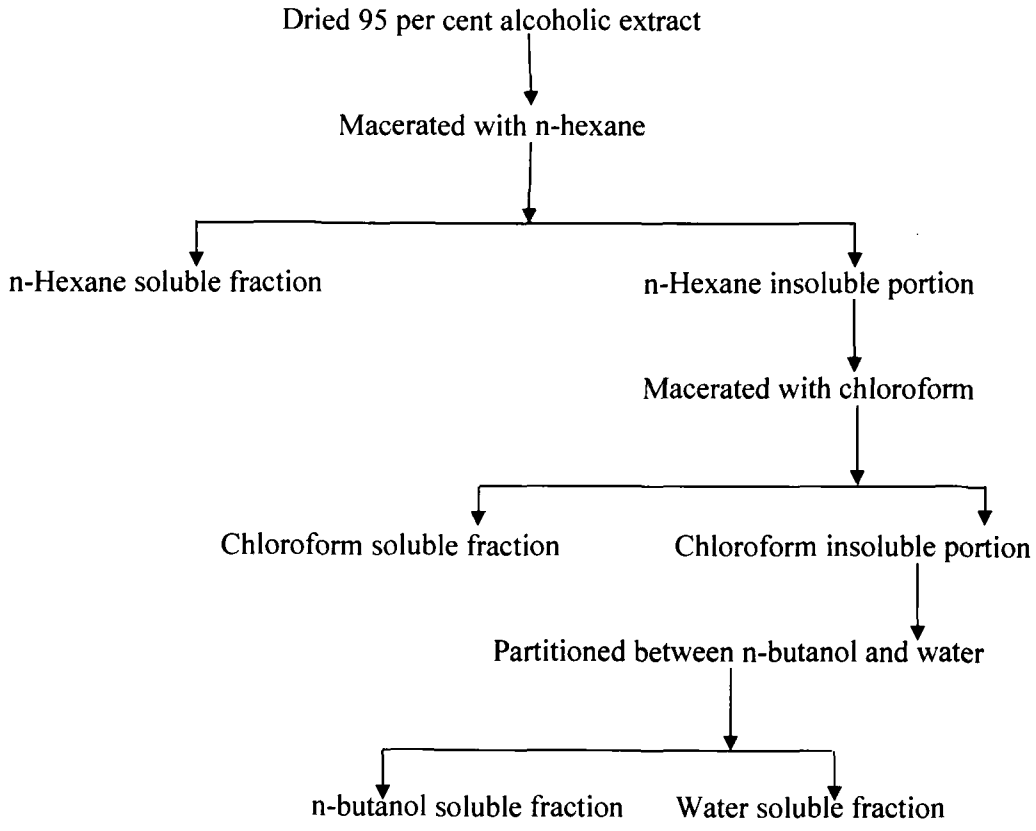


Fig. 3.7: Flow chart for the preparation of various fractions from 95 per cent alcoholic extracts

Thus, employing the procedures described above, following extracts and their fractions were prepared from the respective plant parts.

Table 3.1: Extracts and fractions prepared from the plant parts

Plant	Part	Extract/Fraction
Azadirachta indica	leaves	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Asparagus racemosus	roots	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Ipomoea nil	aerial parts	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Costus speciosus	aerial parts	95 per cent alcoholic extract
		50 per cent hydro-alcoholic extract
		Aqueous extract
Butea monosperma	flowers	95 per cent alcoholic extract
		50 per cent hydro-alcoholic extract
		Aqueous extract

Only those extracts of the specified plant parts that showed promising *in vitro* cytotoxicity against a panel of twelve cancer cell lines (95 per cent alcoholic extracts of *Azadirachta indica* leaves, *Asparagus racemosus* roots and *Ipomoea nil* aerial parts) were further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions so as to separate the compounds on the basis of their polarity. The active extracts and their respective fractions were then chemically characterized by High Performance Liquid Chromatography (HPLC).

3.5 High Performance Liquid Chromatography (HPLC) of active extracts and their fractions

3.5.1 Preparation of samples for HPLC analysis

Following solutions were prepared in HPLC grade methanol and filtered through 0.45 µm Millipore filter.

- 10 mg/ml of 95 per cent alcoholic extract
- 10 mg/ml of n-hexane fraction from 95 per cent alcoholic extract
- 10 mg/ml of chloroform fraction from 95 per cent alcoholic extract
- 10 mg/ml of n-butanol fraction from 95 per cent alcoholic extract
- 10 mg/ml of aqueous fraction from 95 per cent alcoholic extract

3.5.2 Solvent phase

The mobile phase consisting of filtered degassed methanol and water (2:3) was pumped at the flow rate of 0.7 ml/min. in a Waters HPLC system consisting of two pumps model 515 with Waters pump control module, an automatic sampling unit (Waters 717 plus auto sampler), a column oven, a photodiode array detector Waters 2996 and temperature control module II. Waters Empower software was used for data analysis and data processing.

3.5.3 Analysis of HPLC profile

10 µl of each sample was analysed at 30°C on a merck RP-18 Column (5µm, 250 mm x 4.00, ID) by UV detection at 210 nm. The HPLC chromatograms of following isolates are presented in Fig. 3.8 – 3.22.

Table 3.2: Extracts and fractions chracterised by HPLC

Plant	Part	Extract/Fraction
Azadirachta indica	leaves	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
Asparagus racemosus	roots	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
Ipomoea nil	aerial parts	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction

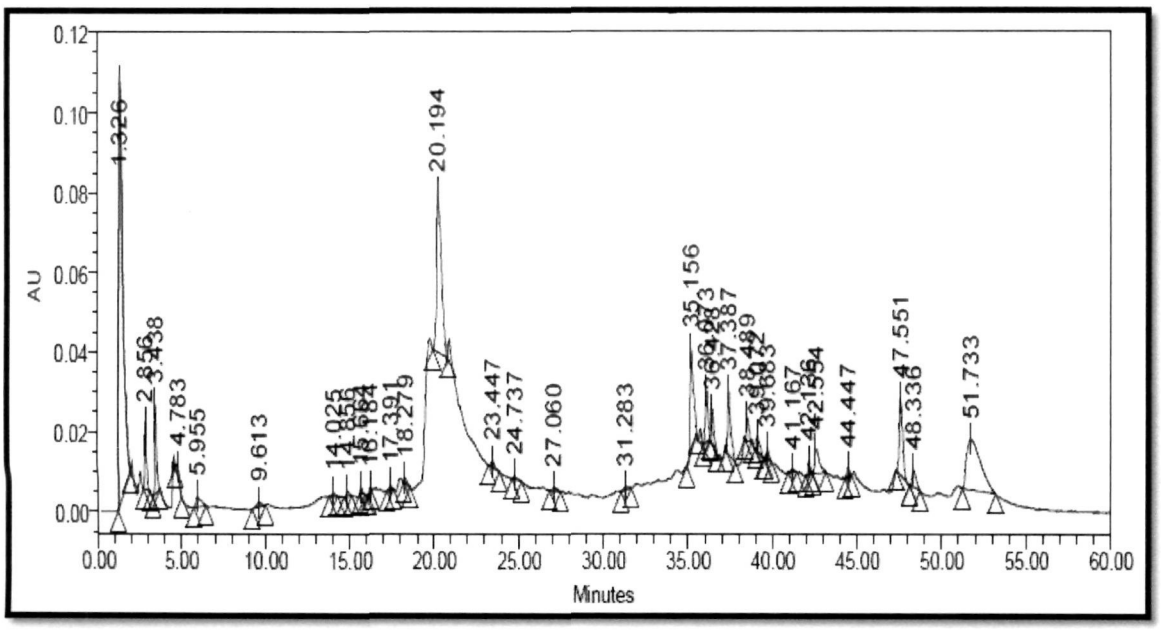


Fig. 3.8: HPLC chromatogram of 95per cent alcoholic extract of *Azadirachta indica* leaves

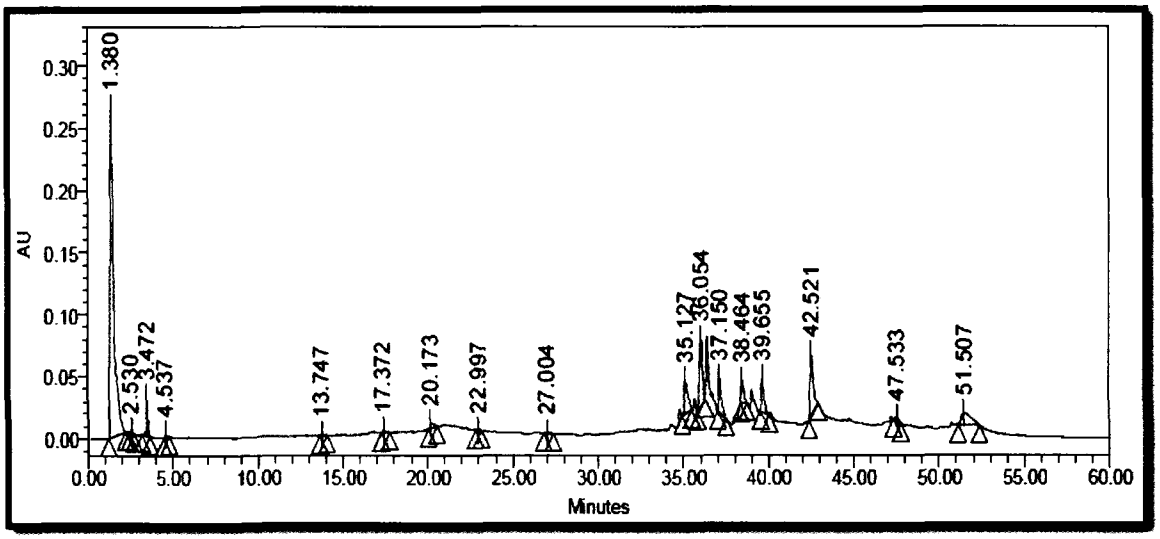


Fig. 3.9: HPLC chromatogram of n-hexane fraction from alcoholic extract of *A. indica* leaves

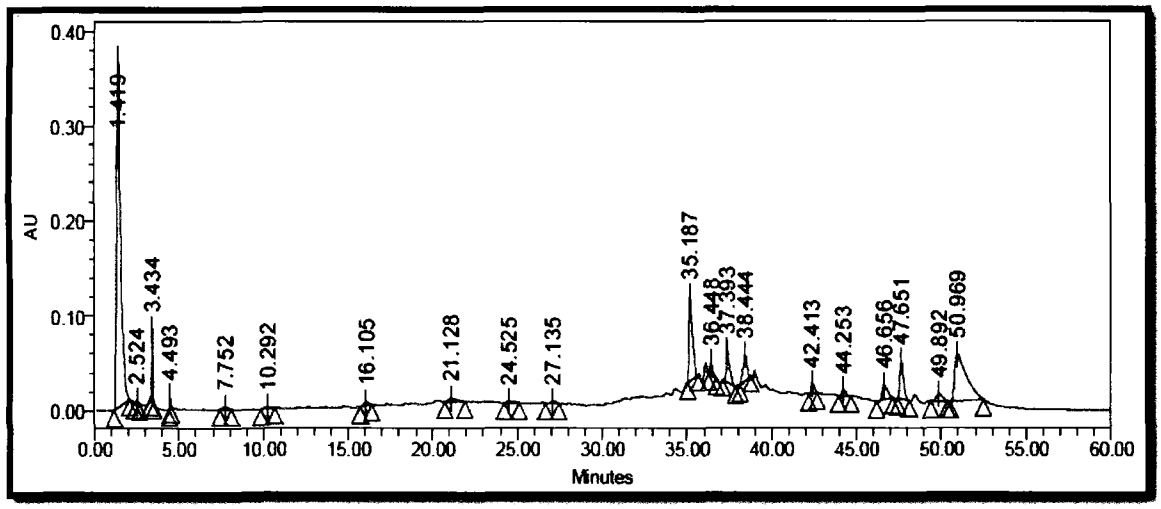


Fig. 3.10: HPLC chromatogram of chloroform fraction from alcoholic extract of *A. indica* leaves

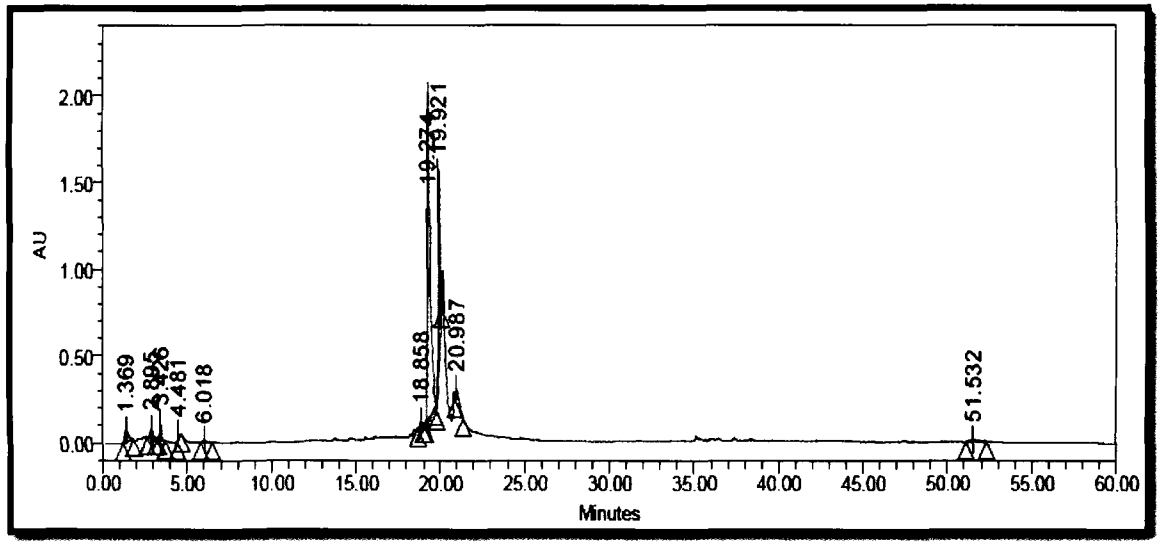


Fig. 3.11: HPLC chromatogram of n-butanol fraction from alcoholic extract of *A. indica* leaves

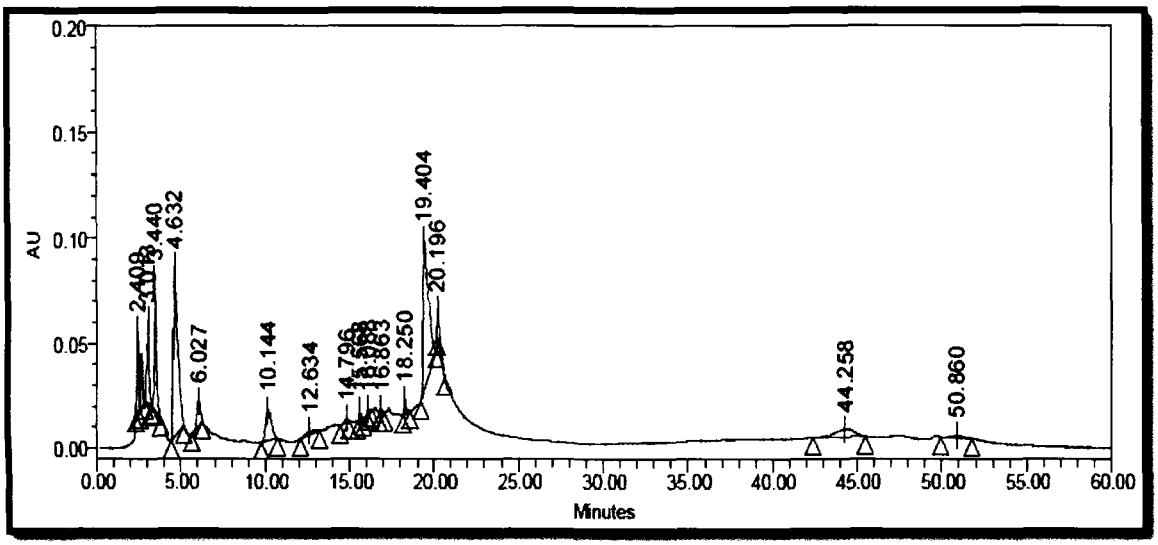


Fig. 3.12: HPLC chromatogram of aqueous fraction from alcoholic extract of *A. indica* leaves

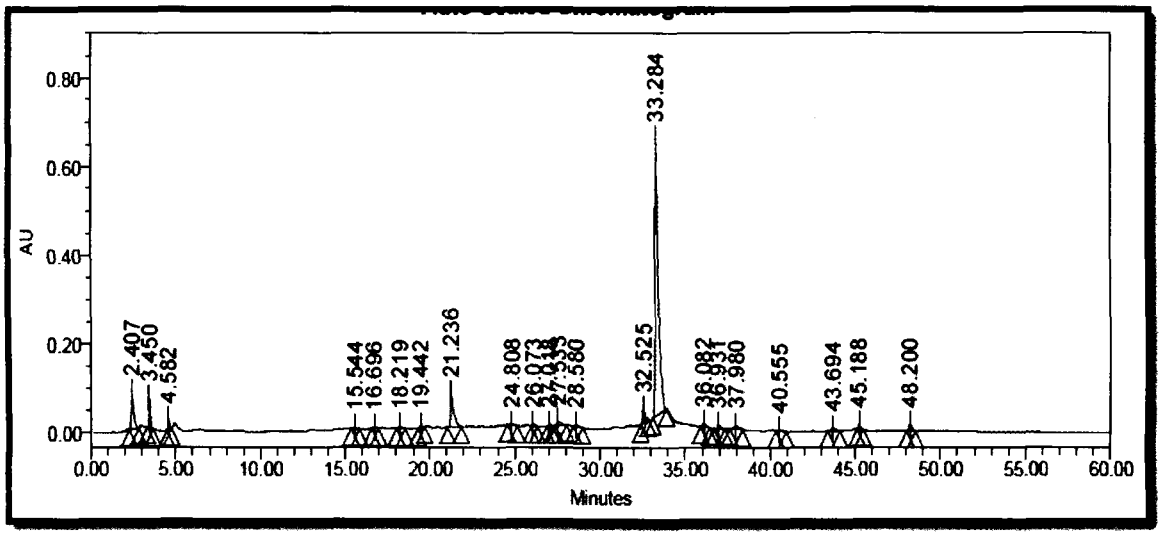


Fig. 3.13: HPLC chromatogram of 95% alcoholic extract of *Asparagus racemosus* roots

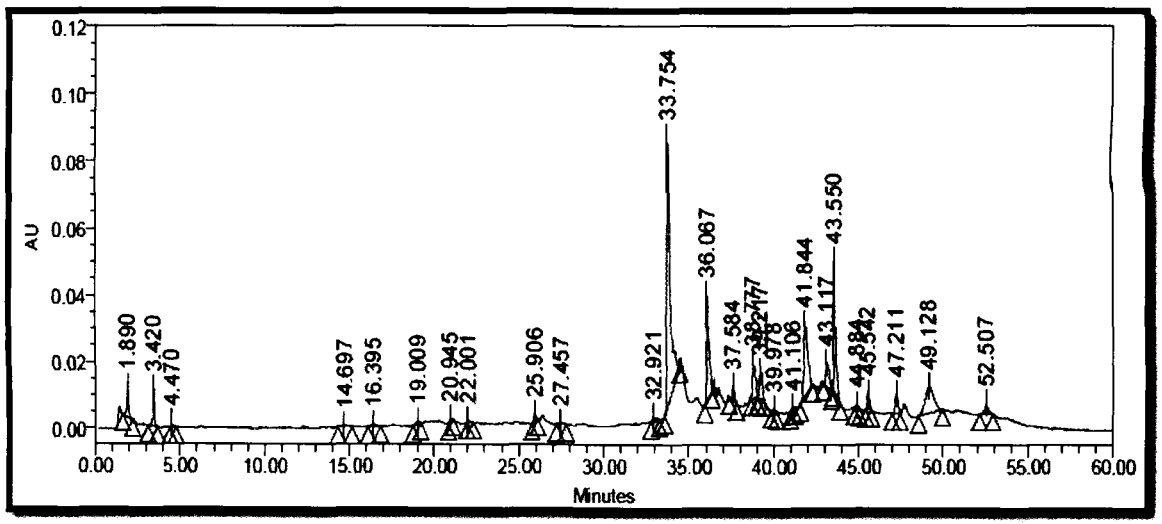


Fig. 3.14: HPLC chromatogram of n-hexane fraction from alcoholic extract of *A. racemosus* roots

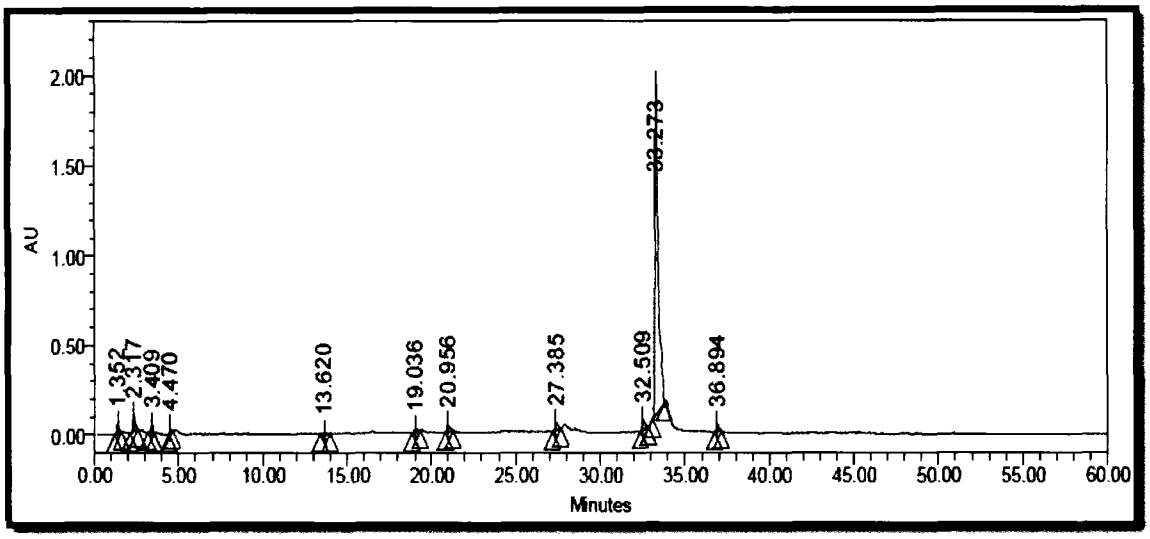


Fig. 3.15: HPLC chromatogram of chloroform fraction from alcoholic extract of *A. racemosus* roots

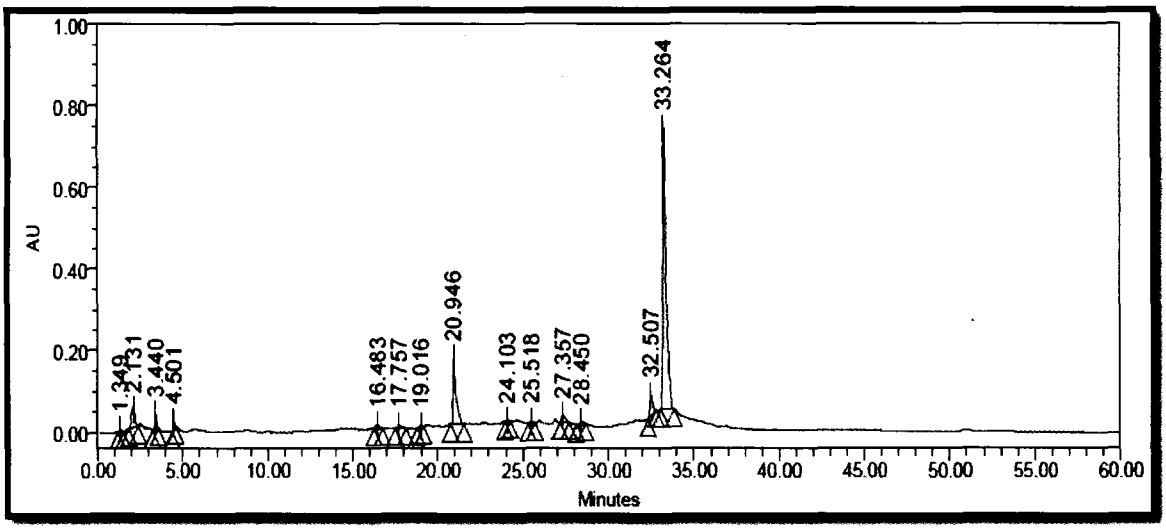


Fig. 3.16: HPLC chromatogram of n-butanol fraction from alcoholic extract of *A. racemosus* roots

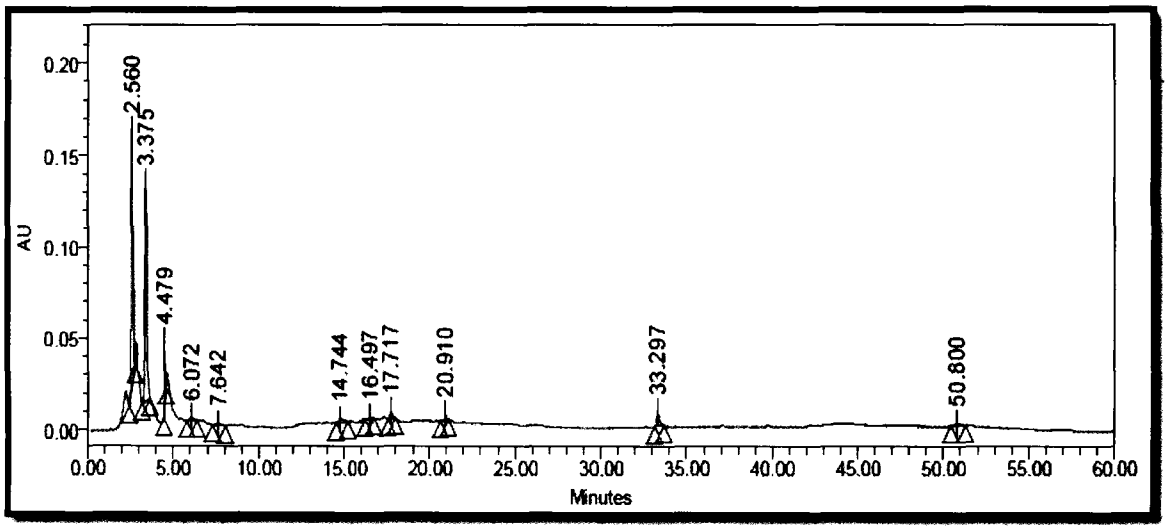


Fig. 3.17: HPLC chromatogram of aqueous fraction from alcoholic extract of *A. racemosus* roots

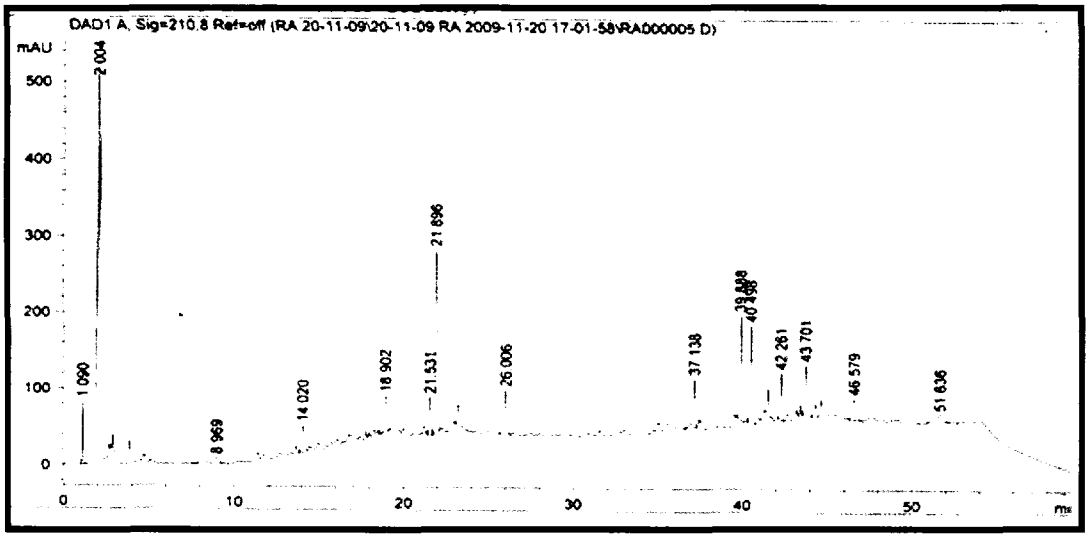


Fig. 3.18: HPLC chromatogram of alcoholic extract of *Ipomoea nil* aerial parts

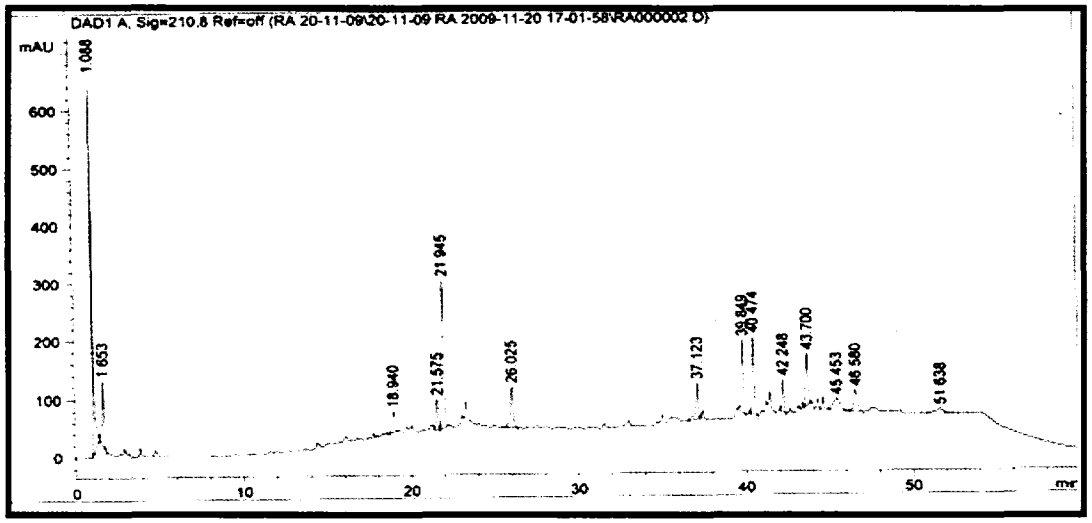


Fig. 3.19: HPLC chromatogram of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts

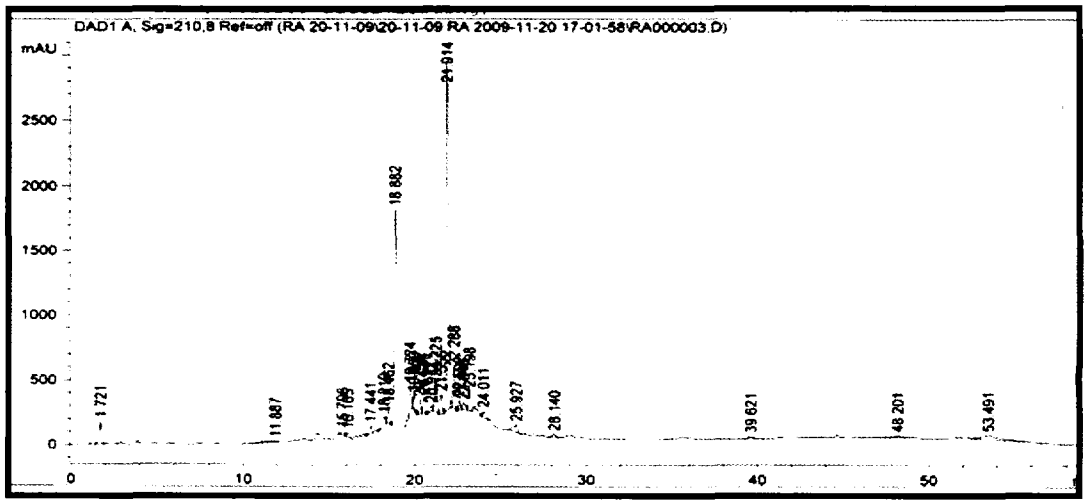


Fig. 3.20: HPLC chromatogram of chloroform fraction from alcoholic extract of *Ipomoea nil* aerial parts

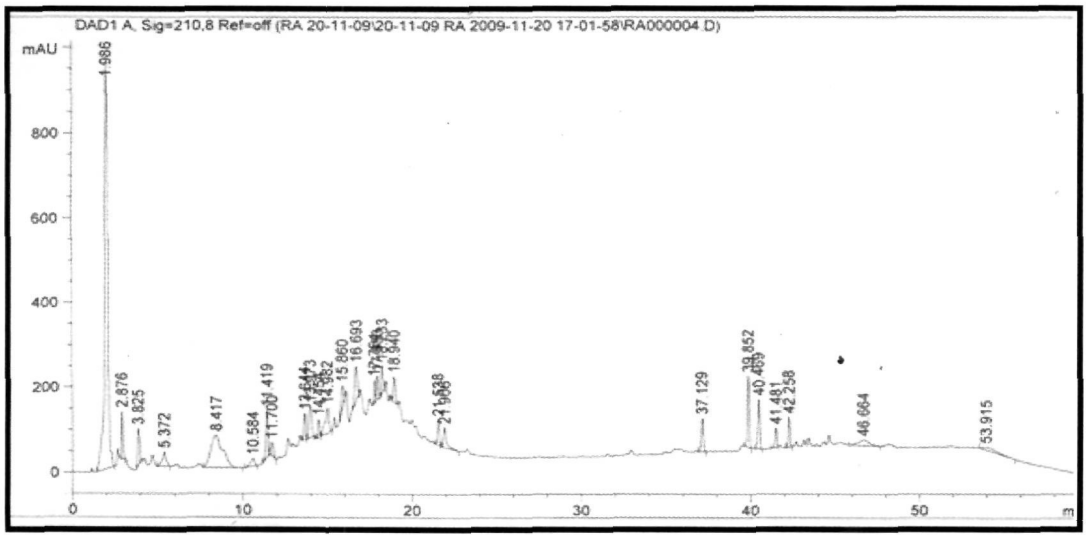


Fig. 3.21: HPLC chromatogram of n-butanol fraction from alcoholic extract of *Ipomoea nil* aerial parts

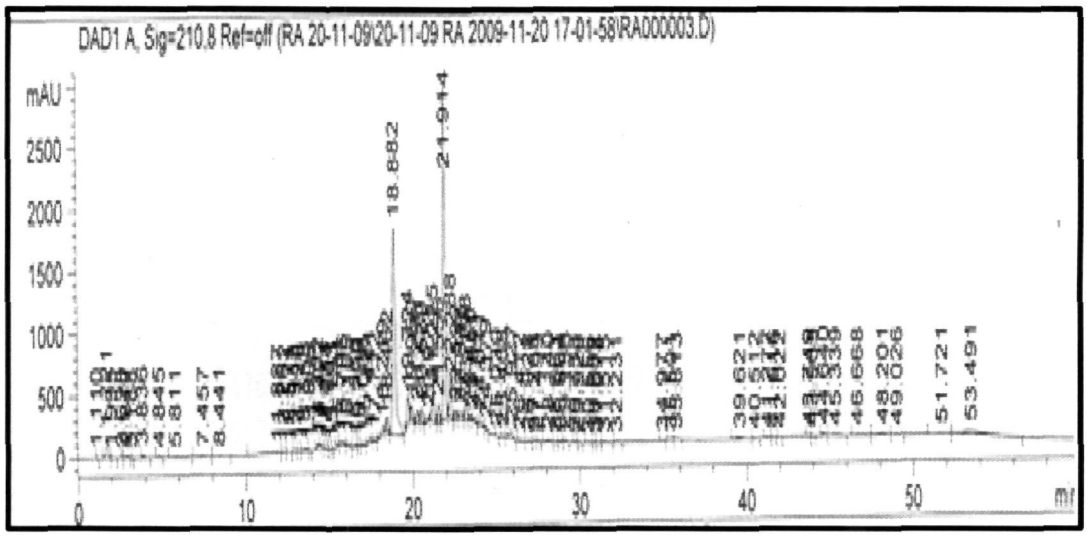


Fig. 3.22: HPLC chromatogram of aqueous fraction from alcoholic extract of *Ipomoea nil* aerial parts

3.6 *In vitro* cytotoxicity against cancer cell lines

For conducting *in vitro* cytotoxicity studies, mammalian cell lines are grown under *in vitro* conditions where they undergo mitotic divisions and multiply rapidly. Cells are cultured in 96 well plates and the rate of multiplication and growth is measured indirectly by staining the cells with a dye and measuring the intensity of colour. Intensity of colour is directly proportional to the number of cells present. The rate of proliferation of cancer cell lines in the presence and absence of the test substance is compared, usually after a specified time. Several different cancer cell lines are used in a

single experiment so that selectivity of the test substance can be assessed which gives an indication of potential usefulness of the test substance in a clinical setting.

The mostly preferred technique for testing cytotoxic potential is Sulphorhodamine B (SRB) assay. This technique relies on the uptake of the negatively charged pink aminoxanthine dye, Sulphorhodamine B (SRB) by basic amino acids in the plasma membrane of the cells. The greater the number of cells, greater is the amount of dye that is taken up by the cells. When the cells are lysed, the released dye gives intense colour. Greater the intensity of colour, greater is the absorbance. The SRB assay is sensitive, simple, reproducible and more rapid than formazan-based assays and gives better linearity. Hence, this assay was used in the present studies for evaluating the cytotoxic potential of various extracts and fractions described above.

3.6.1 Sulphorhodamine B (SRB) assay

3.6.1.1 Chemicals Required

- 5-Fluorouracil (Sigma Chem. Co., USA, Cat No F-6627)
- Dimethyl sulfoxide (DMSO) (Sigma Chem. Co., USA, Cat No D-2650)
- Doxorubicin (Sigma Chem. Co., USA, Cat No D-1515)
- Ethylenediamine tetraacetic acid, disodium salt (EDTA) (HiMedia Laboratories Pvt. Ltd., Mumbai, RM 1036)
- Fetal bovine serum (FBS) (Sigma Chem. Co., USA, Cat No F7524)
- Gentamycin (Sigma Chem. Co., USA, Cat No G-1284)
- Glacial acetic acid (Sisco Research Laboratories. Pvt. Ltd., Mumbai Cat No.012885)
- Isopropyl alcohol (Sisco Research Laboratories. Pvt. Ltd., Mumbai Cat No. 092250)
- Minimum Essential Medium (MEM) (Sigma Chem. Co., USA, Cat No M-0643)
- Mitomycin C (Sigma Chem. Co., USA, Cat No M-4287)
- Paclitaxel (Sigma Chem. Co., USA, Cat NoT-7402)
- Penicillin (Sigma Chem. Co., USA, Cat No P-3032)
- Phosphate Buffer Saline (PBS) (Sigma Chem. Co., USA, Cat No D-5652)
- Roswell Park Memorial Institute medium (RPMI) (Sigma Chem. Co., USA, R-6504)
- Sodium bicarbonate ($NaHCO_3$) (HiMedia Laboratories Pvt. Ltd., Mumbai , RM 447)
- Streptomycin (Sigma Chem. Co., USA, Cat No S-9137)
- Sulphorhodamine Blue (SRB) (Sigma Chem. Co., USA, Cat No S1402)
- Trichloroacetic acid (Sisco Research Laboratories. Pvt. Ltd., Mumbai Cat No. 204842)
- Tris buffer (HiMedia Laboratories Pvt. Ltd., Mumbai, R M 262)
- Trypsin (Sigma Chem. Co., USA, Cat Not-4799)

3.6.1.2 Apparatus

- 96-well flat bottom tissue culture plates (Grenier, Cat No. 655180)
- Cryo 1°C Freezing Container (Mr.Frosty, Sigma Chem Co, USA Cat No. C-1562)
- Cryovials (Grenier, Cat No.122 263)
- Glass Pipettes (10ml, 5ml) (Borosil)
- Glass Strips 25mm x 40cm, 6mm thick (LKB, Sweden)
- Measuring cylinder (25ml, 50 ml, 100 ml) (Borosil)
- Media Glass bottles (2 L, 1L, 500ml, 250ml, 100ml) (Borosil)
- Microcentrifuge Tubes (1.5 ml) (Eppendorf)
- Micropipettes (2-20µl, 20-200µl, 200-1000µl) (BRAND) (Tripette)
- Pipettes Dispensor (BRAND) (accu-jet@pro)
- Sterile Centrifuge Tubes (15ml, 50ml) (Grenier)
- Sterile disposable Syringes (10ml, 5ml, 2ml) (Dispovan)
- Syringe Driven Filter Unit (0.22µ) (Millipore Cat No. SLGV 025 LS)
- Tips for Micropipettes (2-20µl, 20-200µl, 200-1000µl) (Tarson)
- Tissue culture flasks – 25 Cm^2 (T-25) (Grenier, Cat. No. 910010)
- Tissue culture flasks – 75 Cm^2 (T-75) (Nunc, Cat No.156499)
- Tissue culture flasks - 150 Cm^2 (T-150) (Iwaki Cat No.3133-150)

3.6.1.3 Instruments

- Carbondioxide incubator (Heraeus, Germany)
- Centrifuge (Beckman,USA; G S-6R)
- Deep freezer (Scien Temp, USA)
- Filteration assembly (Sartorius, Germany)
- Hemocytometer (Sigma Chem. Co., USA, Cat No Z375357-13A)
- Liquid Nitrogen Container (Thermo Electron Corporation, USA)
- Mechanical shaker (IKA, Germany; MTS 4)
- Microscope (Nikon, Japan; T E 300)
- Robotic Liquid handling system (Tecan, Switzerland; Genesis 150 work station)
- Sunrise ELISA reader (Tecan, Switzerland; Part No. I 137 301)
- Vertical laminar flow - Clean air work station (Klenzaids Model No.1194)
- Weighing balance (Sartorius, Germany; Genius)
- Digital Camera (Olympus Digital Camera (C4000)
- Microscope (Olympus Research Microscope (VANOX)

3.6.1.4 Preparation of Reagents

3.6.1.4.1 Growth Medium:

The contents of the vial of RPMI-1640 with 2 mM L-glutamine or Minimum Essential Medium (MEM) as supplied were dissolved in double distilled water as per supplier's instructions. Streptomycin (100 mg/L) and sodium bicarbonate (1.2 g/L) were also added to the medium. The pH of medium was adjusted to 7.2 and it was sterilized by filtration through 0.2 µ filters in laminar flow under sterile conditions. The medium was stored in refrigerator at 2-8°C.

3.6.1.4.2 Complete Growth Medium:

The above growth medium was supplemented with 10 per cent FCS and Penicillin (100 IU/ml) before use, to make it complete growth medium.

3.6.1.4.3 Freezing medium:

Freezing medium for cryopreservation of cells contained 20 per cent FCS and 10 per cent DMSO (cell culture grade) in growth medium.

3.6.1.4.4 Phosphate buffer saline (PBS):

Contents of a vial of PBS were dissolved in double distilled water, diluted up to 1 liter and filtered with 0.2 micron filter under sterile condition.

3.6.1.4.5 Trypsin-EDTA:

50 mg Trypsin (0.05 per cent) and 20 mg EDTA, disodium salt (0.02 per cent) were dissolved in PBS, diluted up to 100 ml and filtered with 0.2 micron filter under sterile condition.

3.6.1.4.6 Trichloro acetic acid:

50 per cent (w/v) TCA solution was prepared in double distilled water.

3.6.1.4.7 Acetic acid:

Glacial acetic acid was diluted to 1 per cent with double distilled water.

3.6.1.4.8 SRB Dye:

400 mg of SRB was dissolved in 1 per cent acetic acid and diluted up to 100 ml (0.4 per cent).

3.6.1.4.9 Tris-buffer:

1.21 g of Tris (10 mM) was dissolved in 950 ml distilled water, diluted up to 1 L and pH was adjusted to 10.5.

3.6.1.5 Cell lines

3.6.1.5.1 Sources of cell lines:

Original stocks of human cancer cell lines were received in frozen state (dry ice) in cryovials, from National Cancer Institute (NCI), Fredrick (USA). Some of the cell lines

were obtained from National Center for Cell Science (NCCS), Pune (India) in culture flasks.

3.6.1.5.2 Cell lines used:

The following cell lines were used in the present study.

Breast :MCF-7
CNS :SK-N-SH
Colon :Colo-205, HCT-15, SW-620, 502713
Liver :Hep-2
Lungs :A-549
Prostate :DU-145
Ovary :OVCAR-5
Neuroblastoma :IMR-32
Canine osteosarcoma :D17

Most of the cell lines used in the present study are included in the panel recommended by NCI, USA (Monks *et al.,* 1991). The cell lines were grown in RPMI-1640 or MEM growth medium depending on cell type as mentioned in the Table 3.3

Table 3.3: Cell lines, their tissue of origin, required media and cell density

Tissue	Cell lines	Medium	No. of cells/well
Breast	MCF-7	MEM	15,000
Colon	502713	RPMI	10,000
	SW-620	RPMI	10,000
	Colo-205	RPMI	15,000
	HCT-15	RPMI	10,000
Ovary	OVCAR-5	RPMI	20,000
Neuroblastoma	IMR-32	MEM	10,000
CNS	SK-N-SH	MEM	15,000
Lungs	A-549	RPMI	10,000
Liver	Hep-2	MEM	10,000
Prostrate	DU-145	MEM	10,000
Bone	D17	MEM	10.000

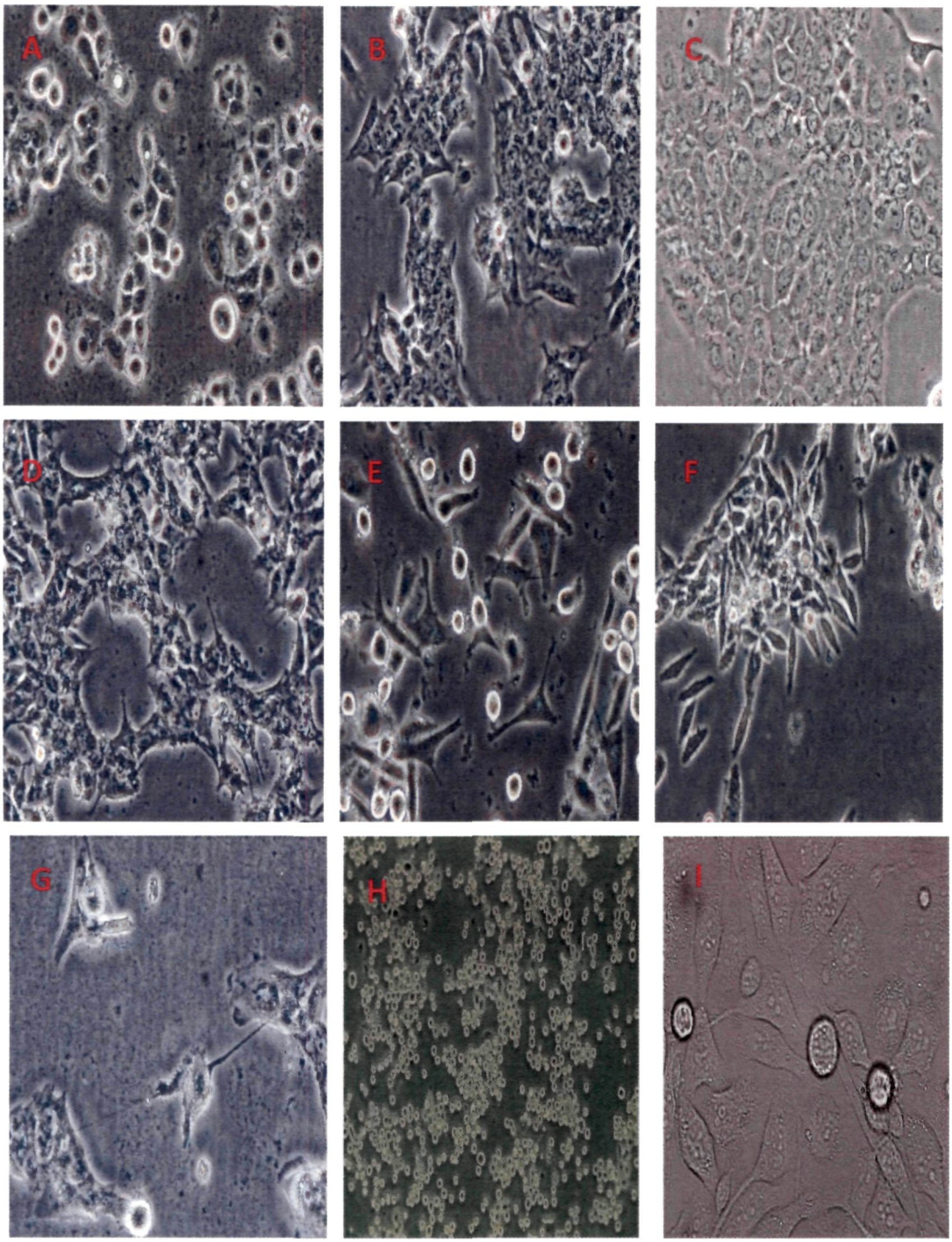


Fig 3.23: Morphology of various cancer cell lines from different tissues

A: HT-29 **B**: 502713

C: OVCAR-5 **D**: SK-N-SH

E: SK-MEL-5 **F**: SW-620

G: CAKI **H**: HL-60

I: PC-3.

3.6.1.5.3 Handling of cell lines on arrival

Cells received in cryovials from NCI, USA were transferred to liquid nitrogen immediately on arrival and were used as per requirement. Cells received in Tissue Culture Flasks from NCCS, Pune were observed under microscope under aseptic conditions. If the flask was found to contain healthy cells without contamination, the contents of the flask were transferred to centrifuge tube and cells with required quantity of the medium were placed in tissue culture flask and grown as described in the later part of this section.

3.6.1.5.4 Revival of cell lines

Cryovials containing cells were removed from the liquid nitrogen container and thawed quickly by shaking in water bath at 37^0C. Cryovials were wiped with 70 per cent alcohol to avoid contamination and transferred to laminar flow. Contents of the vial were transferred into a sterile centrifuge tube containing in 10 ml complete growth medium and centrifuged. Supernatant was discarded and the cells were suspended in fresh complete medium. Cells were mixed properly to ensure uniform distribution in the medium. Cells were transferred to the Tissue Culture Flask -25 containing 7 ml of complete growth medium aseptically and incubated in CO_2 incubator at 37^0C, 5 per cent CO_2 atmosphere and 90 per cent RH.

3.6.1.5.5 Cell culture and maintenance of adherent cell lines

Cancer cells were grown in tissue culture flasks with complete growth medium at 37°C in an atmosphere of 5 per cent CO_2 and 90 per cent RH in a carbon dioxide incubator. Cells were checked daily for their proper growth. The medium of the cells was changed when the color became yellow. To change the medium, the medium in the flask was aspirated with pipette-man and discarded. The fresh medium (5-7 ml in case of TCF-25) was placed in the culture flask under sterile conditions. The flasks were properly marked and incubated in CO_2 incubator. Depending on the mass doubling time of cells, sub-culturing of cells was done, when they were at sub-confluent stage.

3.6.1.5.6 Subculture of the cell lines

It involves detachment of cells from the growth surface of the culture flask and reinoculation of the cells into fresh medium in new culture flask i.e., TCF-25, TCF-75 or

TCF-150 (depending on the number of cells). The medium of the flask at sub-confluent growth was changed one day in advance. The entire medium from the flask was taken out and discarded. Cells were washed with PBS. The minimum quantity of Trypsin-EDTA (Pre-warmed at 37°C) was added just enough to make a thin layer and incubated for approximately 5 min. at 37°C. Cell suspension was made with complete growth medium. An aliquot was taken out, cells were counted and checked for viability with trypan blue. Cell stock with more than 98 per cent cell viability was accepted for determination of *in vitro* cytotoxicity. The cell density was adjusted to $1x10^6$ cells/ml by the addition of more of complete growth medium and inoculated into fresh TCF-75 or TCF-150 and incubated in CO_2 incubator to continue the culture.

3.6.1.5.6.1 Stages of cell growth during culturing

On observation under microscope, cells appear roughly round shaped when seeded in the culture flask. Different stages of cell growth during culture are shown in Fig. 3.24.

3.6.1.5.6.2 Attachment Stage:

Within 24 hours of incubation after seeding the cells get attached to the base of tissue culture flask.

3.6.1.5.6.3 Sub-confluent Stage:

It is a stage of rapid growth of cells. At this stage, some space remains between the growing cells. The cells are in *log phase* of their growth and can be used for experimental purposes as well as for sub-culturing or cryopreservation.

3.6.1.5.6.4 Confluent Stage:

At this stage, cells appear to form a complete monolayer when observed under microscope. The medium turns turbid, cell debris and metabolic wastes accumulate in the medium.

3.6.1.5.7 Cryopreservation

In order to minimize genetic drift in cell lines, senescence or transformation in infinite cell lines and as a guard against accidental loss by contamination or otherwise, it is a common practice to freeze (cryopreserve) aliquots of cells in liquid nitrogen vapours. It

is important to bring the cells at -80°C with the cooling rate of 1°C/min at the time of cryopreservation.

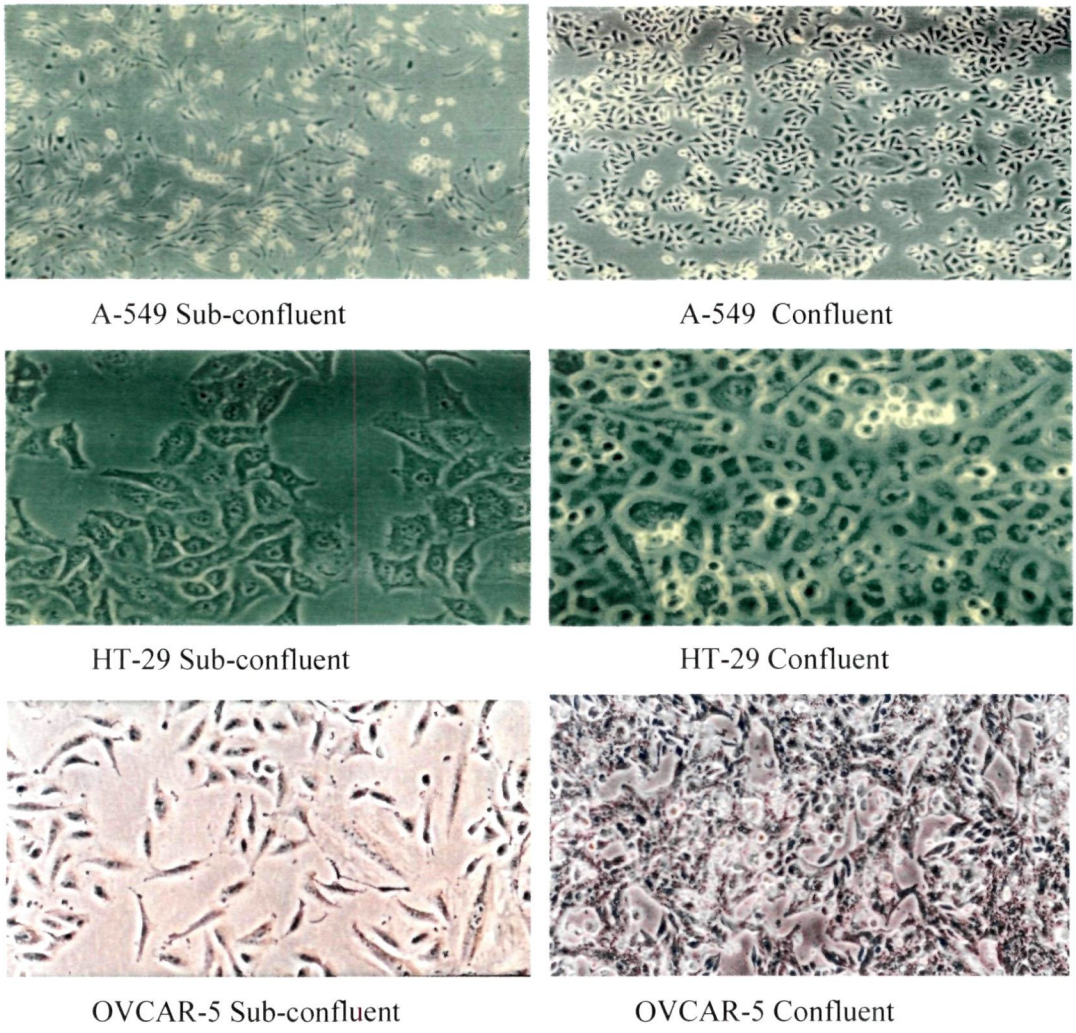


Fig 3.24: Stages of cell growth of various cell lines

The cells of the flask at sub-confluent stage from which medium was changed one day in advance were trypsinised. The cell suspension was made and centrifuged at low speed (500 rpm). Cells with more than 98 per cent viability as determined by trypan blue exclusion technique were cryopreserved. The pellet was suspended in medium for cryopreservation and the cell density was adjusted to $1x10^8$ cells/ml. The aliquots of 1.0 ml were transferred into Cryovials. The temperature of the vials was brought down to -80°C by using a specially designed box (Sigma Chem. Co, USA Cat. No. C-1562) which

is commercially available. The whole box was put into -80°C refrigerator for 24 h. Later, the vials were transferred to liquid nitrogen container.

3.6.1.6 Preparation of test materials

3.6.1.6.1 Stock solution

Dimethyl sulphoxide (DMSO) was used as solvent to dissolve test materials. It is cytotoxic at high concentrations but it has no effect on the cells at concentrations below 1 per cent w/v. For 95 per cent alcoholic, 50 per cent hydro-alcoholic and aqueous extracts, DMSO, 50 per cent aqueous-DMSO and distilled water were used as solvents, respectively. n-Hexane and chloroform fractions were also dissolved in DMSO while n-butanol fractions were dissolved in 50 per cent DMSO and aqueous fractions were dissolved in distilled water. A stock solution of 20 mg/ml of each extract and fraction was prepared.

3.6.1.6.2 Working solution

The stock solutions (20 mg/ml) were serially diluted with complete growth medium containing 50 µg/ml of gentamycin to obtain working test solutions of 200, 60 and 20 µg/ml. The working test solutions were not filtered/ sterilized but microbial contamination was controlled by the addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

3.6.1.7 Preparation of Positive control

3.6.1.7.1 Stock solution

A stock solution of $2x10^{-2}M$ concentration of the positive control was prepared. For Paclitaxel and Doxorubicin DMSO was used as solvent and 5-Fluorouracil and Mitomycin C were dissolved in distilled water and stored at -20°C.

3.6.1.7.2 Working solution

The stock solutions were serially diluted with complete growth medium containing 50 µg/ml of gentamycin to obtain working test solutions of $2x10^{-6}M$ or $2x10^{-5}M$ as per the requirement. The working solutions of positive controls were not filtered/ sterilized but microbial contamination was controlled by the addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working solutions.

3.6.2 Cytotoxicity assay

3.6.2.1 Preparation of cell suspension

Healthy cells at sub-confluent stage showing no signs of contamination were used for *in vitro* cytotoxicity assay (Monks *et al.*, 1991; Skehan *et. al.*, 1990; Peter *et al.*, 2007). The cells were harvested from tissue culture flask after trypsination and single cell suspension was prepared. An aliquot was taken and the cells were counted by haemocytometer in order to determine cell density of the original suspension. The cell suspension was diluted with appropriate growth medium to obtain the different cell densities ($5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$ cells/ml) depending on the cell line.

3.6.2.2 Procedure for SRB exposure assay

100 µl of cell suspension of the desired density was added to each well of 96-well cell culture plates and incubated at 37°C in an atmosphere of 5 per cent CO_2 and 90 per cent RH in a carbon dioxide incubator for 24 h. After 24 hrs of incubation, 100 µl of test sample, positive control or medium was added in the wells containing cells. The cells with the samples were further incubated for 48 h. The cells were fixed with ice-cold 50 µl TCA (50 per cent) for 1 h at 4°C. The plates were then washed five times with distilled water and allowed to dry in the air. Then, 100 µl of sulphorhodamine solution was added to each well of the dry 96-well plates and staining of cells was allowed at room temperature for 30 min. Excess of sulphorhodamine solution was removed by washing the plates with (1 per cent) acetic acid, five times. The washed plates were dried in the air. The adsorbed SRB was solubilised by adding 100 µl of 10 mM Tris Buffer (pH 10.5) to each well and the plates were shaken for 5 min. on a shaker platform. The plates were then read in 96-well plate reader (Molecular Devices, Sunnydale, USA) at 540 nm. Each concentration of the test substance and positive control was tested in triplicate and the average OD was worked out.

3.6.2.3 Calculation

The cell growth was determined by subtracting mean OD values of respective blank from the mean OD value of experimental set. Percentage of growth in the presence of test material was calculated considering the growth in absence of any test material as 100 per cent and in turn per cent growth inhibition in presence of test material was calculated.

Flow chart of activities followed for *in vitro* cytotoxicity assay is presented in Fig. 3.25.

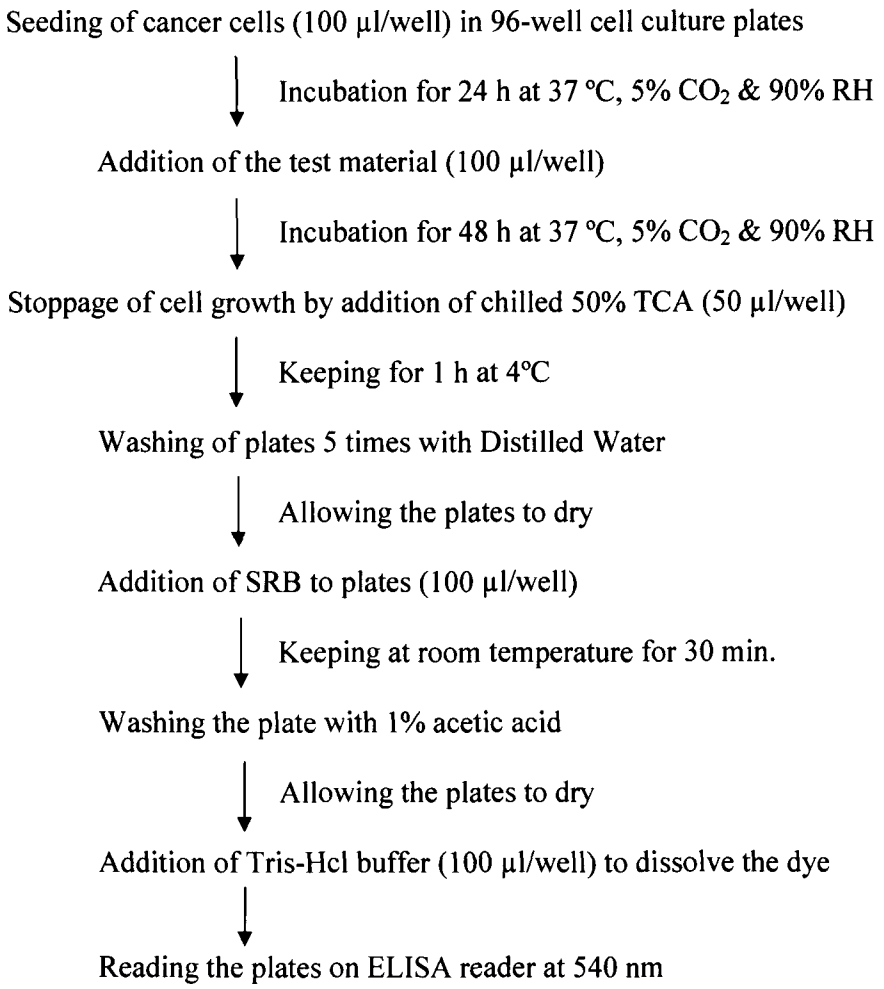


Fig 3.25: Flow chart of *in vitro* Cytotoxicity assay

Thus, employing the procedures described above, following extracts and their fractions were tested for their cytotoxic potential against a panel of twelve cancer cells lines from different tissues of humans and animals.

Table 3.4: Extracts and their fractions tested for cytotoxic potential against cancer cell lines

Plant	Part	Extract/Fraction
Azadirachta indica	leaves	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Asparagus racemosus	roots	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Ipomoea nil	aerial parts	95 per cent alcoholic extract
		n-hexane fraction
		chloroform fraction
		n-butanol fraction
		aqueous fraction
		50 per cent hydro-alcoholic extract
		Aqueous extract
Costus speciosus	aerial parts	95 per cent alcoholic extract
		50 per cent hydro-alcoholic extract
		Aqueous extract
Butea monosperma	flowers	95 per cent alcoholic extract
		50 per cent hydro-alcoholic extract
		Aqueous extract

3.7 *In vivo* anticancer studies

Samples found highly active under *in vitro* cytotoxic assays against a panel of cancer cell lines were further tested for their *in vivo* anticancer activity using various murine tumor models.

3.7.1 Principles

The *in vivo* experiments were based upon the following principles:

- Inhibition in growth of tumor cells implanted in the peritoneal cavity of animals by the test sample.
- Inhibition in growth of solid tumors induced in animals by the test sample.
- Increase in the life span of tumor bearing animals by the test sample.

For comparison, the growth of tumor cells, the growth of solid tumors and the life span in normal saline treated tumor bearing animals were taken to be 100 per cent.

3.7.2 Animals

Following strains/stocks of animals bred and maintained at the Anticancer Vivarium of the Indian Institute of Integrative Medicine (IIIM), Jammu, were used in the present studies.

- Swiss albino mice (outbred)
- BALB/c mice (inbred)
- DBA/2 mice (inbred)
- CD2F1 mice (F1 hybrids of BALB/c females crossed with DBA/2 males)

3.7.2.1 Care and management of animals

All the above strains/stocks of animals were housed and bred in Individually Ventilated Cage (IVC) systems providing HEPA filtered air to each and every cage. Housing of animals in standard polycarbonate cages of IVC systems ensured internationally recommended space for each animal. The animal room temperature and humidity were maintained at 23$\pm$2°C and 50$\pm$5 per cent, respectively. Light : Dark cycle was maintained

at 12 :12 h. Animals were fed with commercially available pelletted mice feed from M/s Ashirwad Industries, Chandigarh (India) and the autoclaved water was given *ad lib*. The animals were cared as per the Guide for the care and use of laboratory animals (1996). All animal experiments and the number of animals used in the present study were approved by the Institutional Animal Ethics Committee of IIIM, Jammu.

3.7.3 Tumor Models used

Following models of murine tumors were used in the present study for evaluating the *in vivo* anticancer potential of test samples.

- Ehrlich Ascites Carcinoma
- Sarcoma-180 (Ascites)
- Methyl cholantherene induced ascites
- Ehrlich Tumor (Solid)
- Sarcoma-180 (Solid)
- L1210 Lymphoid leukemia
- P388 Lymphocytic leukemia

3.7.4 Test materials

Following test materials (extracts and their fractions) that were found to possess significant cytotoxic activity against cancer cell lines under *in vitro* test systems were subjected to *in vivo* studies against murine tumor models mentioned above.

Table 3.5: Extracts and their fractions tested for *in vivo* anticancer activity

Plant	Part	Extract/Fraction
Azadirachta indica	leaves	95 per cent alcoholic extract
		chloroform fraction
Asparagus racemosus	roots	95 per cent alcoholic extract
		chloroform fraction
		n-butanol fraction
Ipomoea nil	aerial parts	95 per cent alcoholic extract
		n-hexane fraction

3.7.4.1 Preparation of test materials

On day one of the experiment, animals in each experimental group were weighed and the average weight of animals in each group was calculated. On the basis of this weight, the amount of test sample required for four days' daily administration in each group was calculated and weighed in separate eppendorf tubes. In case of 95 per cent alcoholic extract and its n-hexane, chloroform and n-butanol fractions from *Asparagus racemosus* (roots) and *Ipomoea nil* (aerial parts), 500 µl of 95 per cent ethanol was added to the weighed quantity of test samples. These test samples were clearly soluble in 95 per cent ethanol. To this solution, sterile normal saline (0.85 per cent w/v) was added to make up the volume up to 5.6 ml. 0.2 ml of this preparation was administered to each animal in a test group daily for four days.

On day 5 of the experiment, animals in each experimental group were again weighed and the average weight of animals in each group was calculated. On the basis of this weight, the amount of test sample required for five days' daily administration in each group was calculated and weighed in separate eppendorf tubes. Following rest of the procedure as described above, 7.0 ml of test solution of each sample was prepared and was administered @ 0.2 ml per animal daily, for the next five days. With this procedure, each dose of the test material administered to each animal contained less than 20 µl of ethanol and thus complied well with procedures described by Geran *et al.*, (1972).

In case of 95 per cent alcoholic extract and its chloroform fraction from *Azadirachta indica* (leaves) the test samples after addition of 95 per cent ethanol and normal saline were added 2 drops of Tween-80 and the suspension was agitated in a porcelain mortar with the help of elliptical movements of pestle, till clear solution was obtained.

3.7.4.2 Administration of test materials to experimental animals

All test materials were administered intraperitoneally in all tumor models. The doses of test material were prepared in such a way that the quantity of test material in each dose was contained in a volume of 0.2 ml.

3.7.4.3 Positive control

5-fluorouracil (5-FU) was used as positive control. The 5-FU was administered to a positive control group of animals in each experiment involving Ehrlich Ascites Carcinoma, Sarcoma-180 (Ascites), Methyl cholantherene induced ascites, L1210 Lymphoid leukemia and P388 Lymphocytic leukemia at a dose of 20 mg/kg body weight. For experiments involving Ehrlich Tumor (Solid) and Sarcoma-180 (Solid) a dose of 22 mg/kg body weight of 5-FU was used.

3.7.4.4 Tumor bearing control

0.2 ml of Normal saline (0.85 per cent w/v) was administered to a group of tumor bearing animals which served as tumor bearing control group in all the *in vivo* experiments.

3.7.5 Protocols

Protocols described by Geran *et al.*, (1972) were followed for all *in vivo* experiments. These protocols are described below.

3.7.5.1 Ehrlich Ascites Carcinoma (EAC)

3.7.5.1.1 Propagation

For propagation of EAC cells, ascitic fluid from an animal bearing 8-10 days old Ehrlich Ascites Carcinoma was withdrawn and diluted with normal saline. Cell number per ml of diluted ascitic fluid was determined with the help of Neubauer's chamber and the volume of ascitic fluid containing $1x10^7$cells was arrived at. This volume of ascitic fluid was injected intraperitoneally in non-inbred swiss mice (4-5 nos.). When the ascites grew 8-10 days old, again peritoneal fluid was collected and EAC cells were transplanted in the peritoneal cavity of fresh non-inbred swiss mice.

3.7.5.1.2 Experimental Animals

Non-inbred swiss mice weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.1.3 Experimental Protocol

Test samples were evaluated for their *in vivo* anticancer activity against Ehrlich Ascites Carcinoma as per the protocol described by Geran *et al.*, (1972). The protocol was as follows:

Day 0: Induction of tumor

Peritoneal fluid was collected from an animal bearing 8-10 days old Ehrlich ascites carcinoma and the number of tumor cells per ml of ascitic fluid was determined by counting the cells with the help of neubauer's chamber. The ascitic fluid was diluted with normal saline in such a way that 0.2 ml of fluid contained $1x10^7$ EAC cells. All animals selected for conducting the experiment were injected intraperitoneally with 0.2 ml of ascitic fluid containing $1x10^7$ EAC cells.

Day 1

On day 1, all animals injected with EAC cells were randomized and divided in different treatment and control groups. Tumor bearing control group contained 15 animals and all other groups (treatment and positive control) contained seven animals each. Animals in each group were weighed and an average body weight of animals in each group was worked out. Based on the body weight, test drugs were prepared for four days' daily administration in such a way that each dose was contained in 0.2 ml volume. First dose was administered at 2.00 PM.

Day 2-4

Animals in the treatment and control groups were treated with respective test drugs at a fixed time (2.00 PM).

Day 5

Animals in each group were weighed and based on the average body weight for each group, test drugs were prepared as described for the next 5 days. All animals were administered a dose at the same fixed time as earlier.

Day 6-9

Animals in the treated and control groups were treated with respective test solution at a fixed time.

Day 12: Evaluation

For evaluation of the experiment, animals in each group were sacrificed by cervical dislocation. The ascitic fluid from each animal was collected in a pre-weighed graduated centrifuge tube with the help of funnel. Thus, volume and weight of ascitic fluid from each animal was recorded. Number of tumor cells in ascitic fluid was counted with the help of neubauer's chamber and the total number of tumor cells present in the ascitic fluid of each animal was calculated. The per cent tumor growth inhibition was calculated as follows.

Calculations

The per cent tumor growth inhibition was calculated using the following formula.

Percent tumor growth inhibition =

$$\frac{\text{Av. no. of cells in control group} - \text{Av. no. of cells in treated group}}{\text{Av. no. of cells in control group}} \times 100$$

Evaluation only on the basis of cell number was carried out which takes care of other parameters like tumor weight and volume.

3.7.5.2 Sarcoma-180 (Ascites)

3.7.5.2.1 Propagation

For propagation of Sarcoma-180 cells, ascitic fluid from an animal bearing 8-10 days old Sarcoma-180 (Ascites) was withdrawn and diluted with normal saline. Cell number per ml of diluted ascitic fluid was determined with the help of Neubauer's chamber and the volume of ascitic fluid containing $1x10^7$cells was arrived at. This volume of ascitic fluid was injected intraperitoneally in BALB/c mice (4-5 nos.). When the ascites grew 8-10 days old, again peritoneal fluid was collected and Sarcoma-180 cells were transplanted in the peritoneal cavity of fresh BALB/c mice.

3.7.5.2.2 Experimental Animals

BALB/c mice weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.2.3 Experimental Protocol

Experimental protocol including calculations followed was essentially the same as described for Ehrlich ascites carcinoma except that BALB/c mice and Sarcoma-180 cells were used instead of non-inbred swiss mice and EAC cells, respectively.

3.7.5.3 Methyl cholantherene induced ascites

3.7.5.3.1 Propagation

Methyl cholanthrene is a chemical carcinogen. An ascitic tumor was induced in BALB/c mice elsewhere by intraperitoneal injections of Methyl cholanthrene. Animals bearing Methyl cholanthrene induced ascites were procured from Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India and maintained at Anticancer Vivarium of the Indian Institute of Integrative Medicine (IIIM), Jammu. For further propagation of methyl cholanthrene induced ascites cells, ascitic fluid from an animal bearing 8-10 days old ascites was withdrawn and diluted with normal saline. Cell number per ml of diluted ascitic fluid was determined with the help of Neubauer's chamber and the volume of ascitic fluid containing $1x10^7$cells was arrived at. This volume of ascitic fluid was injected intraperitoneally in BALB/c mice (4-5 nos.). When the ascites grew 8-10 days old, again peritoneal fluid was collected and Methyl cholanthrene induced ascites cells were transplanted in the peritoneal cavity of fresh BALB/c mice.

3.7.5.3.2 Experimental Animals

BALB/c mice weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.3.3 Experimental Protocol

Experimental protocol including calculations followed was essentially the same as described for Ehrlich ascites carcinoma except that BALB/c mice and Methyl cholanthrene induced ascites cells were used instead of non-inbred swiss mice and EAC cells, respectively.

3.7.5.4 Ehrlich Tumor (solid)

3.7.5.4.1 Propagation

EAC cells propagated in non-inbred swiss mice were used for inducing solid Ehrlich tumor in non-inbred swiss mice.

3.7.5.4.2 Experimental Animals

Non-inbred swiss mice weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.4.3 Experimental Protocol

Day 0: Induction of tumor

Peritoneal fluid was collected from an animal bearing 8-10 days old Ehrlich Ascites carcinoma. The number of tumor cells per ml of ascitic fluid was determined by counting the cells with the help of neubauer's chamber. The ascitic fluid was diluted with normal saline in such a way that 0.2 ml of the fluid contained $1x10^7$ EAC cells. All animals selected for conducting the experiment were injected intramuscularly in right thigh with 0.2ml of ascitic fluid containing $1x10^7$ EAC cells.

Day 1

On day 1, all animals injected with EAC cells were randomized and divided in different treatment and control groups. Tumor bearing control group contained 15 animals and all other groups (treatment and positive control) contained seven animals each. Animals in each group were weighed and an average body weight of animals in each group was worked out. Based on this body weight, test drugs were prepared for four days' daily administration in such a way that each dose was contained in 0.2ml volume. First dose was administered at 2.00 PM.

Day 2-4

Animals in the treatment and control groups were treated with respective test drugs at a fixed time (2.00 PM).

Day 5

Animals in each group were weighed and based on the average body weight for each group, test drugs were prepared as described for the next 5 days' daily administration. All animals were administered a dose at the same fixed time as earlier.

Day 6-9

Animals in the treated and control groups were treated with respective test solutions at a fixed time.

Day 13

The evaluation was done on day 13. Before evaluation, hair over the tumor-bearing thigh were removed.

Evaluation

Shortest and longest diameters of the tumor were measured with the help of vernier caliper and the weight of tumor was calculated using the following formula.

$$\text{Tumor weight (mg)} = \frac{\text{length (mm)} \times (\text{width[mm]})^2}{2}$$

Per cent tumor growth inhibition was calculated using the following formula.

$$\text{Per cent tumor growth inhibition} = \frac{\text{Av. Tumor wt. of Control group} - \text{Av. Tumor wt. of treated group}}{\text{Av. Tumor wt. of Control group}} \times 100$$

3.7.5.5 Sarcoma-180 (solid)

3.7.5.5.1 Propagation

Sarcoma-180 cells propagated in BALB/c mice were used for inducing Sarcoma-180 (solid) tumor in BALB/c mice.

3.7.5.5.2 Experimental Animals

BALB/c mice weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.5.3 Experimental Protocol

Experimental protocol including calculations followed was essentially the same as described for Ehrlich tumor (solid) except that BALB/c mice and Sarcoma-180 cells were used instead of non-inbred swiss mice and EAC cells, respectively.

3.7.5.6 L1210 Lymphoid leukemia

3.7.5.6.1 Propagation

L1210 Lymphoid leukemia cells were procured in frozen state from National Cancer Institute, USA. These cells were injected intraperitoneally in DBA/2 female mice and allowed to grow for the next seven days. On day 7, ascitic fluid from tumor bearing animal was withdrawn and diluted with normal saline. Cell number per ml of diluted ascitic fluid was determined with the help of Neubauer's chamber and the volume of ascitic fluid containing $1x10^6$ cells was arrived at. This volume of ascitic fluid was injected intraperitoneally in DBA/2 female mice (4-5). When the tumor grew 7 days old, again peritoneal fluid was collected and L1210 lymphoid leukemia cells were transplanted in the peritoneal cavity of fresh DBA/2 female mice.

3.7.5.6.2 Experimental Animals

CD2F1 mice (F1 hybrids of BALB/c females crossed with DBA/2 males) weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

3.7.5.6.3 Experimental Protocol

Test samples were evaluated for their *in vivo* anticancer activity against L1210 lymphoid leukemia as per the protocol described by Geran *et al.* (1972). The protocol was as follows:

Day 0: Induction of tumor

Peritoneal fluid was collected from an animal bearing 7 days old L1210 lymphoid leukemia and the number of tumor cells per ml of ascitic fluid was determined by counting the cells with the help of neubauer's chamber. The ascitic fluid was diluted with normal saline in such a way that 0.2 ml of fluid contained $1x10^6$ cells. All animals

selected for conducting the experiment were injected intraperitoneally with 0.2 ml of diluted ascitic fluid containing $1x10^6$ L1210 Lymphoid leukemia cells.

Day 1

On day 1, all animals injected with L1210 lymphoid leukemia cells were randomized and divided in different treatment and two control groups. All groups contained 6 animals each. Animals in each group were weighed and an average body weight of animals in each group was worked out. Based on the body weight, test drugs were prepared for four days' daily administration in such a way that each dose was contained in 0.2 ml volume. First dose was administered at 2.00 PM. One of the control groups (tumor bearing control) was treated with normal saline (0.2 ml) intraperitoneally and the other control group (positive control) was treated with 5-Fluorouracil (20 mg/kg) intraperitoneally.

Day 2-4

Animals in the treatment and control groups were treated with respective test drugs at a fixed time (2.00 PM).

Day 5

Animals in each group were weighed and based on the average body weight for each group, test drugs were prepared as described for the next 5 days. All animals were administered a dose at the same fixed time as earlier.

Day 6-9

Animals in the treated and control groups were treated with respective test solution at a fixed time and were observed daily for the mortality. Any mortality noticed was recorded on daily basis.

Day 12-18

Animals in all the groups were observed daily for the mortality. Any mortality noticed was recorded on daily basis.

Day 19: Evaluation

For evaluation of the experiment, Mean Survival Time (MST) of animals in each group was calculated using the following formula.

$$\text{Mean Survival Time (days)} = \frac{\Sigma S + AS_{(A-1)} - (B+1)NT}{S_{(A-1)} - NT}$$

Where,

A : Day on which deaths are no longer considered to be due to drug toxicity. For survival systems like L1210, P388, etc., Day A = Day 6.

B : Day beyond which control group survivors are considered 'no takes'. For survival systems like L1210, P388, etc., Day B = Day 18.

ΣS : Sum of daily survivors from Day A through Day B.

$S_{(A-1)}$: Number of survivors at the end of Day (A-1).

NT : Number of 'no takes'.

After calculating the Mean Survival Time in days, per cent T/C was calculated using the following formula for all test groups with more than 65 per cent survivors on Day 5.

$$\text{Per cent T/C} = \frac{\text{Mean Survival Time of treated group}}{\text{Mean Survival Time of tumor bearing control group}} \times 100$$

A T/C value of ≤ 85 per cent was taken to be a toxic test. A T/C ≥ 125 per cent was considered moderate activity and a T/C of > 150 per cent was considered significant activity.

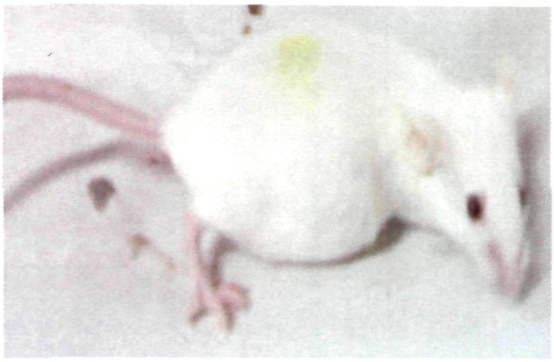
3.7.5.7 P388 Lymphocytic leukemia

3.7.5.7.1 Propagation

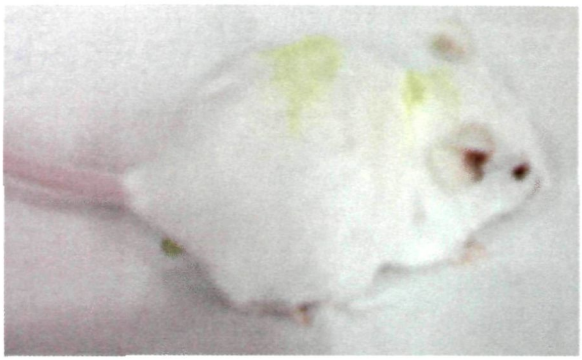
P388 Lymphocytic leukemia cells were procured in frozen state from National Cancer Institute, USA. These cells were injected intraperitoneally in DBA/2 female mice and allowed to grow for the next seven days. On day 7, ascitic fluid from tumor bearing animal was withdrawn and diluted with normal saline. Cell number per ml of diluted ascitic fluid was determined with the help of Neubauer's chamber and the volume of ascitic fluid containing $1x10^6$ cells was arrived at. This volume of ascitic fluid was injected intraperitoneally in DBA/2 female mice (4-5). When the tumor grew 7 days old, again peritoneal fluid was collected and P388 Lymphocytic leukemia cells were transplanted in the peritoneal cavity of fresh DBA/2 female mice.

3.7.5.7.2 Experimental Animals

CD2F1 mice (F1 hybrids of BALB/c females crossed with DBA/2 males) weighing 18-23 g were used for experimentation. All the animals used in a single experiment were of the same sex and were clinically free from any disease.

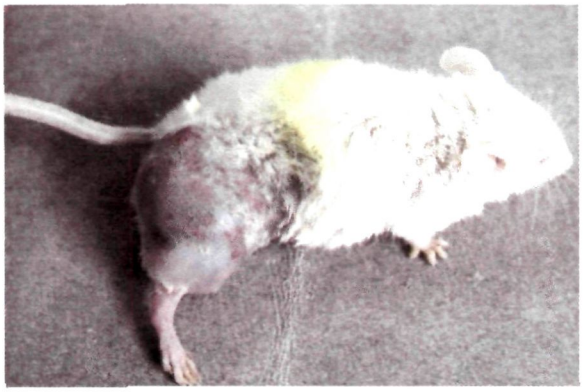


Ehrlich Ascitic Carcinoma bearing swiss mouse



Sarcoma-180 (ascites) bearing BALB/c mouse

Mehyl cholanthrene induced ascites bearing BALB/c mouse



Ehrlich tumor (solid) bearing swiss mouse

Sarcoma-180 (solid) bearing BALB/c mouse

L1210 lymphoid leukemia bearing DBA/2 mouse

P388 Lymphocytic leukemia bearing CD2F1 mouse

Fig. 3.26: Animals bearing transplantable tumors

3.7.5.7.3 Experimental Protocol

Experimental protocol including calculations followed was essentially the same as described for L1210 Lymphoid leukemia.

3.8 Toxicity studies with reference to myelosuppression

The most promising isolates identified on the basis of *in vitro* and *in vivo* anticancer studies as described above were evaluated for their myelosuppressive effects in Sarcoma-180 (solid) bearing animals by estimating the effect on femoral bone marrow cellularity. The animals transplanted with Sarcoma-180 cells ($1x10^7$) intramuscularly on day 0 were treated with test substances at doses yielding best anticancer activity daily for 9 consecutive days. On day 13, femur bones were obtained from these animals and their ends were snipped open with scissors. The marrow plugs were flushed out by forcefully injecting cold HBSS (Ca^{++} and Mg^{++} free) through the bone cavity by inserting a 26 gauze needle. The marrow plugs were dissociated into single cell suspension by repeatedly passing this suspension through 22 gauze needles and the total volume of single cell suspension was measured. Number of nucleated cells per ml of the single cell suspension was counted in a hemocytometer after treating the cells with 2 per cent glacial acetic acid in order to bring about lysis of the mature non-nucleated erythrocytes. Total number of nucleated cells per femur was arrived at by multiplying the volume (in ml) of single cell suspension by the number of cells per ml. Average number of cells per femur in treated animals were compared with 5-Fluorouracil treated positive control, normal saline treated tumor bearing control and normal untreated (without tumor) animals. All the operations were carried out aseptically.

3.9 Mechanistic (Apoptotic) studies

Apoptosis is a tightly regulated pathway responsible for the ordered removal of superfluous, aged and damaged cells. It not only plays an important role in the development and maintenance of tissue homeostasis, but it also represents an effective mechanism by which harmful cells can be eliminated (Thompson, 1995). Cancer cells are known to avoid apoptosis thus enabling themselves flourish at the cost of healthy neighbouring cells. Therefore, induction of apoptosis in cancer cells has been a novel approach for innovative mechanism based anticancer drug discovery (Workman, 1996).

Thus, the natural products capable of inducing apoptosis in cancer cells could be valuable anti-cancer therapeutics (Debatin, 2000).

Apoptosis is characterized by morphological and biochemical changes in cells occurring at different stages. Once triggered, it proceeds with different kinetics depending on cell types and culminates in cell disruption and formation of apoptotic bodies. In the present investigation, promising isolates identified on the basis of *in vitro* and *in vivo* studies were further studied for their apoptotic potential employing multi-paramertic assays such as detection of morphological and ultra-structural changes in cancer cells induced by the test samples through light microscopy, scanning electron microscopy and transmission electron microscopy and changes in mitochondrial membrane potential and cell cycle analysis through flow-cytometry.

3.9.1 Scanning electron microscopic studies

HL-60 cells were seeded in complete growth medium and treated with 25 µg/ml of the n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots and 10 µg/ml of n-hexane fraction from alcoholic extract of aerial parts of *Ipomoea nil* for 5 h and processed for scanning electron microscopy (SEM). Under *in vivo* studies, Ehrlich ascites carcinoma (EAC) bearing mice were treated with 0.2 ml of normal saline, 80 mg/kg of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots and 2 mg/kg of n-hexane fraction from alcoholic extract of aerial parts of *Ipomoea nil* from day 1-9. On day 12, cells collected from the ascitic fluid of treated animals were processed for scanning electron microscopy. The cells were sedimented at 1800 rpm for 10 min. Cell pellets were fixed instantly with 2.5 per cent glutaraldehyde in 0.1M Phosphare buffer (pH 7.2) at 4°C for 1 h, post-fixed with 1per cent OsO_4 in the same buffer, dehydrated with acetone, followed by amyl acetate and critical point dried using liquid CO_2. The samples were coated with gold using Sputter coater (Polaron) and observed under ASID at 40 KV in Electron microscope (JEOL, JEM-100CXII, Japan) as per the method described by Sharma *et al.*, (2009).

3.9.2 Light microscopy and transmission electron microscopic studies

Light microscopy and transmission electron microscopy studies were performed on Sarcoma-180 (solid) tumor tissue from animals treated with 80 mg/kg of n-butanol

fraction from alcoholic extract of *Asparagus racemosus* roots and 2 mg/kg of n-hexane fraction from alcoholic extract of *Ipomoea nil* (aerial parts). Small sized pieces of tumor tissue were dissected out from the treated and tumor bearing control animals and fixed immediately in a mixture of 2 per cent glutaraldehyde and 2 per cent paraformaldehyde in 0.1M Phosphate buffer (pH 7.2) at 4°C for 3 h, post-fixed with 1per cent OsO_4 for 3 h in the same buffer, dehydrated in acetone, followed by propylene oxide and embedded in epon-812 (Renó *et al.,* 1999; Sharma *et al.,* 2009). Semi-thin sections were cut using an ultramicrotome (Ultrotome-IV, LKB, Bromma, Sweden), stained with 1 per cent aqueous toluidine blue at 40°C and observed with a Vanox light microscope (Olympus). Ultrathin sections obtained with ultramicrotome were stained with 2 per cent aqueous uranyl acetate and lead citrate and examined with an electron microscope (JEOL, JEM-100CXII, Japan) at 60 KV. Minimum of 50 cells per sample were observed from three independent experiments to evaluate cellular and mitochondrial morphological alterations.

3.9.3 Flow-cytometric studies

In a flow cytometry system, large number of cells or particles flow within a laminar fluid stream in a single file passing through a laser beam where they are individually evaluated. As the focused laser beam interacts with a cell, it scatters light and in the case of fluorescent antibodies or dyes, fluorescence signals are created at the same time. The electronic signals are converted into digital values and are illustrated in dot plot or histogram plot. In the present studies, all measurements were performed on FACS-BD-LSR™ (Becton Dickinson USA), equipped with 488 nm argon-ion laser, using cell quest software.

3.9.3.1 Cell Cycle Analysis

Cell cycle phase specificity can be made use of for the measurement of DNA content in the apoptotic cells. Most commonly used dye for DNA content/cell cycle analysis is Propidium iodide (PI). It can be used to stain whole cells or isolated nuclei. The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centered around 600 nm. PI binds to DNA in cells at all stages of the cell cycle and the intensity with which a cell nucleus emits fluorescent light is directly proportional to its DNA content.

Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for optimal DNA resolution. Propidium iodide can also be excited in the U.V. light (351-364 nm) which should be considered when performing multi-color analysis on the multi-beam cell sorters (Singh *et al.*, 2007).

In the present investigation, method described by Kumar *et al.*, (2007) was employed. HL-60 cells ($1x10^6$ cells/ml/well) were seeded in 6 well plates and treated with 50 µg/ml concentrations of alcoholic extract of *Asparagus racemosus* roots and its chloroform and n-butanol fractions and 3 and 5 µg/ml concentrations of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts and incubated for 24 h. Cells were then washed with PBS and 3 ml of cold absolute ethanol was added to it in order to fix the cells for at least 1 hour at 4°C. Cells may be stored in 70 per cent ethanol at -20°C for several weeks prior to PI staining and flow-cytometric analysis. Cells were again washed with PBS, subjected to proteinase-K and RNase digestion followed by staining of clean nuclear materials (nuclei) with propidium iodide using procedures described in the instruction manual of the Cycle Test plus DNA reagent kit (Becton Dickinson, USA). The preparations were analysed for DNA content using BD-LSR Flow-cytometer. Data were collected in list mode on 10,000 events for FL2-A vs. FL2-W. Apoptotic nuclei appear as a broad hypodiploid DNA peak at lower fluorescence intensity as compared to nuclei in G0/G1 phase.

3.9.3.2 Mitochondrial Membrane Potential

The mitochondrial respiratory chain produces energy which is stored as an electrochemical gradient and consists of a transmembrane electric potential, negative inside (about 180-200 mV) and a proton gradient of about 1 unit. This energy is then able to drive the synthesis of ATP, a crucial molecule for a variety of intracellular processes. Mitochondrial dysfunction within the apoptotic process is often associated with loss of mitochondrial transmembrane potential. Several membrane permeable lipophilic cations accumulated by living cells, organelles and liposomes exhibiting a negative interior membrane potential, have been used to study Mitochondrial Membrane Potential ($\Delta\psi$). Such probes include those which exhibit optical and fluorescence activity after accumulation into energized systems, such as 3,3'-diehexiloxadicarbocyanine iodide [$DiOC_6(3)$], nonylacridine orange (NAO), safranine O, rhodamine-123 (Rh-123) etc. Rhodamine-123 is a fluorescent probe that selectively enters mitochondria with an intact

membrane potential and is retained in the mitochondria. The mitochondrial fluorescence intensity of Rhodamine 123 decreases quantitatively in response to dissipation of the mitochondrial membrane potential. In the present studies, Rhodamine-123 was used to evaluate perturbations in mitochondrial membrane potential induced by the test sample(s).

HL-60 cells ($1x10^6$/1.5ml/well) were seeded in 6 well plates and were treated with 50 µg/ml concentrations of alcoholic extract of *Asparagus racemosus* roots and its chloroform and n-butanol fractions and 3 and 5 µg/ml concentrations of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts and incubated for 24 h. Rhodamine-123 (10 nM) was added to it 1 hr before the termination of experiment. Cells were washed with PBS and suspended in sheath fluid. Propidium iodide (5 µg/ml) was added just before analysis to the samples. Intensity of Fluorescence was analyzed from 10,000 events in FL-1 channel on Flow cytometer.

3.10 Statistical analysis

Data were expressed as means ± SE unless otherwise indicated. Comparisons were made between control and treated groups using Student's unpaired *t*-test and p values <0.05 were considered significant.

Chapter IV

Results

CHAPTER – IV

RESULTS

Anticancer activity of *Azadirachta indica* (leaves), *Asparagus racemosus* (roots), *Ipomoea nil* (aerial parts), *Costus speciosus* (aerial parts) and *Butea monosperma* (flowers) was systematically studied in the present investigation. After collection and authentication of the plants/parts, three extracts (alcoholic, hydro-alcoholic and aqueous) were prepared from the specified plant parts and these extracts were studied for their *in vitro* cytotoxic potential against a panel of twelve cancer cell lines of different tissue origin (colon, CNS, prostate, lungs, breast, ovary, liver, neuroblastoma and bone). On the basis of *in vitro* cytotoxic activity, the promising plant extracts were identified and fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and investigated for their *in vitro* cytotoxic potential against the same panel of twelve cancer cell lines of different tissue origin and the most promising fractions were identified. The most promising fractions along with their respective extracts were further studied for their *in vivo* anticancer activity against seven models of murine tumors (Ehrlich ascites carcinoma, Sarcoma-180 (ascites), Methyl cholanthrene induced ascites, Ehrlich tumor (solid), Sarcoma-180 (solid), L1210 Lymphoid leukemia and P388 Lymphocytic leukemia) and myelosuppressive effects in tumor bearing animals. The most active fractions identified on the basis of *in vivo* studies were further studied for their apoptotic potential employing light microscopic, electron microscopic and flow-cytometric techniques.

4.1 *In vitro* cytotoxic activity of extracts and fractions

All extracts prepared from the specified plant parts were studied for *in vitro* cytotoxic activity against a panel of twelve cancer cell lines of different tissue origin (viz., Colo-205, 502713, HCT-15 and SW-620 from colon, SK-N-SH from CNS, Du-145 from prostate, A-549 from lungs, MCF-7 from breast, OVCAR-5 from ovary, HEP-2 from liver, IMR-32 from neuroblastoma and D17 from bone) at three different concentrations (10, 30 and 100 μg/ml) in terms of per cent growth inhibition of cancer cells in culture. More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 μg/ml respectively, was considered promising cytotoxic activity. Likewise, less than 10, 20 and 30 per cent growth inhibition at 10, 30 and 100 μg/ml respectively, was considered poor cytotoxic activity and the activity ranging between 10-30, 20-50 and 30-70 per cent at 10, 30 and 100 μg/ml respectively, was considered moderate cytotoxic activity. Fractions prepared from promising extracts were similarly studied for their cytotoxic activity at 10, 30 and 50 μg/ml concentrations and the growth inhibition of 30, 40 and 50 per cent at these concentrations, respectively, was considered promising cytotoxic activity. Extracts and fractions showing promising cytotoxic activity against more than 50 per cent of the cell lines studied were taken up for further investigation with respect to their *in vivo* anticancer activity against murine tumor models.

4.1.1 *Azadirachta indica* (leaves)

The alcoholic extract of *Azadirachta indica* leaves exhibited 11 to 44 per cent growth inhibition in various cell lines except OVCAR-5 which showed no growth inhibition at 10 μg/ml concentration. Only three cell lines showed promising growth inhibition (more than 30 per cent) at this concentration. However, at 30 μg/ml concentration, the cytotoxic activity ranged between 36-76 per cent with seven cell lines exhibiting more than 50 per cent growth inhibition (Table 4.1, Fig. 4.1). The extract at 100 μg/ml was most active showing cytotoxic activity ranging from 57-94 per cent. Except two cell lines (MCF-7 and D17), all the cell lines exhibited more than 70 per cent growth inhibition at this concentration. Hydro-alcoholic extract showed 0-32, 1-45 and 19-77 per cent growth inhibition at 10, 30 and 100 μg/ml, respectively. Only one cell line each showed promising growth inhibition at 10 and 100 μg/ml concentrations.

Table 4.1: ***In vitro*** **cytotoxic activity of different extracts of** ***Azadirachta indica*** **(leaves) against a panel of cancer cell lines**

Extracts of *Azadirachta indica* (leaves)	**Conc. (µg/ml)**	**TISSUES**											
		COLON				**CNS**	**PROSTATE**	**LUNGS**	**BREAST**	**OVARY**	**LIVER**	**Neuroblastoma**	**Bone**
		Cell lines											
		Colo-205	**502713**	**HCT-15**	**SW-620**	**SKNSH**	**Du-145**	**A-549**	**MCF-7**	**OVCAR-5**	**HEP-2**	**IMR-32**	**D17**
		Per cent Growth Inhibition											
Alcoholic extract	10	22	21	43	17	28	11	44	11	0	40	20	18
	30	36	56	76	66	69	49	54	38	48	66	72	37
	100	91	83	78	83	93	81	81	57	94	73	81	67
Hydro-alcoholic extract	10	8	22	18	0	8	4	23	13	5	17	32	7
	30	14	45	25	1	27	6	27	18	11	23	32	22
	100	67	77	62	24	51	19	38	46	61	51	41	27
Aqueous extract	10	6	4	6	0	3	0	7	12	0	3	0	0
	30	11	12	7	2	5	0	10	17	0	5	0	0
	100	15	19	9	5	20	0	11	20	1	5	8	13
Positive Controls													
Paclitaxel	$1x10^{-5}M$	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2 x10^{-5}M$	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}M$	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x 10^{-6}M$	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

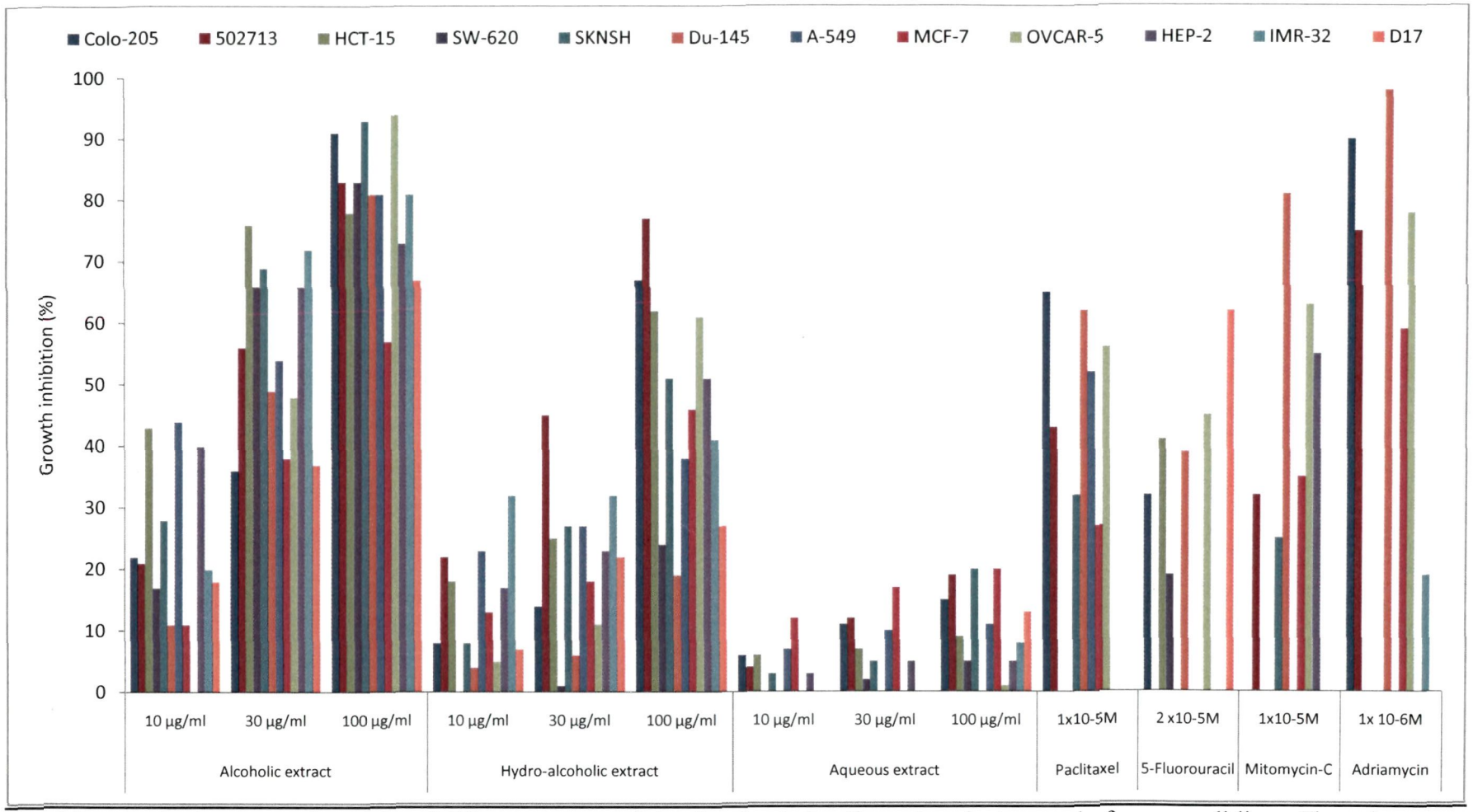


Fig. 4.1: *In vitro* cytotoxic activity of different extracts of *Azadirachta indica* (leaves) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

Similarly, the cytotoxic activity of aqueous extract was 0-12 per cent at 10 μg/ml, 0-17 per cent at 30 μg/ml and 0-20 per cent at 100 μg/ml concentrations with none of the cell lines showing promising growth inhibition at any of the concentrations studied.

Out of three extracts of leaves of *Azadirachta indica* tested, only the alcoholic extract significantly inhibited the growth in more than 50 percent of the cell lines studied at 30 and 100 μg/ml concentrations and therefore was considered to possess promising cytotoxic activity. It was further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and all the fractions were again evaluated for their cytotoxic activity against the same panel of twelve cancer cell lines at 10, 30 and 50 μg/ml concentrations. Results of this cytotoxic activity are presented in Table 4.2 and Fig. 4.2.

n-Hexane fraction did not show much promise as it could inhibit the growth of different cell lines to the extent of 30 and 53 per cent at 10 and 30 μg/ml, respectively. At these concentrations only one cell line exhibited promising cytotoxicity. With 50 μg/ml concentration of n-hexane fraction although cytotoxic activity ranged between 0-79 per cent, only one cell line (502713) was inhibited from growing more than 50 per cent. Thus, only one cell line (502713) showed promising activity at all the concentrations used.

Chloroform fraction showed 0-41 per cent growth inhibition of cancer cells at 10 μg/ml concentration. However, this fraction at 30 μg/ml concentration exhibited cytotoxic activity in the range of 0-71 per cent against different cell lines with five cell lines showing 40 per cent or more growth inhibition (Table 4.2, Fig. 4.2). This fraction at 50 μg/ml concentration was the most active exhibiting cytotoxicity in the range of 0-97 per cent against different cell lines and eight cell lines showing more than 50 per cent growth inhibition. It was particularly effective against colon (Colo-205, 502713) and prostate (Du-145) cancer cell lines.

n-Butanol fraction from alcoholic extract of *Azadirachta indica* leaves showed 0-45 per cent cytotoxic activity against different cell lines with only one of the cell lines exhibiting more than 30 per cent growth inhibition at 10 μg/ml concentration. At 30 and 50 μg/ml, two and three cell lines respectively showed more than 40 and 50 per cent

Table 4.2: ***In vitro*** **cytotoxic activity of different fractions from alcoholic extract of** ***Azadirachta indica*** **(leaves) against a panel of cancer cell lines**

Fractions from *Alcoholic Extract of Azadirachta indica* (leaves)	Conc. (µg/ml)	TISSUES											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	502713	HCT-15	SW-620	SKNSH	Du-145	A-549	MCF-7	OVCAR-5	HEP-2	IMR-32	D17
		Per cent Growth Inhibition											
n-hexane fraction	10	0	30	8	4	4	9	13	16	0	0	0	0
	30	0	53	12	4	18	21	22	32	15	7	16	0
	50	0	79	23	0	32	36	39	48	32	19	34	0
Chloroform fraction	10	20	36	22	0	6	41	11	22	0	0	8	16
	30	56	66	40	0	24	71	37	52	21	4	32	38
	50	88	96	58	0	46	97	55	79	35	8	50	62
n-butanol fraction	10	0	45	10	6	28	16	7	4	0	6	0	0
	30	11	70	23	11	42	39	18	13	0	16	7	20
	50	19	84	38	22	59	54	33	29	10	27	14	34
Aqueous fraction	10	9	0	5	0	8	0	0	0	10	5	0	8
	30	26	0	12	0	19	0	0	8	12	10	0	14
	50	41	0	21	0	33	0	0	15	17	15	1	27
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 40 and 50 per cent growth inhibition at 10, 30 and 50 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

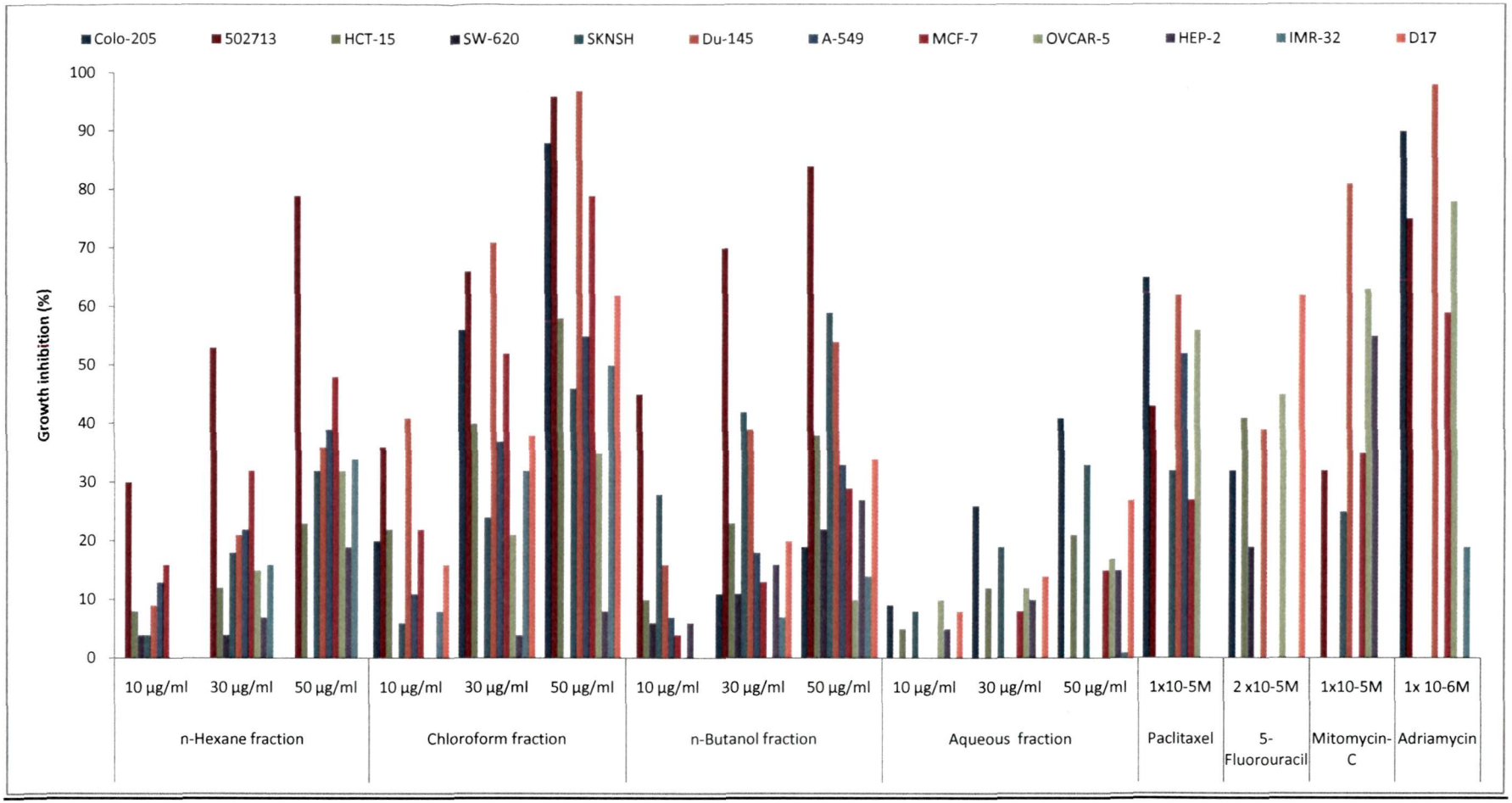


Fig. 4.2: *In vitro* cytotoxic activity of different fractions from alcoholic extract of *Azadirachta indica* (leaves) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

growth inhibition. The aqueous fraction had almost negligible cytototxic activity. It showed 0-10, 0-26 and 0-41 per cent growth inhibition of different cell lines at 10, 30 and 50 μg/ml concentrations respectively with none of the cell lines showing promising cytotoxicity at any of the concentrations studied.

Out of the four fractions from alcoholic extract of *Azadirachta indica* leaves only the chloroform fraction showed promising cytotoxic activity against eight cell lines at 50 μg/ml concentration. Therefore, the alcoholic extract of *Azadirachta indica* leaves and its chloroform fraction were considered to be of promise and selected for further *in vivo* investigations against murine cancer models.

4.1.2 *Asparagus racemosus* (roots)

The alcoholic extract of *Asparagus racemosus* roots exhibited moderate (0-80 per cent) cytotoxicity against different cell lines at 10 μg/ml concentration with five cell lines showing more than 30 per cent growth inhibition. At 30 μg/ml, the cytotoxicity ranged between 38-95 per cent and ten cell lines showed more than 50 per cent growth inhibition. Higher concentration (100 μg/ml) was the most active one showing 49-98 per cent cytotoxicity against different cell lines with nine of the twelve cell lines exhibiting more than 70 per cent growth inhibition (Table 4.3, Fig. 4.3).

Hydro-alcoholic extract showed either poor or moderate (0-27 per cent) cytotoxic activity at 10 μg/ml and none of the cell lines exhibited promising cytotoxicity. At 30 μg/ml, the cytotoxicity ranged from 35-86 per cent and eight of the cell lines studied showed more than 50 per cent growth inhibition. Cytotoxicity ranged between 63-98 per cent at 100 μg/ml concentration with seven cell lines exhibiting more than 70 per cent growth inhibition. The cytotoxic activity of aqueous extract of *Asparagus racemosus* roots was either poor or moderate as it ranged from 0-25, 0-31 and 0-49 per cent against different cell lines at 10, 30 and 100 μg/ml concentrations, respectively and none of the cell lines studied showed more than 30, 50 or 70 per cent growth inhibition at respective concentrations. Perusal of results given in Table 4.3 and Fig. 4.3 showed that most of the cytotoxic activity was confined in alcoholic extract of *Asparagus racemosus* roots and therefore, this extract was studied further by fractionating it into

Table 4.3: ***In vitro*** **cytotoxic activity of different extracts of** ***Asparagus racemosus*** **(roots) against a panel of cancer cell lines**

Extracts of *Asparagus racemosus* (roots)	Conc. (µg/ml)	TISSUES											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	502713	HCT-15	SW-620	SKNSH	Du-145	A-549	MCF-7	OVCAR-5	HEP-2	IMR-32	D17
		Per cent Growth Inhibition											
Alcoholic extract	10	16	19	13	45	23	0	80	14	21	52	35	46
	30	83	47	66	87	38	68	95	55	80	66	77	72
	100	93	49	76	89	62	70	98	69	96	90	82	88
Hydro-alcoholic extract	10	9	17	10	0	24	0	19	0	6	27	8	12
	30	63	57	45	35	47	51	48	61	86	62	82	52
	100	82	63	71	64	78	67	68	69	98	95	87	72
Aqueous extract	10	12	13	7	6	17	0	25	4	0	11	0	8
	30	18	14	15	9	23	2	31	5	0	18	0	29
	100	22	15	20	18	29	6	32	9	1	33	0	49
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2 x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x 10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

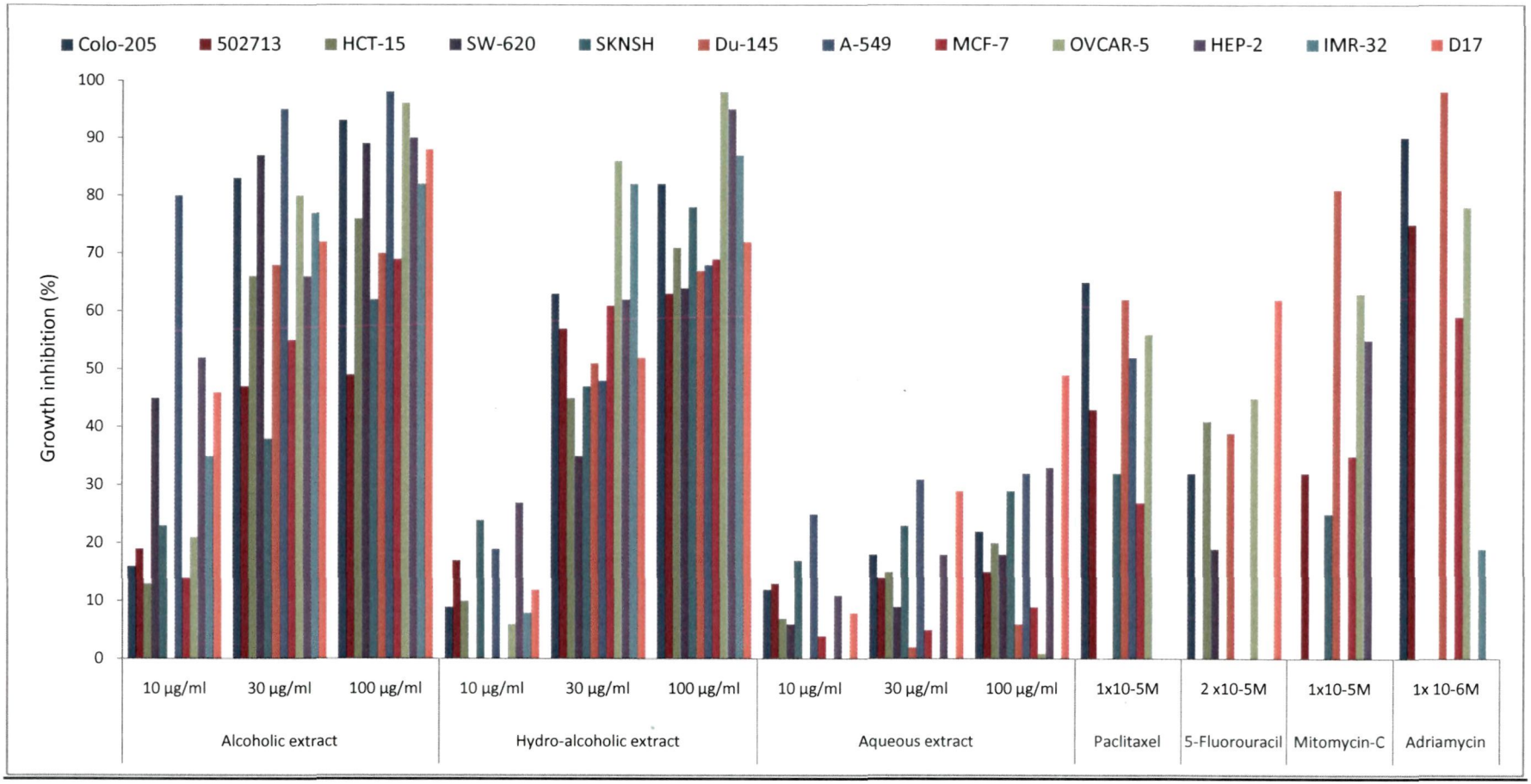


Fig. 4.3: *In vitro* cytotoxic activity of different extracts of *Asparagus racemosus* (roots) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

n-hexane, chloroform, n-butanol and aqueous fractions. All these fractions were again evaluated for their cytotoxic potential against the same panel of twelve cancer cell lines and the results are summarized in Table 4.4 and Fig. 4.4.

n-Hexane fraction could maximally inhibit the growth of different cell lines to the extent of 33, 48 and 67 per cent at 10, 30 and 50 μg/ml concentrations, respectively. Only 1, 2 and 2 cell lines exhibited more than 30, 40 and 50 per cent growth inhibition at respective concentrations. Cytotoxicity observed with chloroform fraction ranged between 12-49 per cent at 10 μg/ml concentration with six cell lines showing more than 30 per cent growth inhibition. At 30 μg/ml, it exhibited 24-74 per cent cytotoxicity with seven cell lines showing more than 40 per cent growth inhibition (Table 4.4, Fig. 4.4). At 50 μg/ml concentration, the cytotoxicity varied between 36-99 per cent and seven cell lines exhibited more than 50 per cent growth inhibition. n-Butanol fraction was observed to be little more active. At 10 μg/ml, cytotocity was up to 59 per cent with eight cell lines showing more than 30 per cent growth inhibition. With 30 μg/ml, the cytotoxicity ranged between 16-81 per cent and nine cell lines exhibited more than 40 per cent growth inhibition. Higher concentration (50 μg/ml) was even more cytotoxic (25-99 per cent) as eight cell lines exhibited more than 50 per cent growth inhibition at 50 μg/ml.

The aqueous fraction did not show any promise as far as its cytotoxic potential against different cell lines is concerned. It exhibited 0-14, 0-21 and 0-39 per cent cytotoxicity at 10, 30 and 50 μg/ml concentrations, respectively with none of the cell lines showing more than 30, 40 or 50 per cent growth inhibition at respective concentrations (Table 4.4, Fig. 4.4).

Thus, it can be deduced that out of the four fractions from alcoholic extract of *Asparagus racemosus* roots the n-butanol fraction was the most promising and enriched with excellent cytotoxicity. Chloroform fraction also possessed good cytotoxic activity against little less number of cell lines than that of n-butanol fraction. Therefore, the alcoholic extract of *Asparagus racemosus* roots and its chloroform and n-butanol fractions were selected for further *in vivo* investigations against murine cancer models.

Table 4.4: ***In vitro*** **cytotoxic activity of different fractions from alcoholic extract of** ***Asparagus racemosus*** **(roots) against a panel of cancer cell lines**

Fractions from *Alcoholic Extract of Asparagus racemosus* (roots)	Conc. (µg/ml)	TISSUES											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	502713	HCT-15	SW-620	SKNSH	Du-145	A-549	MCF-7	OVCAR-5	HEP-2	IMR-32	D17
		Per cent Growth Inhibition											
n-hexane fraction	10	0	27	4	6	8	10	0	6	17	0	33	3
	30	0	41	12	16	15	21	9	14	32	0	48	17
	50	0	67	19	22	25	39	15	30	47	10	67	31
Chloroform fraction	10	28	39	12	30	24	33	40	21	14	49	31	23
	30	42	69	24	50	36	66	51	38	26	74	49	34
	50	63	89	36	70	48	99	66	49	43	95	78	47
n-butanol fraction	10	49	43	11	24	15	56	0	46	51	48	45	59
	30	76	67	22	42	34	81	16	70	79	80	76	79
	50	99	98	33	48	43	98	25	88	90	93	94	91
Aqueous fraction	10	0	6	9	0	4	5	0	10	13	0	4	14
	30	5	8	17	0	8	10	0	20	21	0	7	18
	50	13	17	37	9	8	15	0	30	39	0	12	26
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 40 and 50 per cent growth inhibition at 10, 30 and 50 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

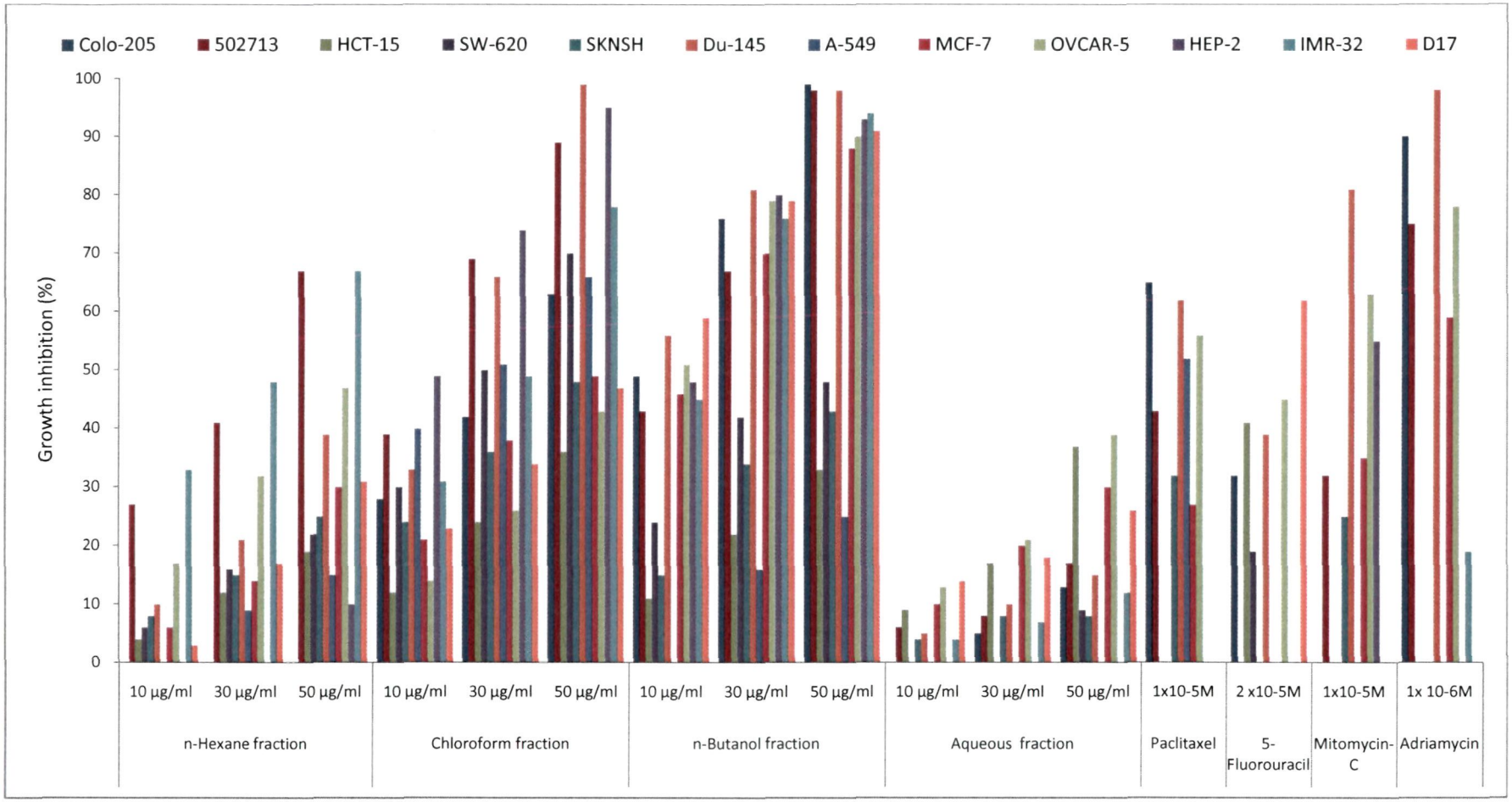


Fig. 4.4: *In vitro* cytotoxic activity of different fractions from alcoholic extract of *Asparagus racemosus* (roots) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

4.1.3 *Ipomoea nil* (aerial parts)

Alcoholic extract of *Ipomoea nil* aerial parts showed an impressive cytotoxicity even at 10 μg/ml concentration, exhibiting growth inhibition in the range of 4-84 per cent with seven of the cell lines showing more than 30 per cent growth inhibition. 30 μg/ml concentration was quite cytotoxic, inhibiting the growth of ten out of twelve cell lines by more than 50 per cent. The alcoholic extract at 100 μg/ml concentration exhibited 83-100 per cent cytotoxic activity and all the twelve cancer cell lines studied showed more than 70 per cent growth inhibition (Table 4.5, Fig. 4.5).

The cytotoxicity of hydro-alcoholic extract was less than the alcoholic extract. At 10 μg/ml, the growth inhibition was 0-32 per cent. 30 μg/ml concentration showed 0-88 per cent cytotoxicity with five cell lines showing more than 50 per cent growth inhibition. At 100 μg/ml concentration, the cytotoxicity ranged between 20-98 per cent with the same five of the twelve cell lines exhibiting more than 70 per cent growth inhibition.

The aqueous extract had lesser cytotoxic effects as compared to hydro-alcoholic and alcoholic extracts, ranging from 0-39 per cent at 10 μg/ml, 4-53 per cent at 30 μg/ml and 19-89 per cent at 100 μg/ml against different cell lines. Only two, one and three cell lines showed more than 30, 50 and 70 per cent growth inhibition at three respective concentrations.

The alcoholic extract was considered to hold definite promise as maximum cytotoxicity was observed in alcoholic extract followed by hydro-alcoholic extract. Therefore, the alcoholic extract was further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and all the fractions were again evaluated for their cytotoxic potential against the same panel of twelve cancer cell lines.

n-Hexane fraction emerged to be the most cytotoxic one, which inhibited the growth of different cell lines between 32-66 per cent at a concentration of 10 μg/ml. Even at such a low concentration, all the twelve cell lines exhibited promising activity of more than 30 per cent growth inhibition. At 30 μg/ml also, all the twelve cell lines studied showed more than 40 per cent growth inhibition with overall cytotoxicity ranging between 48-82

Table 4.5: ***In vitro*** **cytotoxic activity of different extracts of** ***Ipomoea nil*** **(aerial parts) against a panel of cancer cell lines**

Extracts of *Ipomoea nil* (aerial parts)	Conc. (µg/ml)	TISSUES											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	502713	HCT-15	SW-620	SKNSH	Du-145	A-549	MCF-7	OVCAR-5	HEP-2	IMR-32	D17
		Per cent Growth Inhibition											
Alcoholic extract	10	21	27	84	40	42	12	59	24	4	61	34	59
	30	58	71	94	42	81	82	85	67	43	93	78	81
	100	83	84	95	95	91	96	97	81	100	98	85	97
Hydro-alcoholic extract	10	6	2	19	0	8	0	32	11	0	5	0	28
	30	7	6	88	46	16	0	73	15	0	71	56	51
	100	65	41	92	64	45	43	87	47	20	98	78	71
Aqueous extract	10	11	16	31	7	20	0	39	18	0	26	12	14
	30	14	17	37	20	27	12	53	30	4	41	13	27
	100	62	33	80	59	55	56	89	51	19	87	68	36
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

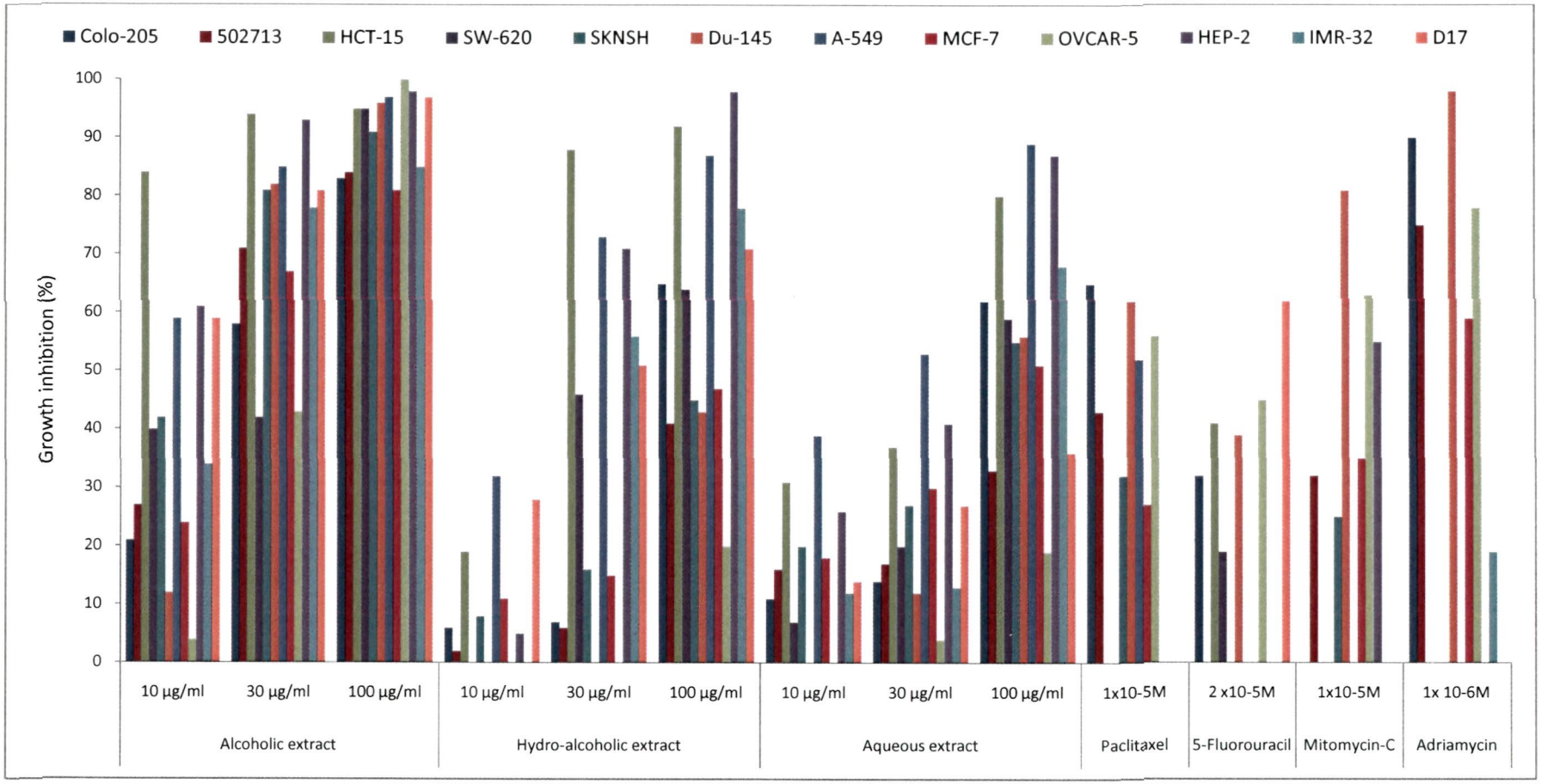


Fig. 4.5: *In vitro* cytotoxic activity of different extracts of *Ipomoea nil* (aerial parts) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

per cent. 50 μg/ml concentration also showed promising cytotoxicity with all the twelve cell lines studied exhibiting more than 50 per cent growth inhibition and the overall cytotoxicity ranging between 62-96 per cent (Table 4.6, Fig. 4.6).

Chloroform fraction also exhibited good cytotoxicity but it was little less than the n-hexane fraction. 10 μg/ml concentration showed cytotoxicity in the range of 18-73 per cent with seven cell lines showing more than 30 per cent growth inhibition. At 30 μg/ml concentration, nine cell lines showed more than 40 per cent growth inhibition with overall cytotoxicity from 33-86 per cent. With 50 μg/ml concentration, the cytotoxicity ranged between 43-94 per cent and seven of the twelve cell lines exbibited more than 50 per cent growth inhibition.

n-Butanol fraction did not show good cytotoxicity as there was 2-39, 11-51 and 19-65 per cent growth inhibition with 10, 30 and 50 μg/ml concentrations, respectively. Only one cell line (SK-N-SH) exhibited more than 30 and 40 per cent growth inhibition at 10 and 30 μg/ml concentrations. At 50 μg/ml concentration also, only four cell lines showed more than 50 per cent cytotoxicity. The aqueous fraction had almost no cytotoxic potential as there was no growth inhibition of any cell line at a concentration of 10 μg/ml. Even at higher concentrations of 30 and 50 μg/ml, the cytotoxicity ranged between 0-8 and 0-16 per cent only (Table 4.6, Fig. 4.6).

From four fractions of alcoholic extract of *Ipomoea nil* aerial parts, the n-hexane fraction had maximum cytotoxic potential and was considered to hold significant promise as far as anticancer activity is concerned. Therefore, the alcoholic extract of *Ipomoea nil* aerial parts and its n-hexane fraction were selected for further *in vivo* studies against murine cancer models.

Table 4.6: ***In vitro*** **cytotoxic activity of different fractions from alcoholic extract of** ***Ipomoea nil*** **(aerial parts) against a panel of cancer cell lines**

Fractions from *Alcoholic Extract of Ipomoea nil* (aerial parts)	Conc. (µg/ml)	**TISSUES**											
		COLON				**CNS**	**PROSTATE**	**LUNGS**	**BREAST**	**OVARY**	**LIVER**	**Neuroblastoma**	**Bone**
		Cell lines											
		Colo-205	**502713**	**HCT-15**	**SW-620**	**SKNSH**	**Du-145**	**A-549**	**MCF-7**	**OVCAR-5**	**HEP-2**	**IMR-32**	**D17**
		Per cent Growth Inhibition											
n-hexane fraction	10	65	55	32	46	70	56	60	57	37	47	66	52
	30	78	72	48	59	82	69	79	80	49	58	81	78
	50	92	87	62	78	96	86	91	94	71	79	91	90
Chloroform fraction	10	73	22	28	18	52	37	46	50	28	32	44	26
	30	86	34	42	33	73	52	58	68	36	43	55	44
	50	94	46	49	43	90	76	78	87	47	49	79	64
n-butanol fraction	10	11	6	18	14	39	4	19	5	2	8	11	18
	30	32	13	33	28	51	16	32	13	11	17	31	37
	50	49	21	47	38	65	29	47	25	19	40	43	49
Aqueous fraction	10	0	0	0	0	0	0	0	0	0	0	0	0
	30	0	4	2	0	0	8	2	0	0	1	0	0
	50	6	10	8	0	0	12	7	0	0	3	2	16
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 40 and 50 per cent growth inhibition at 10, 30 and 50 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

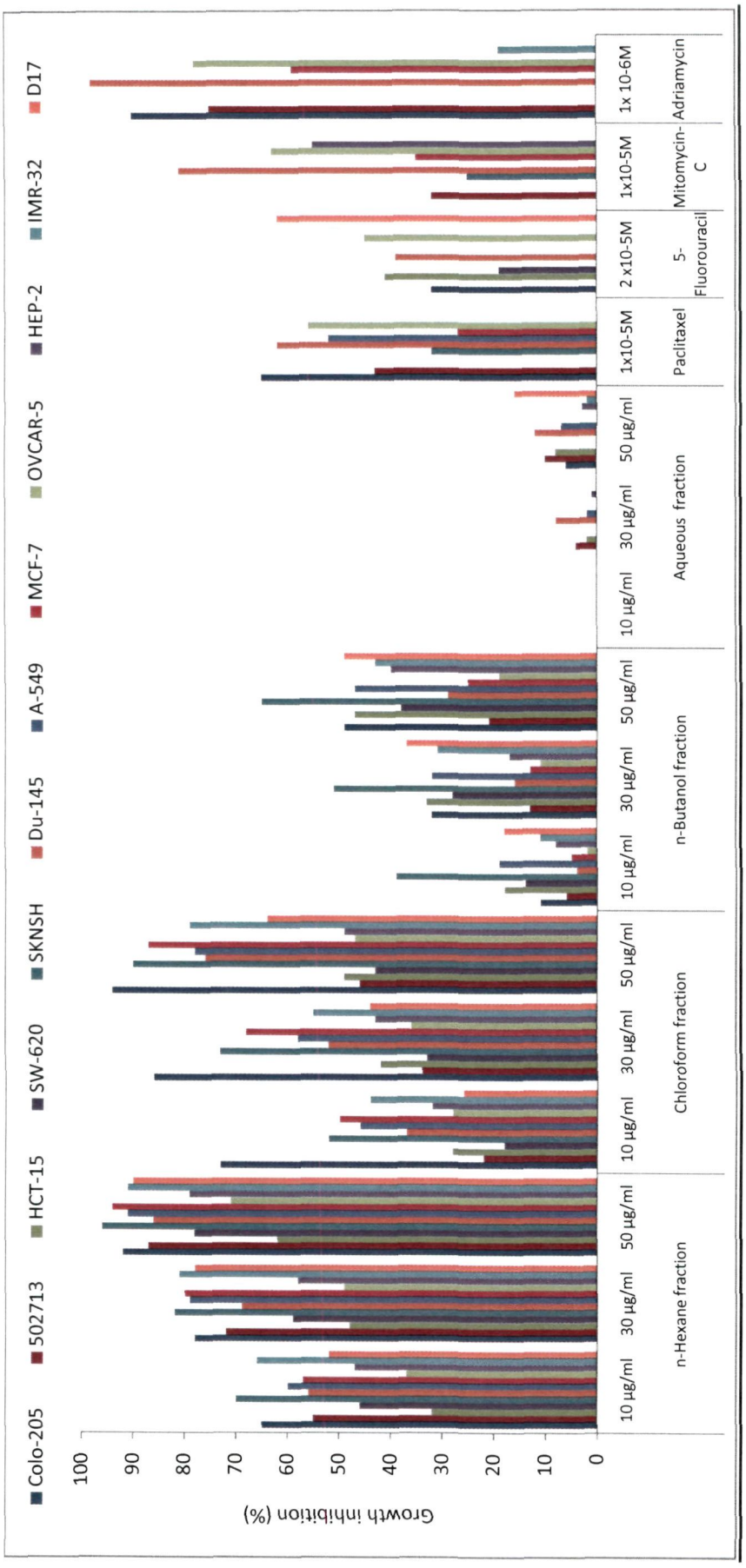


Fig. 4.6: *In vitro* cytotoxic activity of different fractions from alcoholic extract of *Ipomoea nil* (aerial parts) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

4.1.4 *Costus speciosus* (aerial parts)

The alcoholic extract of *Costus speciosus* aerial parts showed 0-50, 0-61 and 0-64 per cent growth inhibition of different cell lines at 10, 30 and 100 μg/ml concentrations, respectively. At 10 and 30μg/ml, only three and two cell lines exhibited more than 30 and 50 per cent cytotoxicity, respectively. Higher concentration of 100 μg/ml could not inhibit the growth of any cell line beyond 64 per cent (Table 4.7, Fig. 4.7). The cytotoxic potential of alcoholic extract was considered to be non-promising as most of the cell lines did not show promising growth inhibition.

Likewise, the hydro-alcoholic extract showed 0-28, 0-35 and 12-61 per cent cytotoxicity at 10, 30 and 100 μg/ml concentrations, respectively. As none of the cell lines exhibited more than 30, 50 or 70 per cent growth inhibition at 10, 30 and 100 μg/ml concentrations, cytotoxic potential of hydro-alcoholic extract too was not considered to be promising. The aqueous extract exhibited 0-23, 0-29 and 0-45 per cent cytotoxic activity at 10, 30 and 100 μg/ml concentrations, respectively and the results were not promising.

None of the extracts of aerial parts of *Costus speciosus* exhibited promising cytotoxic activity at any of the concentrations studied. Therefore, it was concluded that the aerial parts of *Costus speciosus* did not possess significant anticancer potential and was not taken up for further studies.

4.1.5 *Butea monosperma* (flowers)

Perusal of results presented in Table 4.8 and Fig. 4.8 reveals that the alcoholic extract of *Butea monosperma* flowers had 0-8, 0-23 and 0-45 per cent cytotoxic effects against different cell lines at 10, 30 and 100 μg/ml concentrations, respectively. None of the cell lines showed promising results at any of the concentrations. Similarly, the hydro-alcoholic extract exhibited 0-16, 0-28 and 0-58 per cent cytotoxicity at 10, 30 and 100 μg/ml concentrations, respectively and the growth of none of the cell lines was inhibited beyond 58 per cent even at highest concentration of 100 μg/ml. Aqueous extract inhibited the growth of different cell lines to the extent of 9, 36 and 95 per cent only at 10, 30 and 100 μg/ml concentrations, respectively. Only two cell lines (Colo-205 and

Table 4.7: ***In vitro*** **cytotoxic activity of different extracts of** ***Costus speciosus*** **(aerial parts) against a panel of cancer cell lines**

Extracts of *Costus speciosus* (aerial parts)	Conc. (µg/ml)	TISSUES											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	502713	HCT-15	SW-620	SKNSH	Du-145	A-549	MCF-7	OVCAR-5	HEP-2	IMR-32	D17
		Per cent Growth Inhibition											
Alcoholic extract	10	0	14	4	0	11	5	50	16	7	33	13	45
	30	1	15	9	0	16	12	61	19	11	37	18	57
	100	6	21	26	0	26	18	64	31	19	49	22	67
Hydro-alcoholic extract	10	5	6	0	0	2	2	16	16	0	28	4	11
	30	8	14	0	0	11	4	32	22	11	35	4	17
	100	21	21	15	45	27	42	61	22	27	49	12	26
Aqueous extract	10	4	23	19	0	3	2	16	2	8	5	0	0
	30	11	27	19	0	17	4	29	7	10	11	0	8
	100	21	28	22	1	31	16	45	21	17	19	0	11
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5-Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomycin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

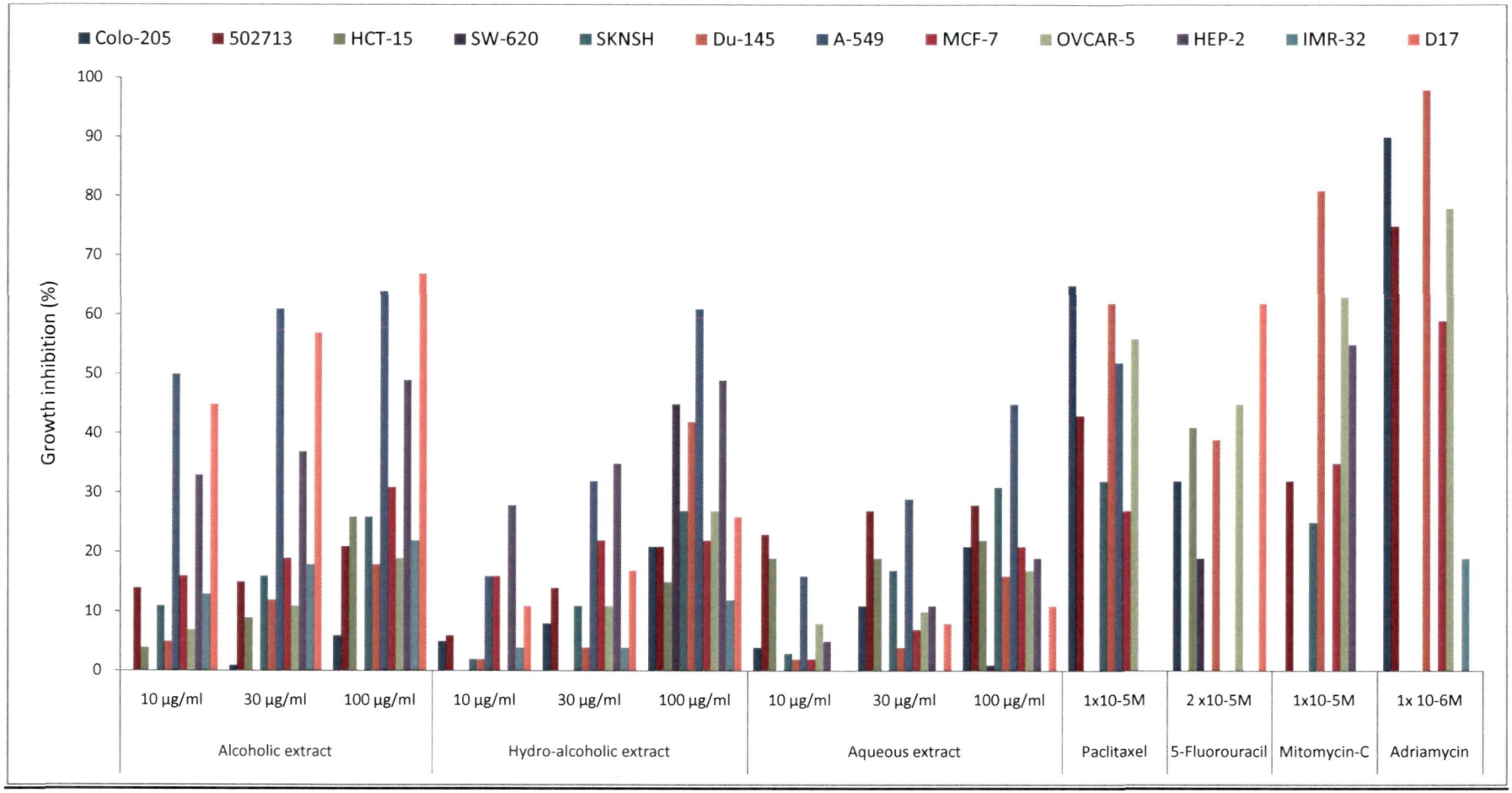


Fig. 4.7: *In vitro* cytotoxic activity of different extracts of *Costus speciosus* (aerial parts) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

Table 4.8: ***In vitro*** **cytotoxic activity of different extracts of** ***Butea monosperma*** **(flowers) against a panel of cancer cell lines**

Extracts of *Butea monosperma* (flowers)	Conc. (µg/ml)	**TISSUES**											
		COLON				CNS	PROSTATE	LUNGS	BREAST	OVARY	LIVER	Neuroblastoma	Bone
		Cell lines											
		Colo-205	**502713**	**HCT-15**	**SW-620**	**SKNSH**	**Du-145**	**A-549**	**MCF-7**	**OVCAR-5**	**HEP-2**	**IMR-32**	**D17**
		Per cent Growth Inhibition											
Alcoholic extract	10	0	0	0	0	8	0	0	0	0	0	5	6
	30	0	0	0	0	23	0	0	0	0	0	12	14
	100	8	12	0	45	42	2	0	0	0	6	18	16
Hydro-alcoholic extract	10	0	0	0	0	4	0	0	0	0	0	16	0
	30	0	0	0	0	18	0	0	0	0	0	28	8
	100	4	22	8	58	37	8	0	8	0	8	37	11
Aqueous extract	10	0	0	0	0	9	0	0	0	0	0	0	0
	30	36	0	0	0	12	0	0	0	0	0	25	0
	100	87	0	0	95	43	0	19	6	5	5	43	5
Positive Controls													
Paclitaxel	$1x10^{-5}$M	65	43	-	-	32	62	52	27	56	-	-	-
5Fluorouracil	$2x10^{-5}$M	32	-	41	19	-	39	-	-	45	-	-	62
Mitomicin-C	$1x10^{-5}$M	-	32	-	-	25	81	-	35	63	55	-	-
Adriamycin	$1x10^{-6}$M	90	75	-	-	-	98	-	59	78	-	19	-

More than 30, 50 and 70 per cent growth inhibition at 10, 30 and 100 µg/ml was considered promising cytotoxic activity and is shown in red font.
The values are means of three similar experiments each carried out in triplicate.

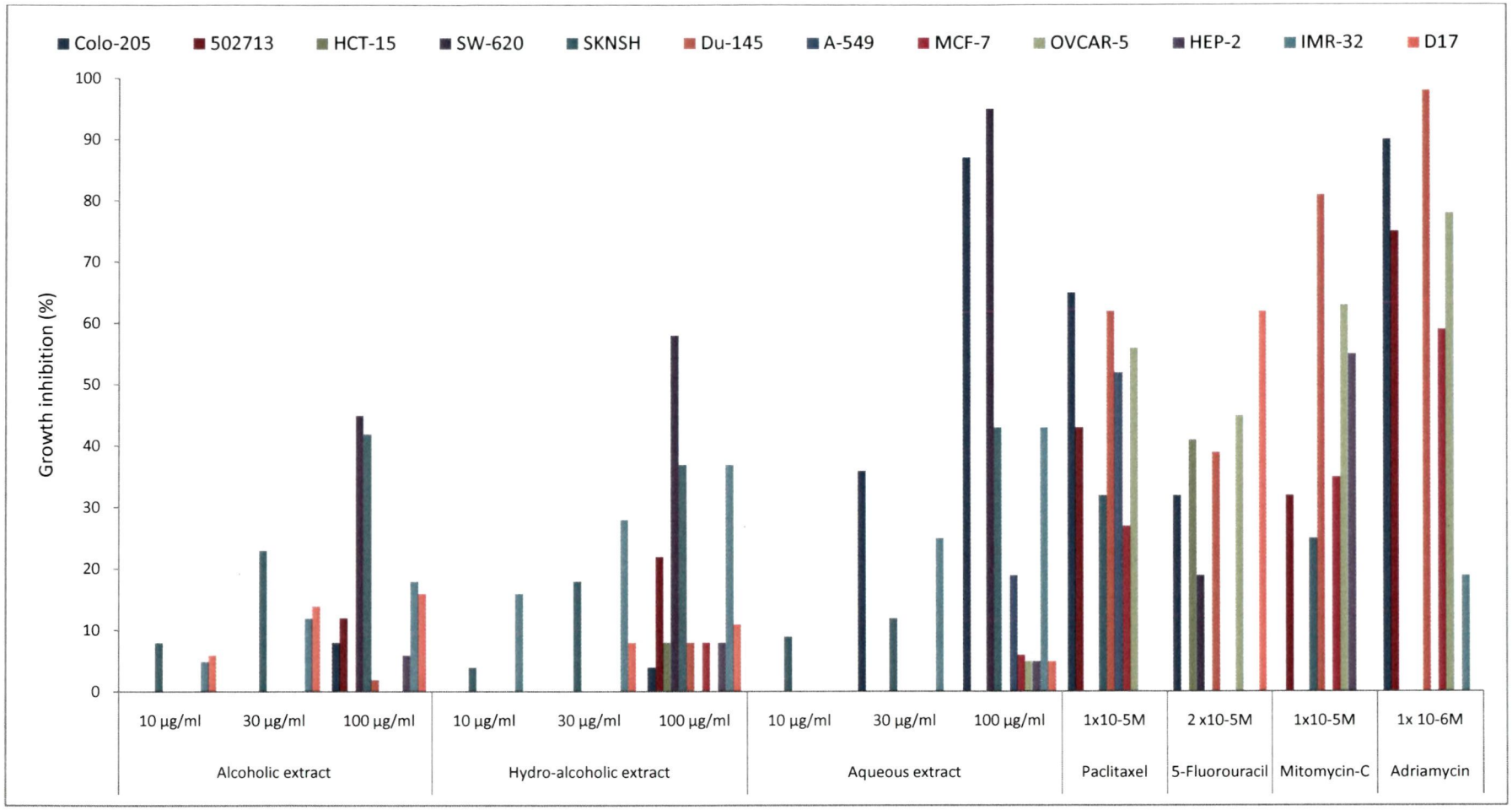


Fig. 4.8: *In vitro* cytotoxic activity of different extracts of *Butea monosperma* (flowers) against a panel of cancer cell lines. The values are means of three similar experiments each carried out in triplicate.

SW-620) showed promising cytotoxicity *i.e.*, more than 70 per cent growth inhibition at 100 μg/ml concentration and all other cell lines had poor or moderate growth inhibition even at this concentration of aqueous extract (Table 4.8, Fig. 4.8). Thus, modt of the cell lines did not show promising activity and the extract was considered non-promising.

In view of the negligible cytotoxic activity exhibited by all the extracts of *Butea monosperma* flowers at the studied concentrations, it was considered that the flowers of *Butea monosperma* also are without any promise as far as anticancer activity is concerned and so it was not investigated further.

4.2 *In vivo* anticancer activity of alcoholic extract and its chloroform fraction from *Azadirachta indica* leaves against murine cancer models

4.2.1 Ehrlich Ascites Carcinoma (EAC)

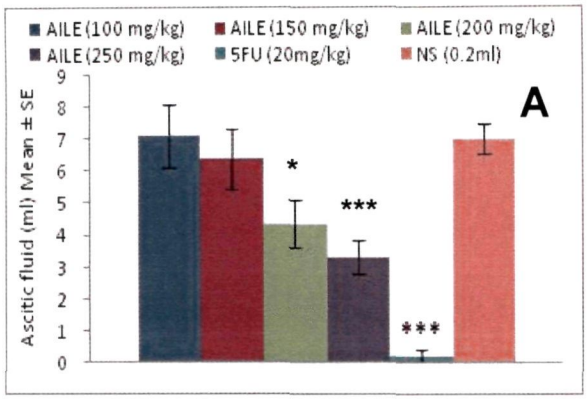
Four treatment groups of animals transplanted with EAC cells were treated with 100, 150, 200 and 250 mg/kg respectively of alcoholic extract of *Azadirachta indica* leaves (AILE) intraperitoneally (i.p.). Fifth group was treated with 20 mg/kg of 5-Fluorouracil (5-FU) i.p. and it served as positive control. The last group (tumor bearing control) was not administered any drug except 0.2 ml normal saline (NS). Data obtained on *in vivo* anticancer activity of alcoholic extract of *Azadirachta indica* (leaves) against Ehrlich Ascites Carcinoma is presented in Table 4.9 and Fig. 4.9.

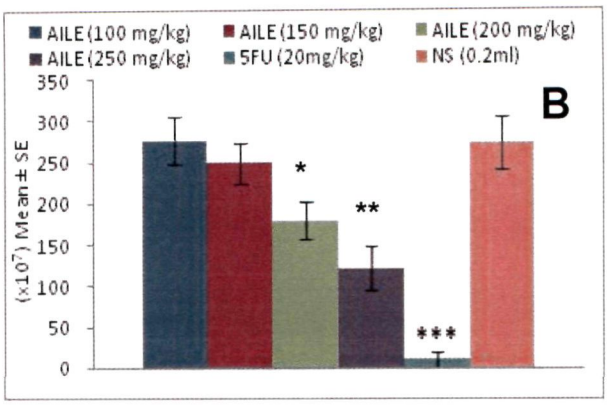
The average volume of ascitic fluid in NS treated control group was 7.03 ± 0.47 ml and in 5-FU treated positive control group it was significantly ($p<0.001$) lower (0.21 ± 0.21 ml). The animals treated with AILE 100, 150, 200 and 250 mg/kg recorded reduction in the volume of ascitic fluid in dose dependent manner (7.1 ± 0.99, 6.4 ± 0.93, 4.38 ± 0.76 and 3.32 ± 0.51 ml, respectively). However, the reduction was statistically significant in groups treated with AILE 200 and 250 mg/kg only ($p<0.05$ and $p<0.001$, respectively) (Fig. 4.9-A). Fig. 4.9-B depicts the number of tumor cells in different groups of animals. Total number of tumor cells in NS treated control group was 273.9 ± 31.51 x 10^7 whereas in 5-FU treated positive control group it was significantly ($p<0.001$) lower

Table 4.9: Anticancer activity of *Azadiracta indica* leaves against murine Ehrlich ascites carcinoma

Treatments	Dose	Aanimals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (AILE)	100 mg/kg	7	0	7.1 ± 0.99	276.71 ± 29.42	-1.0
	150 mg/kg	7	0	6.4 ± 0.93	249.71 ± 25.10	8.83
	200 mg/kg	7	0	4.38 ± 0.76*	179.85 ± 23.18*	34.33
	250 mg/kg	7	0	3.32 ± 0.51***	121.71 ± 26.43**	55.56
5-FU	20 mg/kg	7	0	0.21 ± 0.21***	10.14 ± 10.14***	96.29
NS	0.2 ml	15	0	7.03 ± 0.47	273.9 ± 31.51	-
Chloroform fraction (AILF2)	100 mg/kg	7	0	6.28 ± 0.87	245.42 ± 28.40*	31.51
	150 mg/kg	7	0	5.39 ± 0.79**	227.14 ± 28.12**	36.61
	200 mg/kg	7	0	5.1 ± 0.77**	224.57 ± 28.95**	37.33
	250 mg/kg	7	0	4.25 ± 0.86**	177.85 ± 22.12***	50.36
5-FU	20 mg/kg	7	0	0.34 ± 0.21***	15.57 ± 15.57***	95.65
NS	0.2 ml	15	0	8.52 ± 0.59	358.34 ± 30.97	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





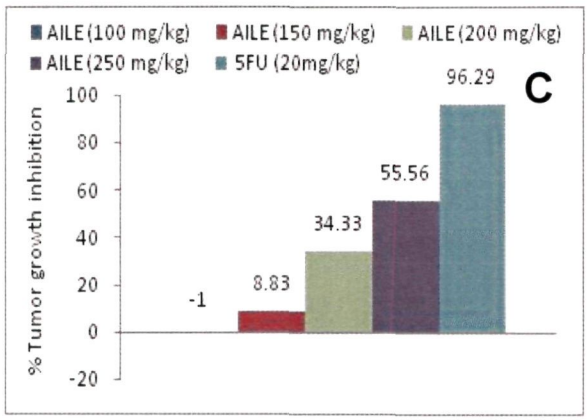
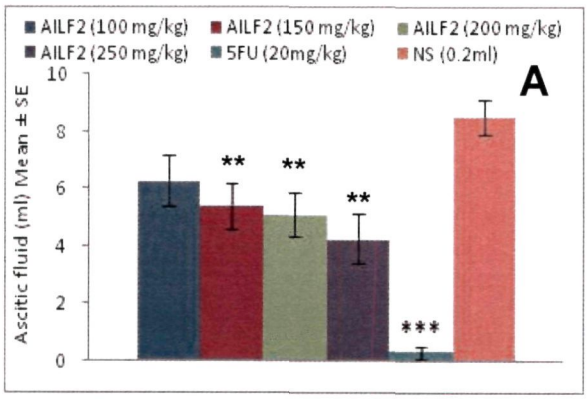
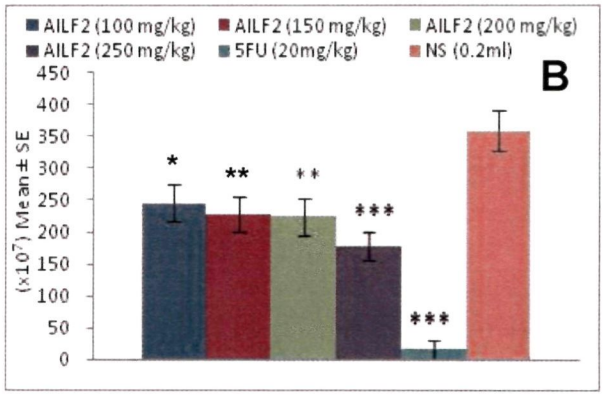


Fig. 4.9: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





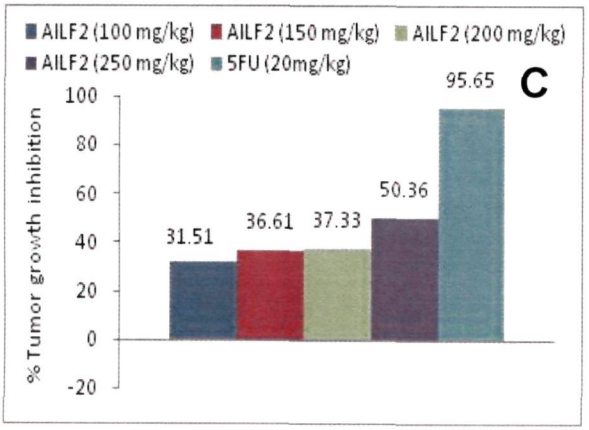


Fig. 4.10: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

($10.14 \pm 10.14 \times 10^7$). But in treated groups of animals the number of tumor cells was found to be $276.71 \pm 29.42 \times 10^7$, $249.71 \pm 25.10 \times 10^7$, $179.85 \pm 23.18 \times 10^7$ and $121.71 \pm 26.43 \times 10^7$, with 100, 150, 200 and 250 mg/kg doses of AILE, respectively. Statistically, the number of tumor cells in animals treated with AILE 250 mg/kg were significantly ($p<0.01$) lesser than that of NS treated control animals. The number of tumor cells had declined significantly ($p<0.05$) also with AILE 200 mg/kg dose.

Based on the total number of tumor cells present in the ascitic fluid of treated animals, effectiveness of AILE in terms of per cent tumor growth inhibition is illustrated in Fig. 4.9-C. In 5-FU treated group, the tumor growth inhibition was 96.29 per cent whereas in groups treated with AILE 100, 150, 200 and 250 mg/kg, it was -1, 8.83, 34.33 and 55.56 per cent, respectively.

Table 4.9 and Fig. 4.10 show the effect of chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2) at 100, 150, 200 and 250 mg/kg dose levels in Ehrlich ascites carcinoma bearing animals. The volume of ascitic fluid in 5-FU treated group (0.34 ± 0.21 ml) had significantly ($p<0.001$) declined as compared to NS treated group (8.52 ± 0.59 ml). AILF2 at 150, 200 and 250 mg/kg also reduced the volume of ascitic fluid significantly as compared to control animals and it was recorded to be 5.39 ± 0.79, 5.1 ± 0.77 and 4.25 ± 0.86 ml, respectively (Fig. 4.10-A).

The number of tumor cells had also declined significantly in the positive control group as well as groups treated with different doses of AILF2. In control animals the number of these cells was $358.34 \pm 30.97 \times 10^7$ and in 5-FU treated group it was $15.57 \pm 15.57 \times 10^7$. In animals treated with 100 mg/kg of AILF2 the number of tumor cells was $245.42 \pm 28.40 \times 10^7$, significantly ($p<0.05$) less than that of NS treated control animals. Groups treated with 150 and 200 mg/kg had even lesser number of tumor cells ($227.14 \pm 28.12 \times 10^7$ and $224.57 \pm 28.95 \times 10^7$, respectively) and on comparison with NS treated control animals, the difference was highly significant ($p<0.01$). Least number of tumor cells ($177.85 \pm 22.12 \times 10^7$) was recorded in a group treated with AILF2 250 mg/kg and its difference from NS treated group proved to be highly significant ($p<0.001$) (Fig. 4.10-B).

Tumor growth inhibition in 5-FU treated group was 95.65 Per cent whereas in other groups treated with AILF2 @ 100, 150, 200 and 250 mg/kg, it was 31.51, 36.61, 37.33 and 50.36 per cent, respectively (Fig. 4.10-C). Overall, per cent tumor growth inhibition in groups treated with 100 and 150 mg/kg doses of AILF2 was higher than similar doses of AILE.

4.2.2 Sarcoma-180 (Ascites)

In vivo anticancer activity of AILE against Sarcoma-180 (ascites) was assessed at 100, 150, 200 and 250 mg/kg dose levels in BALB/c mice transplanted with Sarcoma-180 cells. The positive control and tumor bearing control groups were treated with 20 mg/kg of 5-FU i.p. and 0.2 ml NS i.p., respectively. Data obtained on the *in vivo* anticancer activity of AILE is presented in Table 4.10 and Fig. 4.11.

The volume of ascitic fluid in NS treated group was 6.85 ± 0.72 ml and in 5-FU treated group it was 0.71 ± 0.47 ml, the difference between them being highly significant ($p<0.001$). In other groups of animals treated with AILE 100, 150, 200 and 250 mg/kg, the volume of ascitic fluid recorded was 6.68 ± 0.81 ml, 6.95 ± 0.86 ml, 6.14 ± 0.74 ml and 5.27 ± 0.74 ml, respectively. Statistically, the volumes of ascitic fluid in all the groups treated with AILE were not significantly different than that of NS treated control group (Fig. 4.11-A).

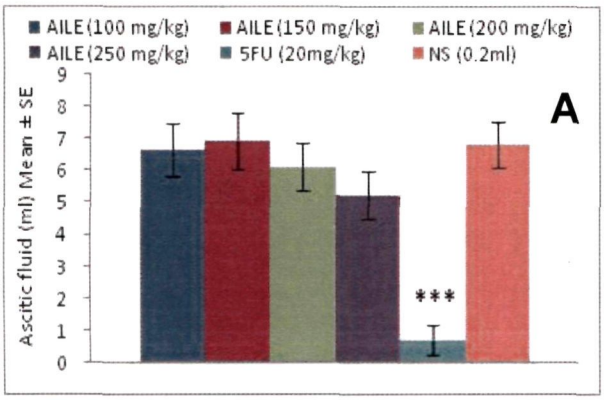
Total number of tumor cells present in the ascitic fluid of 5-FU treated positive control group ($22.0 \pm 15.29 \times 10^7$) was significantly ($p<0.001$) lower than NS treated control group ($257.2 \pm 22.39 \times 10^7$). In animals treated with 100, 150, 200 and 250 mg/kg doses of AILE the number of tumor cells present in the ascitic fluid was found to be $254.71 \pm 27.70 \times 10^7$, $259.85 \pm 28.84 \times 10^7$, $227.71 \pm 29.37 \times 10^7$ and $195.85 \pm 38.52 \times 10^7$, respectively and the same was not significantly different than that of NS treated control animals (Fig. 4.11-B).

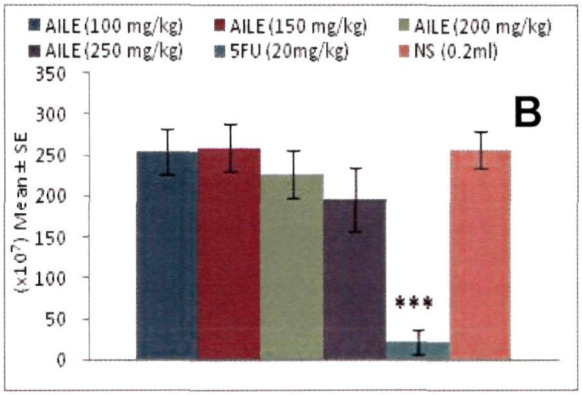
Fig. 4.11-C shows the per cent growth inhibition of Sarcoma-180 (ascites) in treated groups. In 5-FU treated group, the tumor growth was inhibited by 91.44 per cent whereas with 100, 150, 200 and 250 mg/kg doses of AILE, growth inhibition was to the extent of 0.96, -1.03, 11.46 and 23.85 per cent, respectively.

Table 4.10: Anticancer activity of *Azadiracta indica* leaves against murine Sarcoma-180 (ascites)

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (AILE)	100 mg/kg	7	0	6.68 ± 0.81	254.71 ± 27.70	0.96
	150 mg/kg	7	0	6.95 ± 0.86	259.85 ± 28.84	-1.03
	200 mg/kg	7	0	6.14 ± 0.74	227.71 ± 29.37	11.46
	250 mg/kg	7	0	5.27 ± 0.74	195.85 ± 38.52	23.85
5-FU	20 mg/kg	7	0	0.71 ± 0.47***	22.0 ± 15.29***	91.44
NS	0.2 ml	15	0	6.85 ± 0.72	257.2 ± 22.39	-
Chloroform fraction (AILF2)	100 mg/kg	7	0	6.11 ± 0.70	200.57 ± 24.65	1.05
	150 mg/kg	7	0	4.97 ± 0.80	165.57 ± 25.71	18.31
	200 mg/kg	7	0	4.52 ± 0.77	145.14 ± 27.02	28.39
	250 mg/kg	7	0	4.15 ± 0.78	135.0 ± 26.41	33.39
5-FU	20 mg/kg	7	0	0.21 ± 0.21***	5.0 ± 3.61***	97.53
NS	0.2 ml	15	0	6.03 ± 0.57	202.7 ± 19.00	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





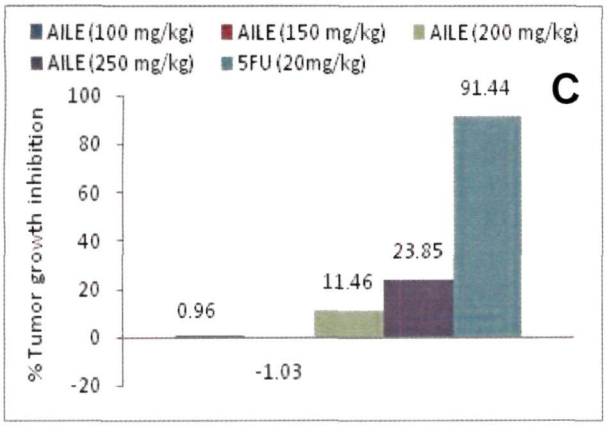
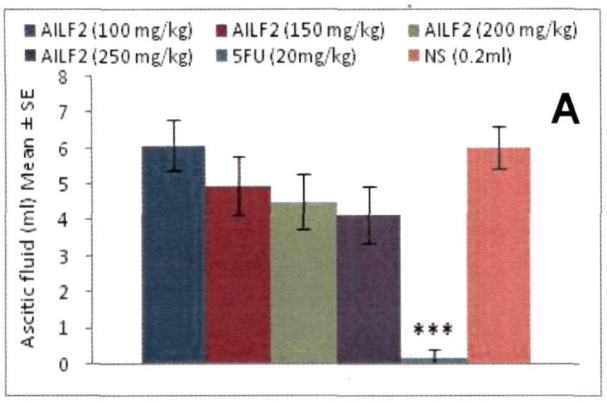
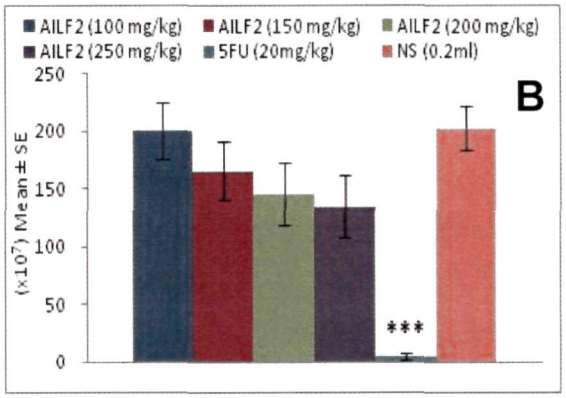


Fig. 4.11: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





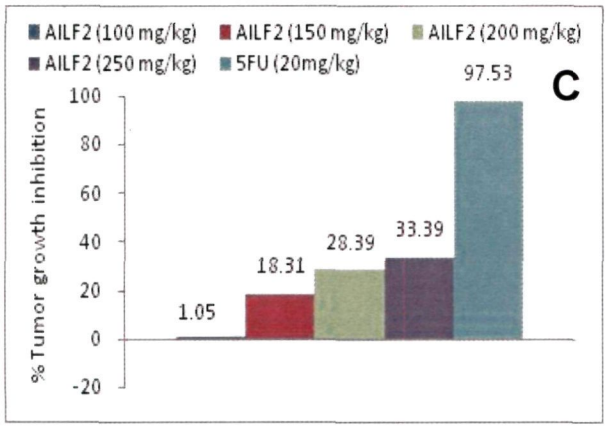


Fig. 4.12: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on murine Sarcoma-180 (Ascites). A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Control vs. treatment * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Different doses of AILF2 resulted in reduction of volumes of ascitic fluid which were recorded as 6.11 ± 0.70, 4.97 ± 0.80, 4.52 ± 0.77 and 4.15 ± 0.78 ml with 100, 150, 200 and 250 mg/kg doses, respectively (Table 4.10 and Fig. 4.12-A). This reduction in ascitic fluid volume was not significant as compared to NS treated control group (6.03 ± 0.57 ml). However, in 5-FU treated animals, it was significant (0.21 ± 0.21 ml) ($p<0.001$). Similarly, the decline in number of tumor cells with different doses of AILF2 was not significant as compared to NS treated control group but it was highly significant in 5-FU treated positive control group (Fig. 4.12-B).

Growth inhibition of Sarcoma-180 (ascites) as presented in Fig. 4.12-C was 1.05, 18.31, 28.39 and 33.39 per cent with AILF2 100, 150, 200 and 250 mg/kg, respectively. However, in 5-FU treated group, growth inhibition was to the extent of 97.53 per cent.

Although, per cent growth inhibition of Sarcoma-180 (ascites) in groups treated with 100, 150, 200 and 250 mg/kg doses of AILF2 was higher than similar doses of AILE, statistically, the growth inhibition in both the cases was not significant. On the other hand, the growth inhibition of Ehrlich ascites carcinoma by AILE and AILF2 was statistically significant.

4.2.3 Methyl cholanthrene induced ascites (Meth-A)

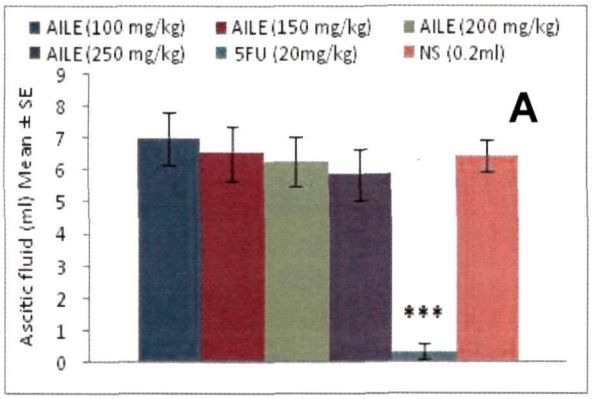
Data obtained on *in vivo* anticancer activity of AILE at 100, 150, 200 and 250 mg/kg dose levels against Methyl cholanthrene induced ascites is presented in Table 4.11 and Fig. 4.13. 5-FU was quite effective in reducing the volume of ascitic fluid from 6.44 ± 0.51 ml (in control animals) to 0.35 ± 0.23 ml. In other groups of animals treated with AILE 100, 150, 200 and 250 mg/kg, the volume of ascitic fluid recorded was 7.02 ± 0.82, 6.55 ± 0.86, 6.29 ± 0.77 and 5.89 ± 0.80 ml, respectively. Statistically, the volumes of ascitic fluid in all the groups treated with AILE were not significantly different than that of NS treated control group (Fig. 4.13-A).

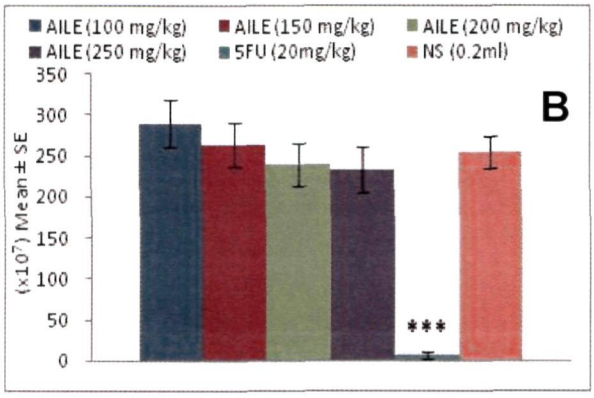
Total number of tumor cells present in the ascitic fluid of 5-FU treated positive control group ($6.42 \pm 4.26 \times 10^7$) was significantly ($p<0.001$) lower than NS treated control group ($254.2 \pm 19.99 \times 10^7$). In animals treated with 100, 150, 200 and 250 mg/kg doses

Table 4.11: Anticancer activity of *Azadiracta indica* leaves against Methyl cholanthrene induced ascites in mice

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (AILE)	100 mg/kg	7	0	7.02 ± 0.82	289.57 ± 29.58	-13.91
	150 mg/kg	7	0	6.55 ± 0.86	264.14 ± 27.54	-3.85
	200 mg/kg	7	0	6.29 ± 0.77	239.85 ± 26.22	5.64
	250 mg/kg	7	0	5.89 ± 0.80	233.42 ± 28.06	8.17
5-FU	20 mg/kg	7	0	0.35 ± 0.23***	6.42 ± 4.26***	97.47
NS	0.2 ml	15	0	6.44 ± 0.51	254.2 ± 19.99	-
Chloroform fraction (AILF2)	100 mg/kg	7	0	5.92 ± 0.85	214.42 ± 34.85	10.95
	150 mg/kg	7	0	5.72 ± 0.79	202.28 ± 33.97	15.99
	200 mg/kg	7	0	5.67 ± 0.79	200.71 ± 33.82	16.64
	250 mg/kg	7	0	5.22 ± 0.72	196.14 ± 32.43	18.54
5-FU	20 mg/kg	7	0	0.32 ± 0.22***	10.14 ± 6.80***	95.78
NS	0.2 ml	15	0	6.37 ± 0.60	245.8 ± 20.46	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





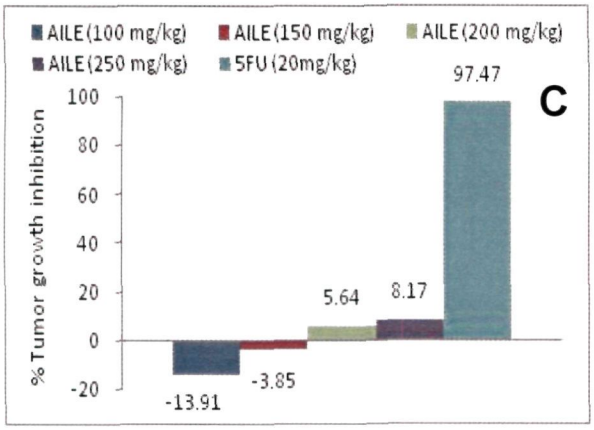
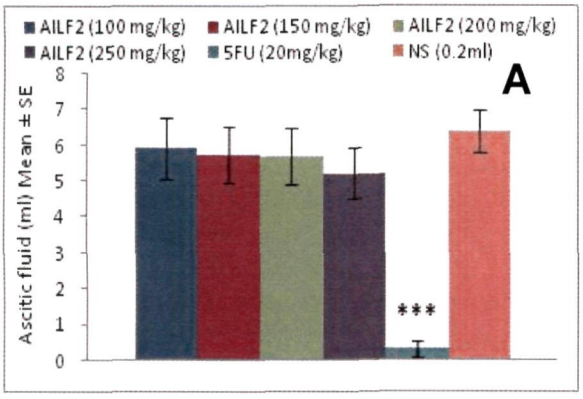
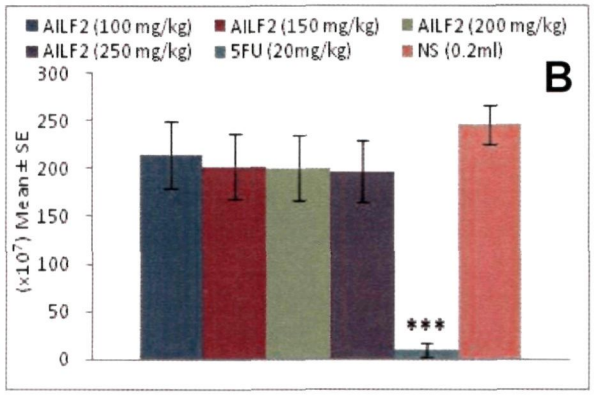


Fig. 4.13: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





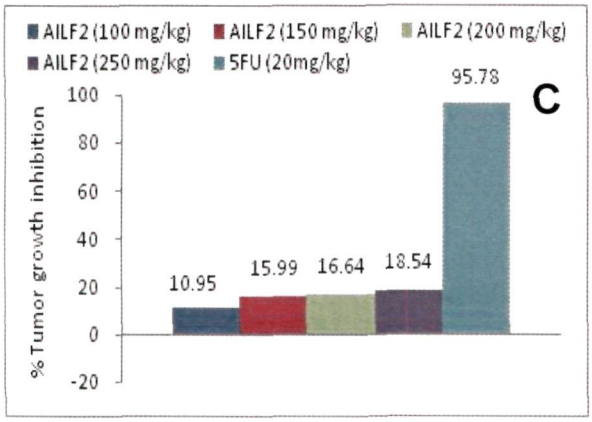


Fig. 4.14: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

of AILE the number of tumor cells present in the ascitic fluid was found to be 289.57 ± 29.58 x 10^7, 264.14 ± 27.54 x 10^7, 239.85 ± 26.22 x 10^7 and 233.42 ± 28.06 x 10^7, respectively and the same was not significantly different than that of NS treated control animals (Fig. 4.13-B).

Fig. 4.13-C shows the per cent growth inhibition of Methyl cholanthrene induced ascites in treated groups. In 5-FU treated group, the tumor growth was inhibited by 97.47 per cent whereas with 100, 150, 200 and 250 mg/kg doses of AILE, growth inhibition was to the extent of -13.91, -3.85, 5.64 and 8.17 per cent, respectively (Fig. 4.13-C) implying thereby AILE to be least effective in suppressing tumor growth in Methyl cholanthrene induced ascites as compared to EAC and Sarcoma-180 (ascites).

AILF2 was marginally effective at all the four doses studied in reducing the volume of ascitic fluid in animals bearing Methyl cholanthrene induced ascites (Fig. 4.14-A). Whereas 5-FU significantly ($p<0.001$) reduced the volume of ascitic fluid from 6.37 ± 0.60 ml seen in control animals to 0.32 ± 0.22 ml, a marginal decrease was seen with 100, 150, 200 and 250 mg/kg doses of AILF2, the respective values being 5.92 ± 0.85, 5.72 ± 0.79, 5.67 ± 0.79 and 5.22 ± 0.72 ml, respectively.

Similarly, AILF2 at all doses was only marginally effective in reducing the number of tumor cells whereas 5-FU had significant effect on this parameter too (Table 4.11, Fig. 4.14-B).

Per cent tumor growth inhibition in 5-FU treated group was 95.78 and in other groups treated with AILF2 100, 150, 200 and 250 mg/kg, it was 10.95, 15.99, 16.64 and 18.54 per cent, respectively (Fig. 4.14-C) but this was still higher than similar doses of AILE.

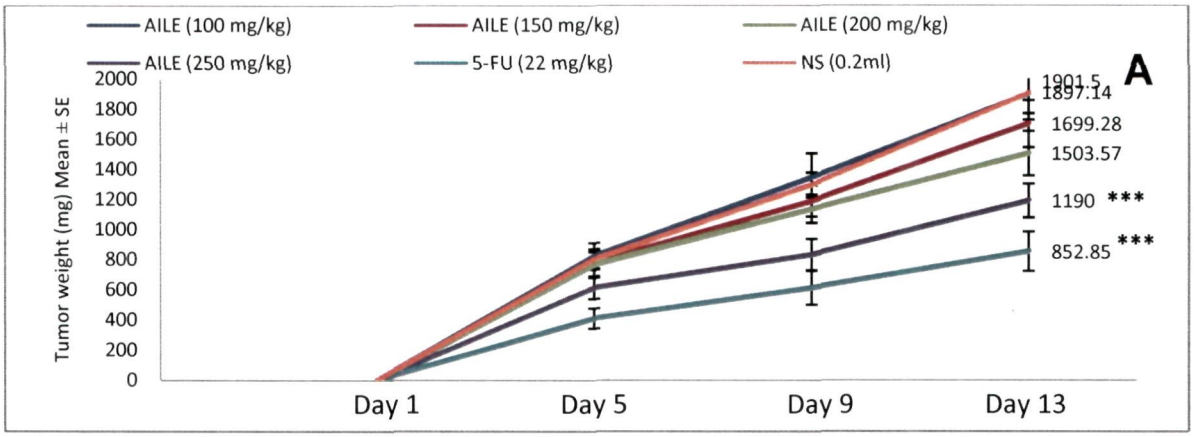
4.2.4 *Ehrlich tumor (solid)*

Data obtained on animals treated with 100, 150, 200 and 250 mg/kg of alcoholic extract of *Azadirachta indica* leaves (AILE), 22 mg/kg of 5-Fluorouracil (5-FU) and tumor bearing control administered 0.2 ml normal saline (NS) against Ehrlich tumor (solid) is presented in Table 4.12 and Fig. 4.15. Only AILE 250 mg/kg and 5-FU treated groups had significantly ($p<0.001$) lower tumor weights as compared to NS treated group.

Table 4.12: Anticancer activity of *Azadiracta indica* leaves against murine Ehrlich tumor (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (AILE)	100 mg/kg	0/7	822.71 ± 87.60	1344.57 ± 158.78	1897.14 ± 170.73	0.22
	150 mg/kg	0/7	779.71 ± 89.98	1188.57 ± 103.32	1699.28 ± 156.78	10.63
	200 mg/kg	0/7	766.14 ± 84.81	1136.57 ± 93.70	1503.57 ± 147.85	20.92
	250 mg/kg	0/7	612.28 ± 71.83	832.14 ± 103.39	1190.0 ± 112.48***	37.41
5-FU	22 mg/kg	0/7	410.42 ± 67.00	613.57 ± 112.84	852.85 ± 130.16***	55.14
NS	0.2 ml	0/15	801.1 ± 61.73	1294.5 ± 81.74	1901.5 ± 133.03	-
Chloroform fraction (AILF2)	100 mg/kg	0/7	725.14 ± 113.62	1107.0 ± 114.48	1674.85 ± 125.22	4.28
	150 mg/kg	0/7	682.57 ± 108.39	1053.57 ± 116.83	1563.42 ± 104.70	10.65
	200 mg/kg	0/7	592.14 ± 89.87	861. 28 ± 86.02	1246.85 ± 98.25**	28.74
	250 mg/kg	0/7	463.71 ± 80.87	705.71 ± 97.10	1049.0 ± 91.23***	40.05
5-FU	22 mg/kg	0/7	377.85 ± 69.96	550.57 ± 77.64	808.85 ± 87.48***	53.74
NS	0.2 ml	0/15	741.1 ± 67.53	1155.7 ± 66.79	1749.8 ± 111.88	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.



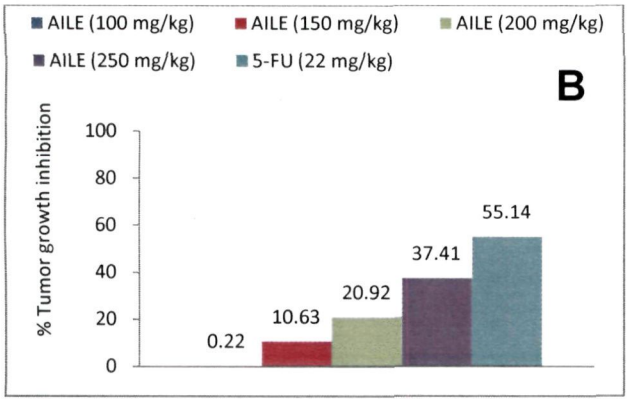
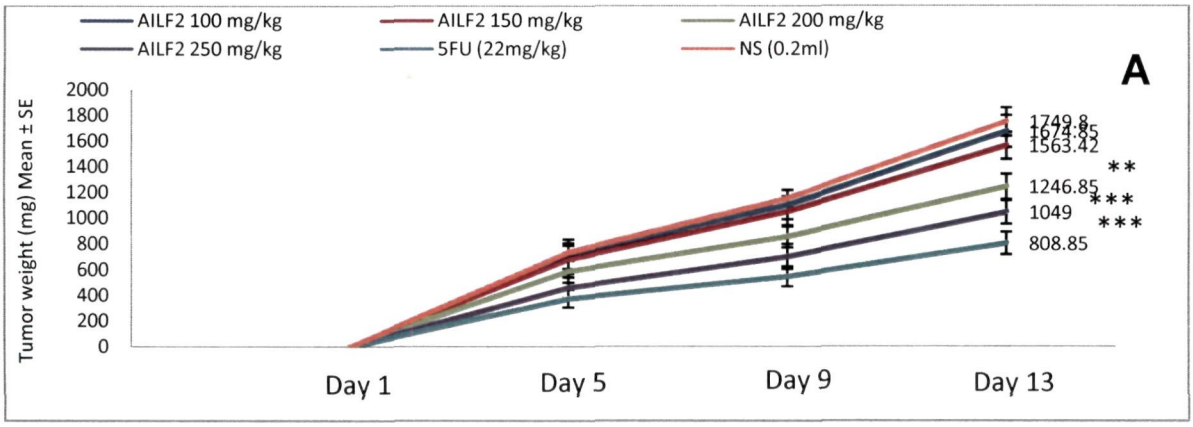


Fig. 4.15: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on murine Ehrlich tumor (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.

Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



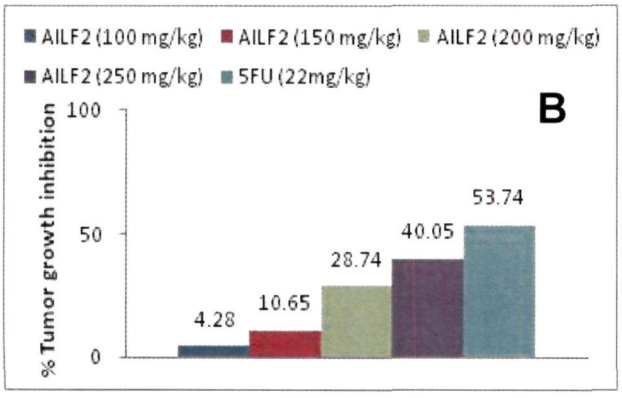


Fig. 4.16: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on murine Ehrlich tumor (solid) in mice. A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.

Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Lower doses of AILE (100, 150 and 200 mg/kg) did reduce the tumor weight but the change was not significant. Growth inhibition in such tumors was 0.22, 10.63 and 20.92 per cent with 100, 150 and 200 mg/kg of AILE respectively, whereas 5-FU produced growth inhibition to the extent of 55.14 per cent (Fig. 4.15-B).

Similarly, chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2) at 250 mg/kg dose suppressed tumor weights significantly ($p<0.001$) as compared to NS treated control group like 5-FU treatment. The tumor growth inhibition at 200 mg/kg of AILF2 was also significant ($p<0.01$). Growth inhibition of Ehrlich tumor (solid) calculated on the basis of tumor weights on day 13 in 5-FU treated group was 53.74 per cent and in other groups treated with AILF2 100, 150, 200 and 250 mg/kg, it was 4.28, 10.65, 28.74 and 40.05 per cent, respectively (Fig. 4.16-B). Overall, tumor growth inhibition in groups treated with 100, 150 and 200 mg/kg doses of AILF2 was better than in groups treated with similar doses of AILE.

4.2.5 Sarcoma-180 (solid)

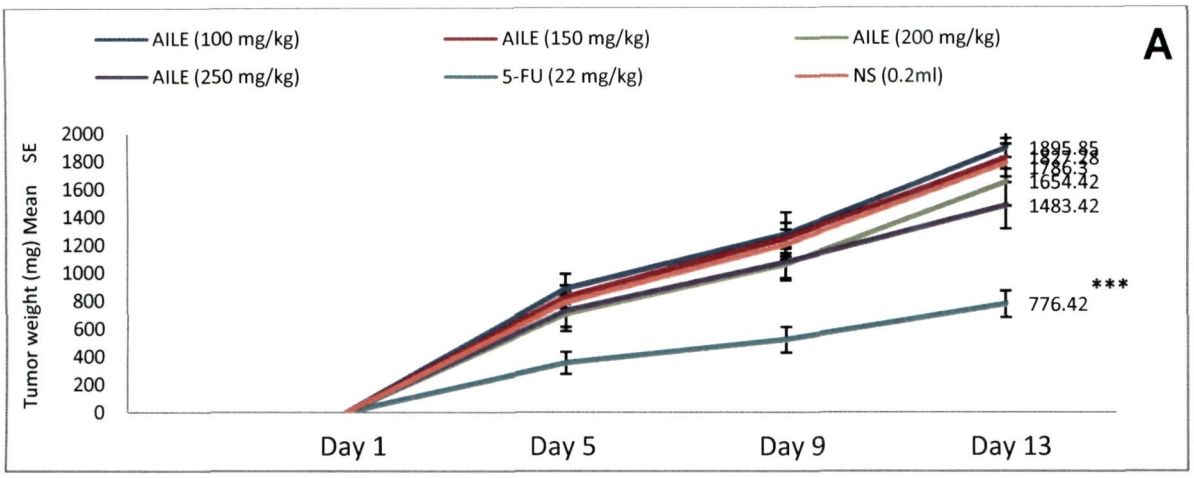
Data on anticancer activity of 100, 150, 200 and 250 mg/kg doses of AILE against Sarcoma-180 (solid) is presented in Table 4.13 and Fig. 4.17. In this case, none of the groups treated with AILE showed any significant growth inhibition as compared to NS treated group. Higher doses (200 and 250 mg/kg) exhibited 7.38 and 16.95 per cent growth inhibition whereas lower doses (100 and 150 mg/kg) had little higher tumor weights than control group thereby producing negative growth inhibition (-6.13, -2.29 per cent, respectively). However, 5-FU treated group had significantly ($p<0.001$) lower tumor weight as compared to NS treated group, exhibiting 56.53 per cent growth inhibition (Fig. 4.17-A & B).

Only the dose of 250 mg/kg of chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2) suppressed the growth of Sarcoma-180 (solid) significantly ($p<0.05$) as compared to NS treated control group. Other doses of AILF2 produced either negative (-5.38 and -3.48 per cent) or poor (9.06 per cent) growth inhibition of Sarcoma-180 (solid). Positive control group however suppressed the tumor growth by 57.24 per cent (Fig. 4.18-A &B). Overall, tumor growth inhibition in groups treated with 200 and

Table 4.13: Anticancer activity of *Azadiracta indica* leaves against murine Sarcoma-180 (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (AILE)	100 mg/kg	0/7	887.71 ± 107.15	1278.42 ± 158.41	1895.85 ± 149.90	-6.13
	150 mg/kg	0/7	830.14 ± 121.63	1251.42 ± 160.44	1827.28 ± 164.50	-2.29
	200 mg/kg	0/7	707.71 ± 124.66	1062.0 ± 113.84	1654.42 ± 172.64	7.38
	250 mg/kg	0/7	730.42 ± 115.30	1077.14 ± 113.72	1483.42 ± 165.53	16.95
5-FU	22 mg/kg	0/7	355.14 ± 79.26	521.14 ± 92.71	776.42 ± 95.07***	56.53
NS	0.2 ml	0/15	790.7 ± 80.95	1203.8 ± 108.83	1786.3 ± 137.67	-
Chloroform fraction (AILF2)	100 mg/kg	0/7	832.57 ± 99.30	1277.42 ± 115.49	1938.28 ± 154.69	-5.38
	150 mg/kg	0/7	817.14 ± 102.62	1264.28 ± 94.03	1903.42 ± 157.33	-3.48
	200 mg/kg	0/7	786.85 ± 112.50	1209.42 ± 97.41	1672.57 ± 125.10	9.06
	250 mg/kg	0/7	727.14 ± 113.63	1038.57 ± 111.53	1439.85 ± 128.97*	21.71
5-FU	22 mg/kg	0/7	338.28 ± 61.22	521.14 ± 85.69	786.42 ± 98.23***	57.24
NS	0.2 ml	0/15	812.1 ± 72.02	1243.9 ± 79.73	1839.3 ± 104.85	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.



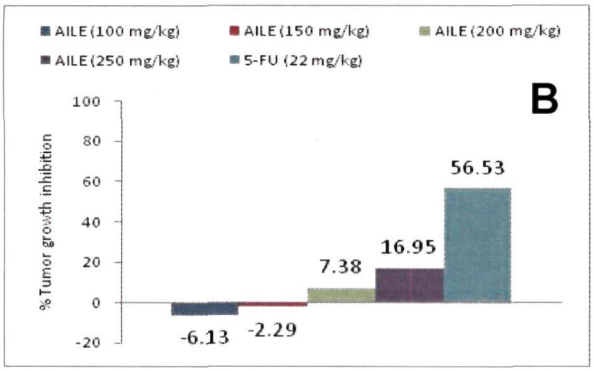
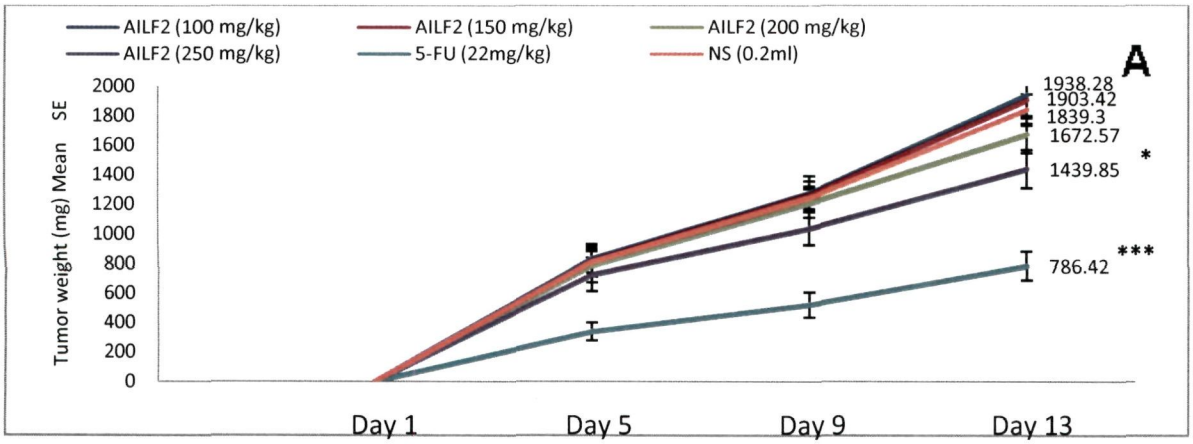


Fig. 4.17: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



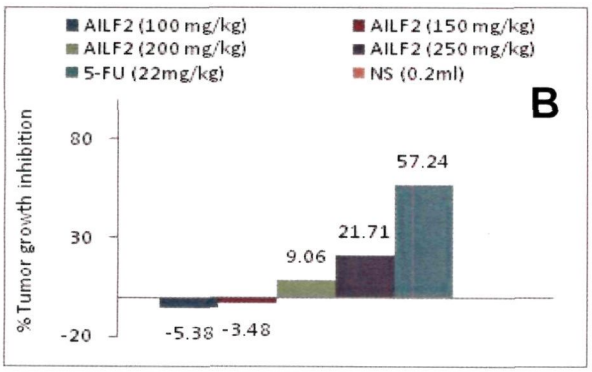


Fig. 4.18: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

250 mg/kg doses of AILF2 was little higher than in groups treated with similar doses of AILE.

4.2.6 L1210 Lymphoid leukemia

Anticancer activity of AILE against CD2F1 mice transplanted with L1210 Lymphoid leukemia cells at 100, 150, 200 and 250 mg/kg doses is presented in Table 4.14 and Fig. 4.19. In NS treated group, the Mean survival time (MST) was 7.33 days and in positive control group it was 16.75 days. In groups treated with AILE 100, 150, 200 and 250 mg/kg, MST was 101.50, 104.50. 95.49 and 81.85 per cent of tumor bearing control group, respectively (Fig. 4.19-B). Net result of different treatments expressed as per cent increase in life span of treated animals over tumor bearing control animals was 128.51 per cent for 5-FU treated group and for other groups treated with AILE 100, 150, 200 and 250 mg/kg, it was 1.50, 4.50, -4.51 and -18.15 per cent, respectively (Fig. 4.19-C).

Chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2) also did not produce significant increase in life span of CD2F1 mice transplanted with L1210 Lymphoid leukemia cells at 100, 150, 200 and 250 mg/kg doses (Table 4.14 and Fig. 4.20). There was only marginal increase in life span (9.14 and 17.32 per cent) with lower doses (100 and 150 mg/kg, respectively) whereas higher doses (200 and 250 mg/kg) shortened the life span of treated animals thereby producing negative increase in life span (-4.51 and -13.65 per cent, respectively). 5-FU treated group however showed 90.99 per cent increase in life span of treated animals over tumor bearing control animals (Fig. 4.20-C).

4.2.7 P388 Lymphocytic leukemia

In vivo anticancer activity of AILE was similarly studied in CD2F1 mice transplanted with P388 Lymphocytic leukemia cells at 100, 150, 200 and 250 mg/kg dose levels. Mean survival time (MST) as depicted in Fig. 4.21-A was 7.66 days in NS treated group whereas it was 19.00 days in positive control group. Groups treated with AILE 100, 150, 200 and 250 mg/kg, had 103.13, 104.04, 114.88 and 98.30 per cent life span as compared to tumor bearing control group, respectively (Fig. 4.21-B). It is thus observed that none of the doses of AILE produced significant increase in life span of P388 Lymphocytic

Table 4.14: Anticancer activity of *Azadiracta indica* leaves against L1210 Lymphoid leukemia in CD2F1 mice

Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (AILE)	100 mg/kg	6	7.44	101.50	1.50
	150 mg/kg	6	7.66	104.50	4.50
	200 mg/kg	6	7.00	95.49	-4.51
	250 mg/kg	6	6.00	81.85	-18.15
5-FU	20 mg/kg	6	16.75	228.51	128.51
NS	0.2 ml	6	7.33	-	-
Chloroform fraction (AILF2)	100 mg/kg	6	8.0	109.14	9.14
	150 mg/kg	6	8.6	117.32	17.32
	200 mg/kg	6	7.0	95.49	-4.51
	250 mg/kg	6	6.33	86.35	-13.65
5-FU	20 mg/kg	6	14.0	190.99	90.99
NS	0.2 ml	6	7.33	-	-

5-FU: 5-fluorouracil; NS: Normal saline.

Table 4.15: Anticancer activity of *Azadiracta indica* leaves against P388 Lymphocytic leukemia in CD2F1 mice

Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (AILE)	100 mg/kg	6	7.9	103.13	3.13
	150 mg/kg	6	7.97	104.04	4.04
	200 mg/kg	6	8.8	114.88	14.88
	250 mg/kg	6	7.53	98.30	-1.7
5-FU	20 mg/kg	6	19.0	248.04	148.04
NS	0.2 ml	6	7.66	-	-
Chloroform fraction (AILF2)	100 mg/kg	6	6.98	102.19	2.19
	150 mg/kg	6	7.2	105.41	5.41
	200 mg/kg	6	7.25	106.14	6.14
	250 mg/kg	6	6.57	96.19	-3.81
5-FU	20 mg/kg	6	11.0	161.05	61.05
NS	0.2 ml	6	6.83	-	-

5-FU: 5-fluorouracil; NS: Normal saline.

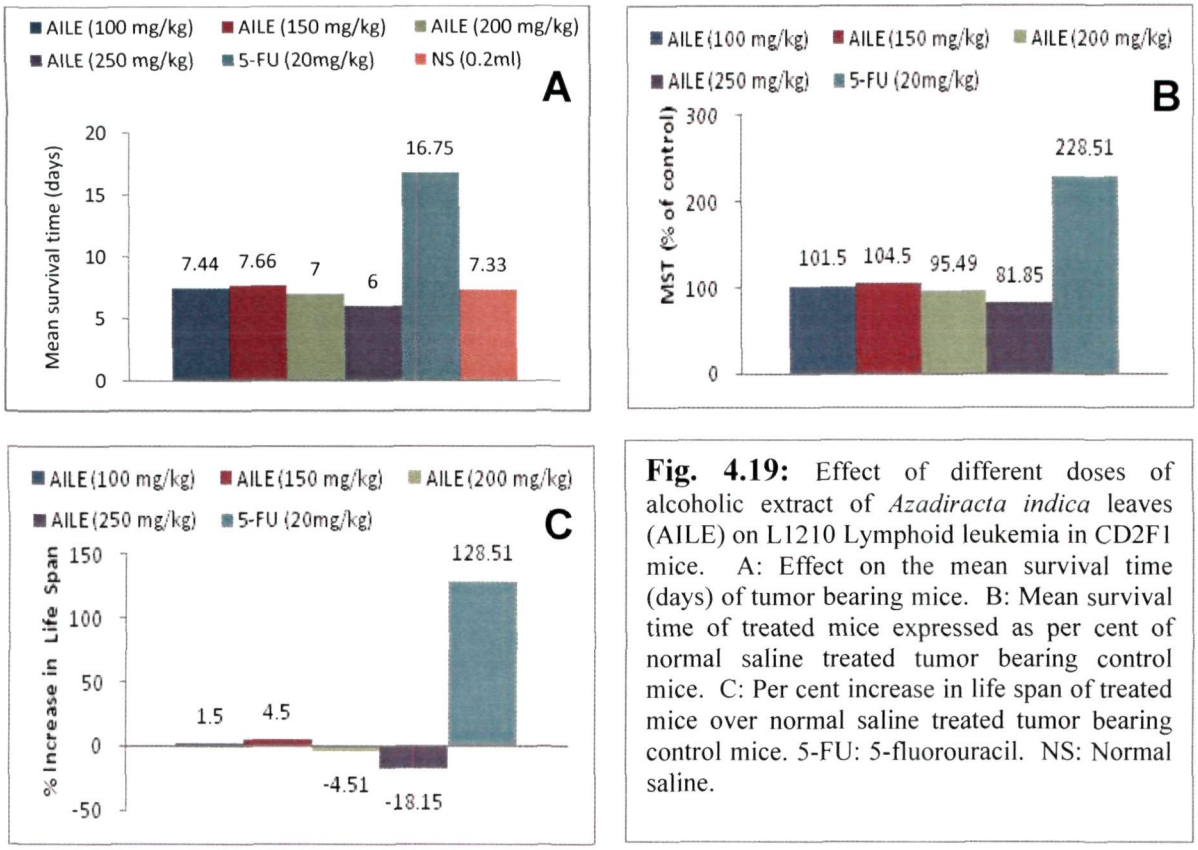


Fig. 4.19: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.

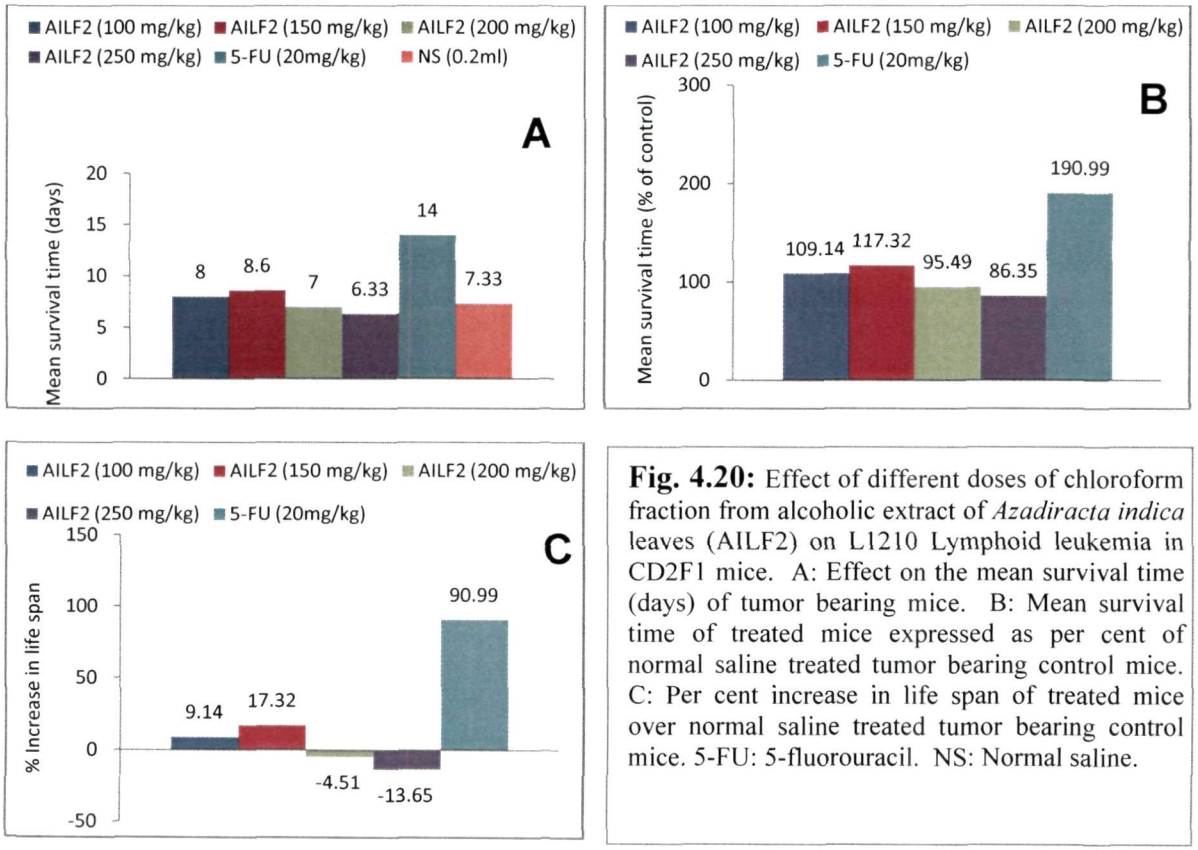


Fig. 4.20: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.

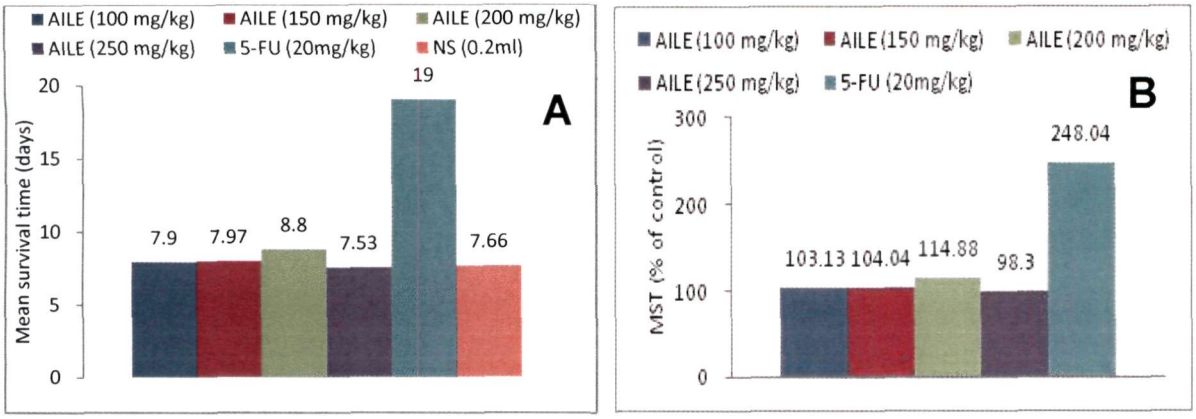


Fig. 4.21: Effect of different doses of alcoholic extract of *Azadiracta indica* leaves (AILE) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.

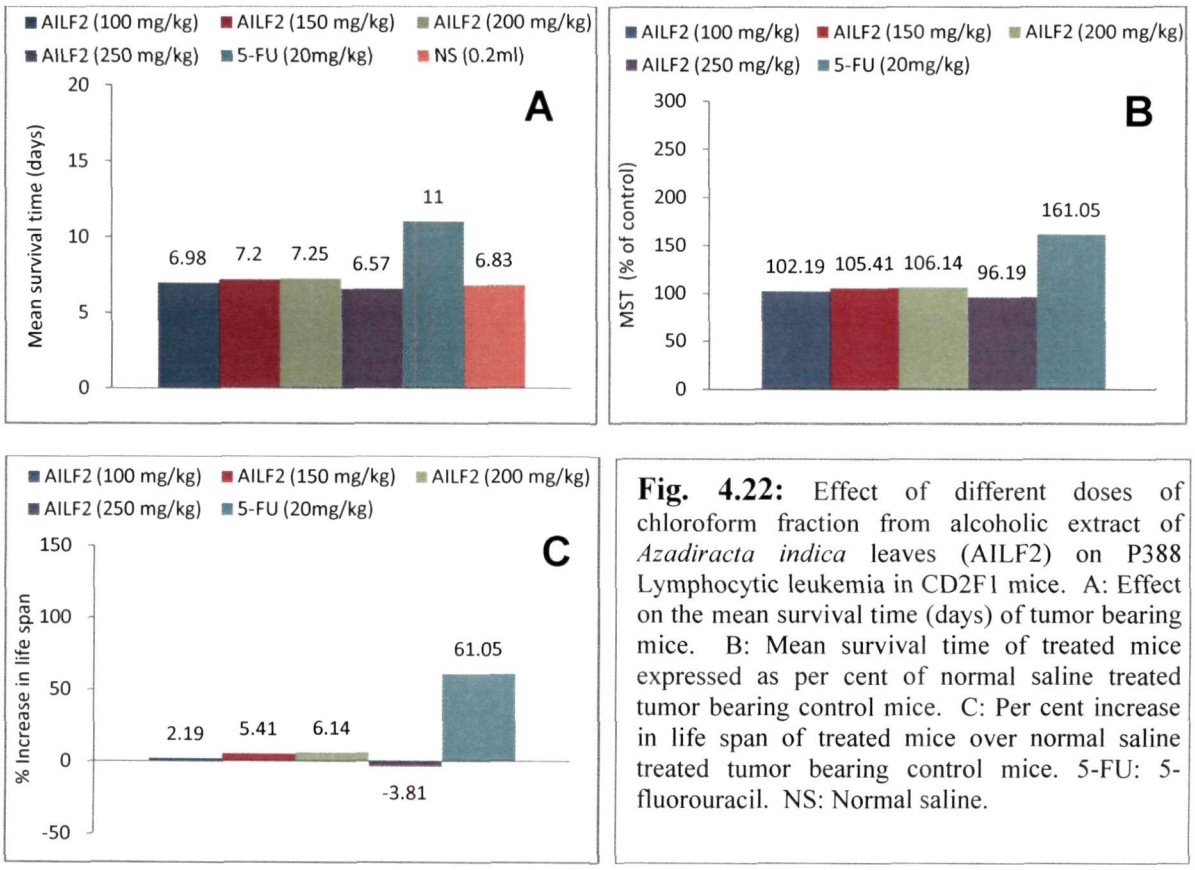


Fig. 4.22: Effect of different doses of chloroform fraction from alcoholic extract of *Azadiracta indica* leaves (AILF2) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.

Leukemia bearing animals whereas 5-FU treatment resulted in 148.04 per cent increase in life span of treated animals (Fig. 4.21-C).

Anticancer activity of chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2) against P388 Lymphocytic leukemia at 100, 150, 200 and 250 mg/kg dose levels was also not very different. As depicted in Table 4.15 and Fig. 4.22, MST was 6.83 days in NS treated group whereas in positive control group it was 11.0 days. Groups treated with AILF2 100, 150, 200 and 250 mg/kg, had 102.19, 105.41, 106.14 and 96.19 per cent life span as compared to NS treated control group (Fig. 4.22-B). Thus, none of the doses of AILF2 could significantly increase the life span of treated animals whereas 5-FU treated group had 61.05 per cent increase in life span over tumor bearing control animals (Fig. 4.22-C).

4.3 *In vivo* anticancer activity of alcoholic extract and its chloroform and n-butanol fractions from *Asparagus racemosus* roots against murine cancer models

4.3.1 Ehrlich ascites carcinoma (EAC)

After transplantation of EAC cells in non-inbred Swiss mice, four groups were treated with 80, 100, 120 and 140 mg/kg respectively of alcoholic extract of *Asparagus racemosus* roots (ARRE). Positive control group was treated with 20 mg/kg of 5-Fluorouracil (5-FU) and the tumor bearing control group was administered 0.2 ml normal saline (NS). As depicted in Table 4.16 and Fig. 4.23-A, the average volume of ascitic fluid was significantly ($p<0.001$) lower in 5-FU treated group (0.34 ± 0.22 ml) than NS treated control group (8.80 ± 0.61 ml). Likewise, treatment with ARRE 120 and 140 mg/kg also significantly ($p<0.001$) reduced the volume of ascitic fluid (4.21 ± 0.73 and 0.41 ± 0.21 ml, respectively) as compared to NS treatment. More or less the similar pattern was observed for the number of tumor cells in different groups of animals (Fig. 4.24-B). AILE 140 mg/kg and 5-FU treatments significantly ($p<0.001$) reduced the total number of tumor cells (9.98 ± 8.45 x 10^7 and 9.14 ± 7.60 x 10^7, respectively) as compared to NS treatment (197.6 ± 24.28 x 10^7). Tumor growth inhibition as illustrated in Fig. 4.24-C was 21.77, 33.05, 53.29 and 94.94 per cent in groups treated with ARRE

Table 4.16: Anticancer activity of *Asparagus racemosus* roots against murine Ehrlich ascites carcinoma

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (ARRE)	80 mg/kg	7	0	6.97 ± 0.76	154.57 ± 42.31	21.77
	100 mg/kg	7	0	6.14 ± 0.73*	132.28 ± 49.94	33.05
	120 mg/kg	7	0	4.21 ± 0.73***	92.28 ± 35.60*	53.29
	140 mg/kg	7	0	0.41 ± 0.21***	9.98 ± 8.45***	94.94
5-FU	20 mg/kg	7	0	0.34 ± 0.22***	9.14 ± 7.60***	95.37
NS	0.2 ml	15	0	8.80 ± 0.61	197.6 ± 24.28	-
Chloroform fraction (ARRF2)	80 mg/kg	7	0	6.45 ± 0.77	212.0 ± 32.37*	31.85
	100 mg/kg	7	0	4.8 ± 0.67**	189.57 ± 29.26**	39.06
	120 mg/kg	7	0	3.08 ± 1.45**	47.71 ± 19.28***	84.66
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.41 ± 0.23***	18.28 ± 10.47***	94.12
NS	0.2 ml	15	0	8.31 ± 0.54	311.1 ± 24.87	-
n-Butanol fraction (ARRF3)	80 mg/kg	7	0	0.36 ± 0.16***	28.42 ± 13.48***	89.00
	100 mg/kg	7	1	0.36 ± 0.16***	15.16 ± 8.90***	94.13
	120 mg/kg	7	4	0.5 ± 0.5***	22.33 ± 5.89***	91.36
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.28 ± 0.22***	12.14 ± 12.14***	95.28
NS	0.2 ml	15	0	7.04 ± 0.66	258.45 ± 28.51	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.

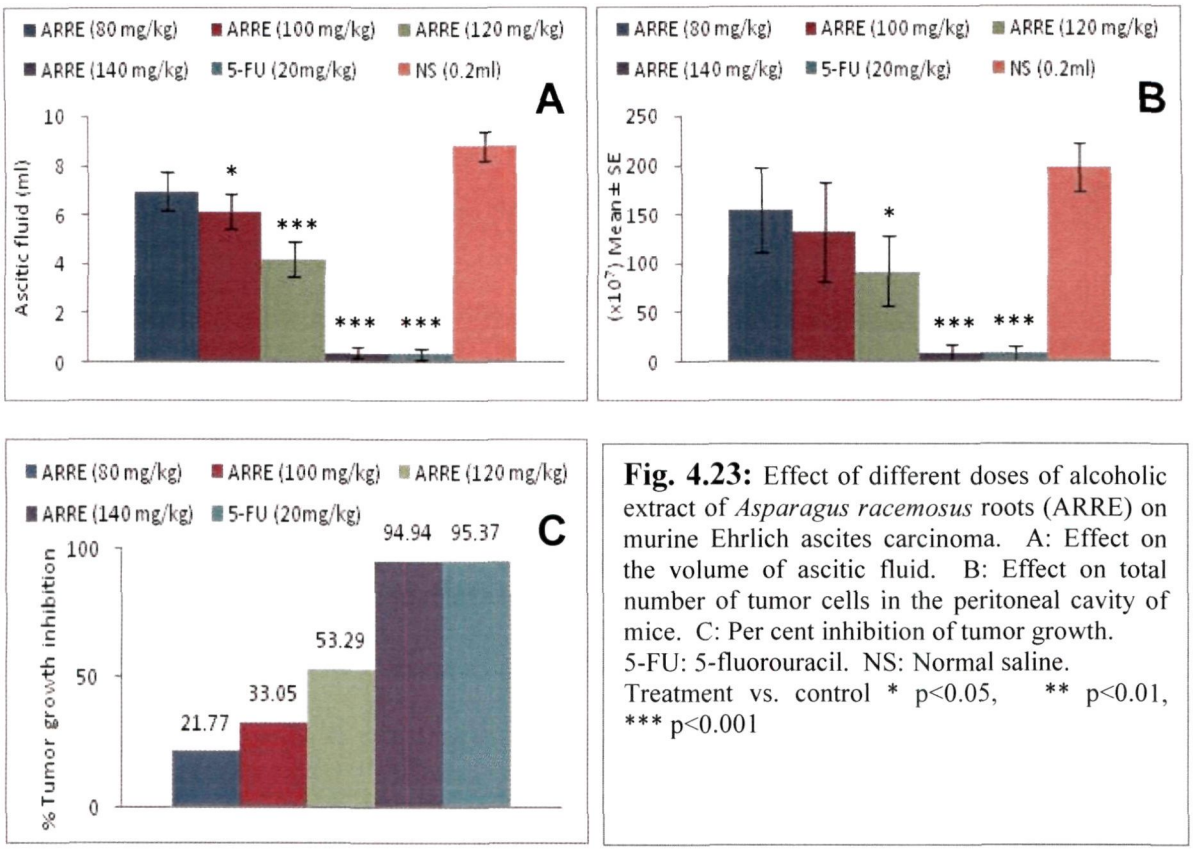


Fig. 4.23: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

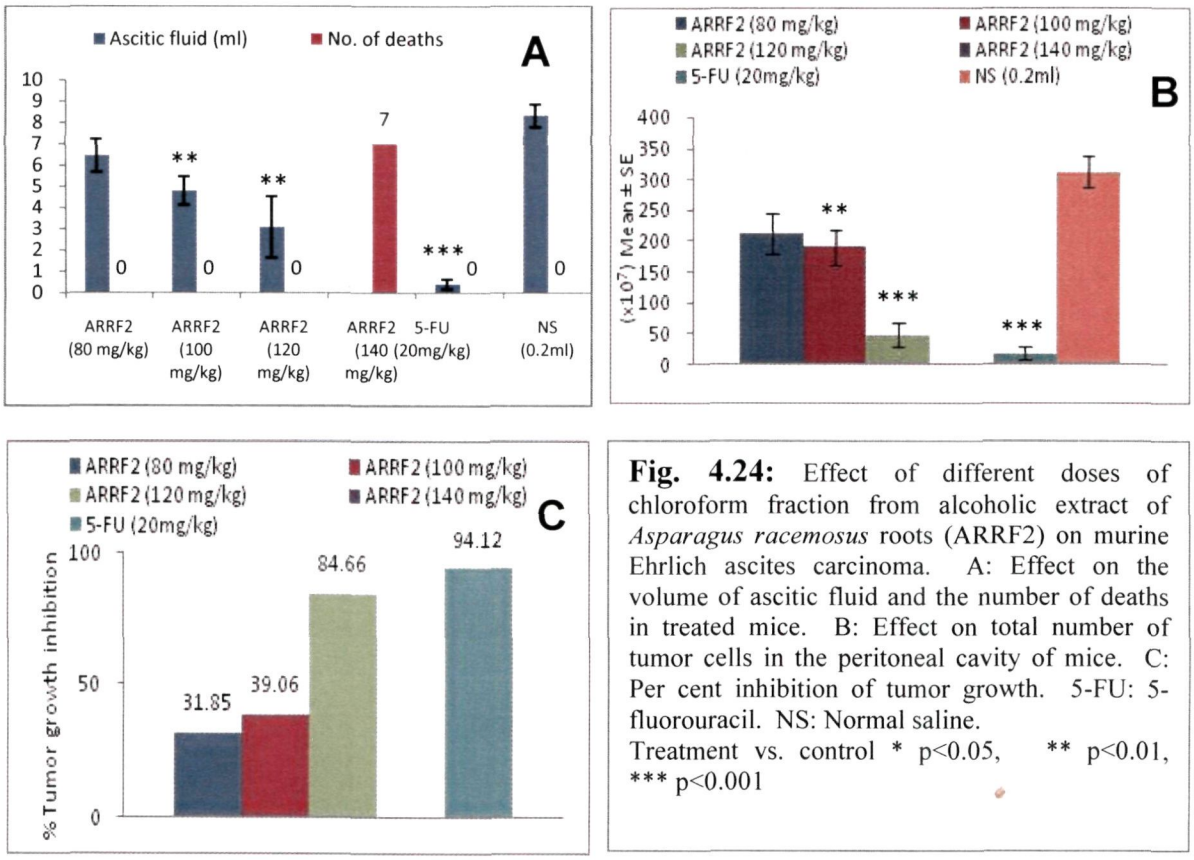
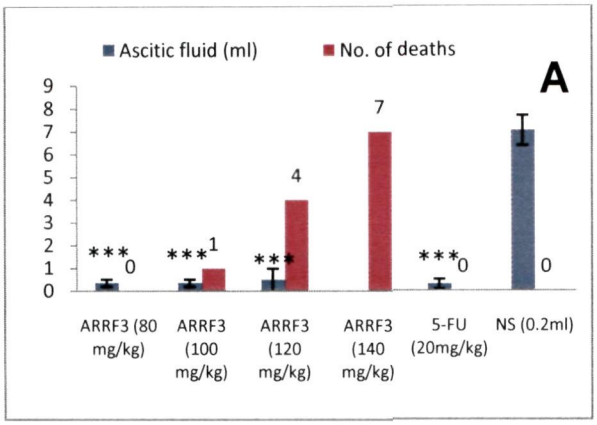


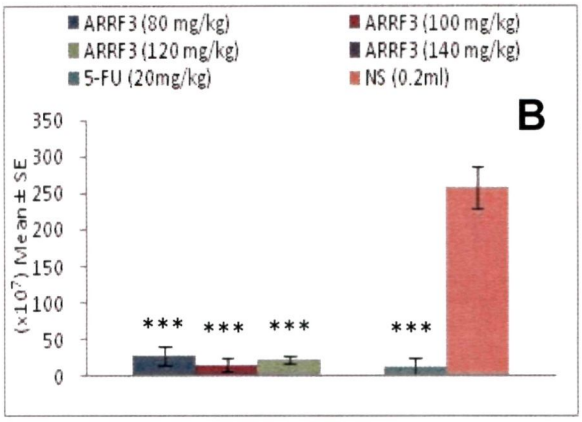
Fig. 4.24: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

80, 100, 120 and 140 mg/kg, respectively whereas in positive control group it was 95.37 per cent.

In vivo anticancer activity of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) against Ehrlich ascites carcinoma was also studied at 80, 100, 120 and 140 mg/kg dose levels. Statistical analysis showed the volume of ascitic fluid in 5-FU treated group (0.41 ± 0.23 ml) to be significantly less ($p<0.001$) than that of NS treated group (8.31 ± 0.54 ml). Groups treated with ARRF2 100 and 120 mg/kg also recorded significantly ($p<0.01$) less volumes of ascitic fluid (4.8 ± 0.67 and 3.08 ± 1.45 ml, respectively). All animals in ARRF2 140 mg/kg treated group died during the course of experiment. Similarly, the number of tumor cells in the positive control and ARRF2 120 mg/kg treated groups (18.28 ± 10.47 x 10^7 and 47.71 ± 19.28 x 10^7, respectively) were significantly less ($p<0.001$) than that of NS treated group (311.1 ± 24.87 x 10^7). Animals treated with ARRF2 80 mg/kg also had significantly ($p<0.05$) lower number of tumor cells (212.0 ± 32.37 x 10^7) than that of NS treated control animals (Fig. 4.24-B). Thus, treatment with ARRF2 80, 100 and 120 mg/kg resulted in 31.85, 39.06 and 84.66 per cent tumor growth inhibition respectively, whereas ARRF2 140 mg/kg proved toxic to animals. 5-FU treatment produced 94.12 per cent inhibition in the growth of Ehrlich ascites carcinoma (Fig. 4.24-C).

Data on anticancer activity of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) studied on similar lines against Ehrlich ascites carcinoma at 80, 100, 120 and 140 mg/kg dose levels is presented in Table 4.16 and Fig. 4.25. Significantly ($p<0.001$) lesser volumes of ascitic fluid were recorded in groups treated with 5-FU and ARRF3 80, 100 and 120 mg/kg (0.28 ± 0.22, 0.36 ± 0.16, 0.36 ± 0.16 and 0.5 ± 0.5 ml, respectively) as compared to NS treated group (7.04 ± 0.66 ml). However, a dose of ARRF3 140 mg/kg proved highly toxic to animals as all animals in this group died during the experiment. There were 1 and 4 deaths also in groups treated with 100 and 120 mg/Kg, respectively. Exactly same pattern was observed for the number of tumor cells in 5-FU and ARRF3 treated groups (Fig. 4.25-B) which were significantly ($p<0.001$) lesser than in NS treated control group. Growth inhibition of Ehrlich ascites carcinoma in positive control group was 95.28 per cent and in groups treated with





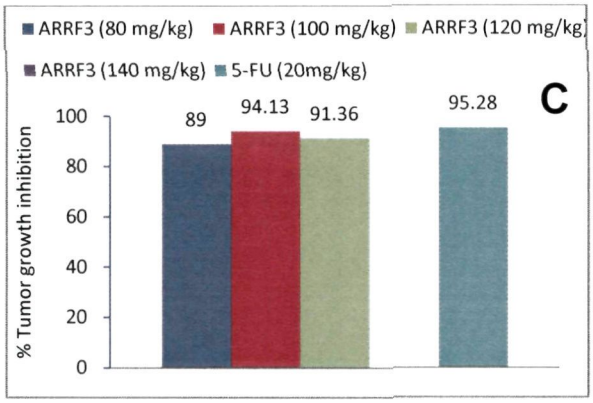
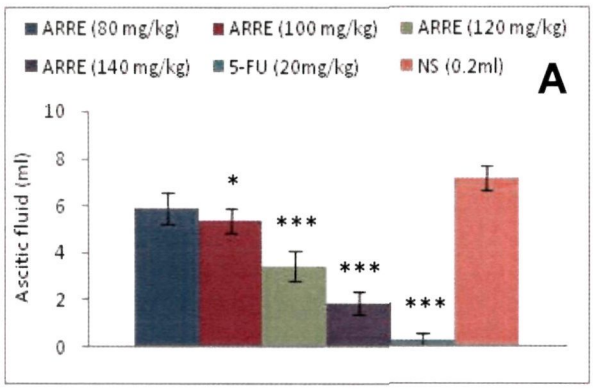
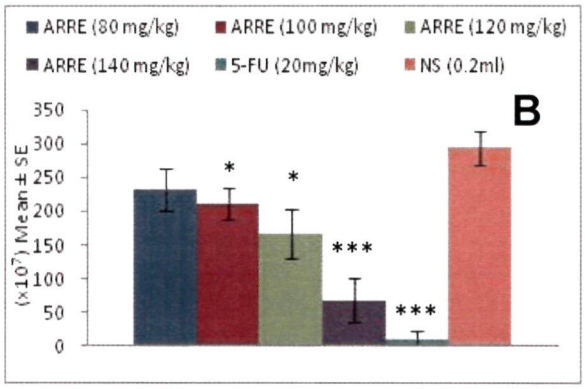


Fig. 4.25: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on mrine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





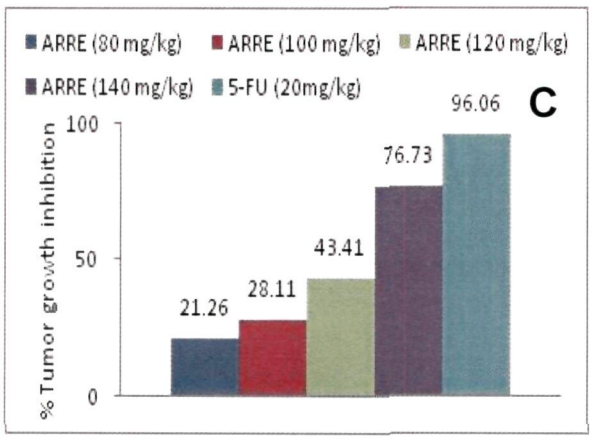


Fig. 4.26: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

ARRF3 80, 100 and 120 mg/kg it was 89.0, 94.13 and 91.36 per cent, respectively (Fig. 4.25-C).

It is thus observed that overall tumor growth inhibition in groups treated with 80, 100 and 120 mg/kg doses of ARRF3 was quite higher than similar doses of ARRF2 and ARRF2 has yielded higher growth inhibition than ARRE. However, a dose of 140 mg/kg of ARRF2 and ARRF3 was highly toxic to animals.

4.3.2 Sarcoma-180 (ascites)

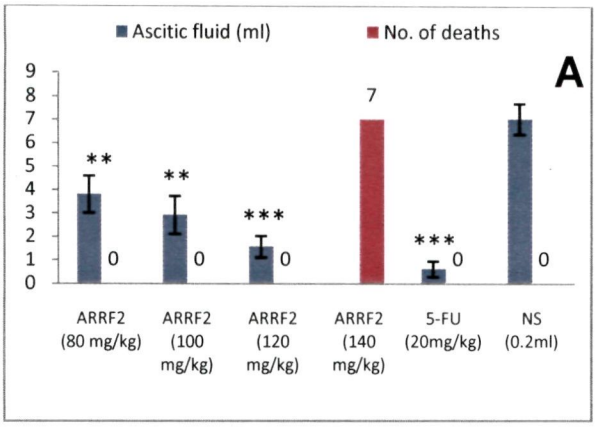
Anticancer activity of ARRE, ARRF2 and ARRF3 was assessed against Sarcoma-180 (ascites) at 80, 100, 120 and 140 mg/kg dose levels in BALB/c mice transplanted with Sarcoma-180 cells. 5-FU and ARRE 120 and 140 mg/kg doses reduced the volume of ascitic fluid (0.32 ± 0.24, 3.45 ± 0.61 and 1.87 ± 0.47 ml, respectively) significantly ($p<0.001$) as compared to NS treated control group (7.19 ± 0.52 ml). Number of tumor cells also declined significantly ($p<0.001$) in 5-FU treated and ARRE 140 mg/kg treated animals (11.57 ± 11.57 x 10^7 and 68.42 ± 32.34 x 10^7, respectively). 100 and 120 mg/kg doses of ARRE also showed significant ($p<0.05$) reduction in the number of tumor cells (211.42 ± 24.16 x 10^7 and 166.42 ± 37.07 x 10^7 respectively) as compared to control animals (294.1 ± 24.39 x 10^7) (Table 4.17; Fig. 4.26). The treatment of Sarcoma-180 bearing animals with 80, 100, 120 and 140 mg/kg doses of ARRE resulted in 21.26, 28.11, 43.41 and 76.73 per cent tumor growth inhibition, respectively whereas 5-FU treatment yielded 96.06 per cent growth inhibition of Sarcoma-180 (ascites) (Fig. 4.26-C).

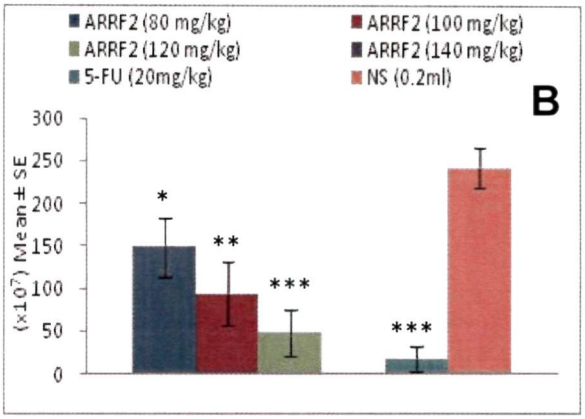
In vivo anticancer activity of ARRF2 against Sarcoma-180 (ascites) studied is presented in Table 4.17 and Fig. 4.27. The volumes of ascitic fluid in 5-FU (0.62 ± 0.33 ml) and ARRF2 120 mg/kg (1.57 ± 0.46 ml) treated groups were significantly ($p<0.001$) lesser than that of NS treated control (7.02 ± 0.66) group. There was significant ($p<0.01$) decline in the volume of ascitic fluid in 80 and 100 mg/kg ARRF2 treated groups also (3.80 ± 0.79 and 2.92 ± 0.81 ml, respectively). However, 140 mg/kg dose of ARRF2 proved toxic to animals as all animals in this group died during the course of experiment. Total number of tumor cells in the ascitic fluid also declined significantly ($p<0.001$) in 5-FU treated positive control group (18.0 ± 15.21 x 10^7) and 120 mg/kg ARRF2 treated

Table 4.17: Anticancer activity of *Asparagus racemosus* roots against murine Sarcoma-180 (ascites)

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (ARRE)	80 mg/kg	7	0	5.88 ± 0.66	231.57 ± 31.61	21.26
	100 mg/kg	7	0	5.37 ± 0.54*	211.42 ± 24.16*	28.11
	120 mg/kg	7	0	3.45 ± 0.61***	166.42 ± 37.07*	43.41
	140 mg/kg	7	0	1.87 ± 0.47***	68.42 ± 32.34***	76.73
5-FU	20 mg/kg	7	0	0.32 ± 0.24***	11.57 ± 11.57***	96.06
NS	0.2 ml	15	0	7.19 ± 0.52	294.1 ± 24.39	-
Chloroform fraction (ARRF2)	80 mg/kg	7	0	3.80 ± 0.79**	149.28 ± 34.87*	38.54
	100 mg/kg	7	0	2.92 ± 0.81**	94.14 ± 37.21**	61.24
	120 mg/kg	7	0	1.57 ± 0.46***	48.71 ± 26.68***	79.94
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.62 ± 0.33***	18.0 ± 15.21***	92.58
NS	0.2 ml	15	0	7.02 ± 0.66	242.9 ± 24.24	-
n-Butanol fraction (ARRF3)	80 mg/kg	7	0	1.4 ± 0.56***	48.85 ± 18.92***	82.78
	100 mg/kg	7	2	0.88 ± 0.48***	33.5 ± 16.81***	88.19
	120 mg/kg	7	3	0.82 ± 0.45***	29.57 ± 23.33***	89.58
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.67 ± 0.35***	20.0 ± 16.90***	92.95
NS	0.2 ml	15	0	7.58 ± 0.55	283.8 ± 23.77	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





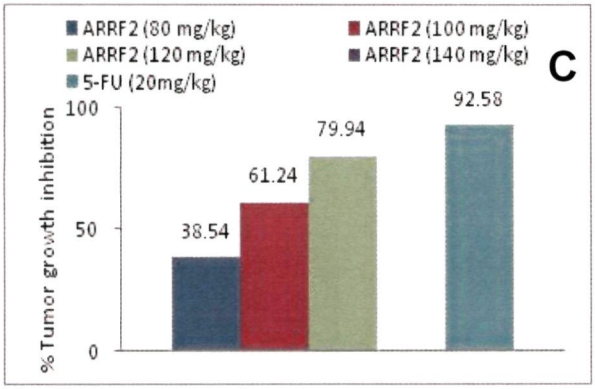
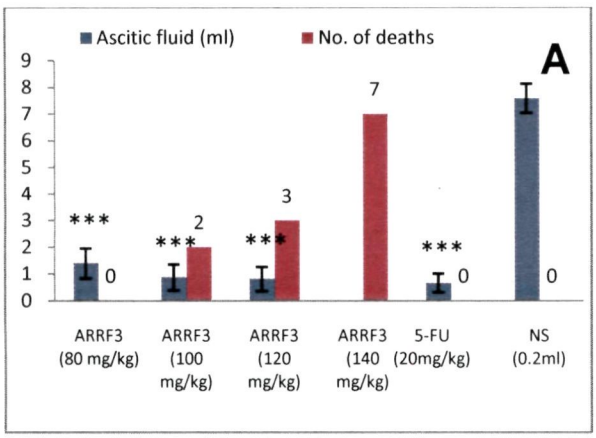
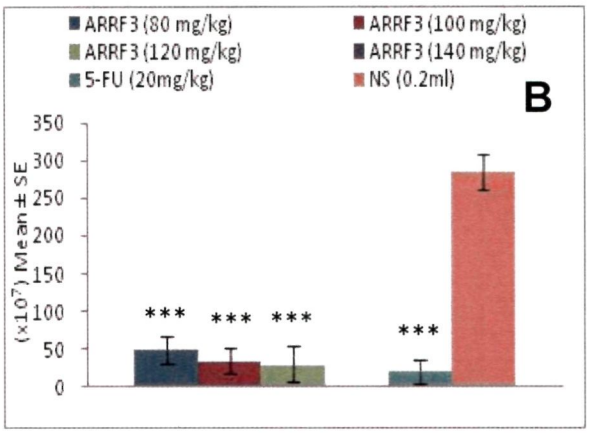


Fig. 4.27: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





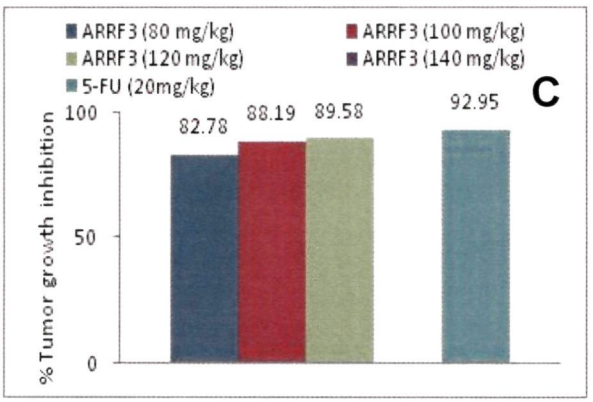


Fig. 4.28: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

group (48.71 ± 26.68 x 10^7) as compared to control group (242.9 ± 24.24 x 10^7). In other groups treated with ARRF2 80 and 100 mg/kg also, the number of tumor cells was greatly reduced (149.28 ± 34.87 x 10^7 and 94.14 ± 37.21 x 10^7, respectively). As depicted in Fig. 4.27-C, ARRF2 80, 100 and 120 mg/kg doses produced 38.54, 61.24 and 79.94 per cent growth inhibition of Sarcoma-180 (ascites) respectively, whereas, 5-FU yielded 92.58 per cent tumor growth inhibition.

It can be seen from Fig. 4.26-C and 4.27-C that the per cent growth inhibition of Sarcoma-180 (ascites) in groups treated with 80, 100 and 120 mg/kg doses of ARRF2 was quite higher than the similar doses of ARRE and 140 mg/kg dose of ARRF2 has been quite toxic to animals.

Perusal of the anticancer activity of ARRF3 against Sarcoma-180 (ascites) illustrated in Table 4.17 and Fig. 4.28 reveals that while ARRF3 has been highly effective in controlling the tumor growth at very low doses, increasing doses were associated with proportionate increase in toxicity. Whereas all animals in ARRF3 140 mg/kg group died during the experiment, 2 and 3 deaths were recorded also in groups treated with 100 and 120 mg/kg of ARRF3, respectively. Nevertheless, there was highly significant ($p<0.001$) reduction in the ascitic fluid of animals treated with 80, 100 and 120 mg/kg of ARRF3 and 5-FU (1.4 ± 0.56, 0.88 ± 0.48, 0.82 ± 0.45 and 0.67 ± 0.35 ml, respectively) as compared to NS treated control group (7.58 ± 0.55 ml) (Fig. 4.28-A). Exactly same pattern was observed for the number of cells also (Fig. 4.28-B). ARRF3 80, 100 and 120 mg/kg treatments resulted in 82.78, 88.19 and 89.58 per cent inhibition in the growth of Sarcoma-180 (ascites) which was comparable to growth inhibition in positive control group (92.95 per cent) (Fig. 4.28-C).

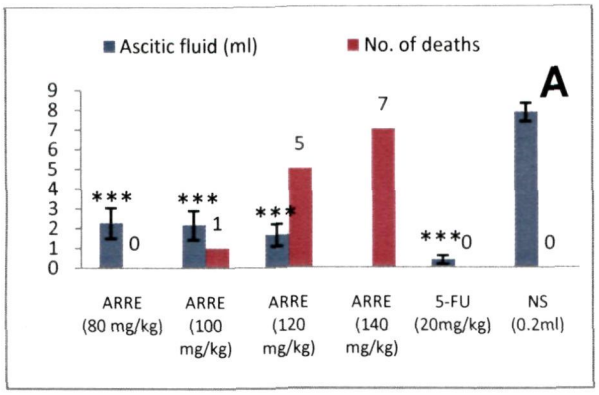
4.3.3 Methyl cholanthrene induced ascites

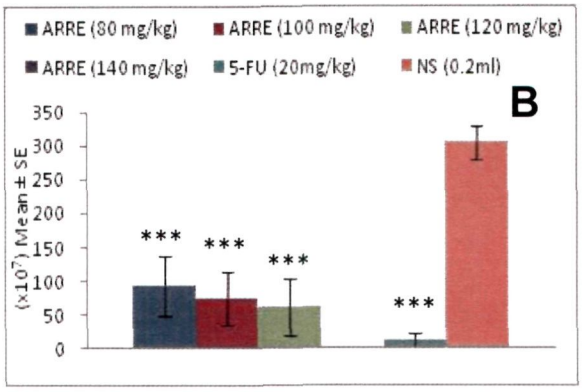
ARRE, ARRF2 and ARRF3 were studied for their anticancer activity against Methyl cholanthrene induced ascites at 80, 100, 120 and 140 mg/kg doses. Data presented in Table 4.18 and Fig. 4.29 illustrates that ARRE at 80, 100 and 120 mg/kg doses significantly ($p<0.001$) reduced the volume of ascitic fluid and the number of tumor cells as well. This resulted in 69.21, 75.47 and 79.74 per cent inhibition in the growth of

Table 4.18: Anticancer activity of *Asparagus racemosus* roots against Methyl cholanthrene induced ascites in mice

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (ARRE)	80 mg/kg	7	0	2.27 ± 0.77***	93.71 ± 44.51***	69.21
	100 mg/kg	7	1	2.17 ± 0.73***	74.66 ± 38.82***	75.47
	120 mg/kg	7	5	1.65 ± 0.56***	61.66 ± 42.43***	79.74
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.38 ± 0.22***	12.14 ± 10.28***	96.01
NS	0.2 ml	15	0	7.85 ± 0.46	304.4 ± 24.52	-
Chloroform fraction (ARRF2)	80 mg/kg	7	0	4.54 ± 0.61**	101.14 ± 33.68***	62.04
	100 mg/kg	7	2	5.42 ± 0.55	58.4 ± 26.64***	78.08
	120 mg/kg	7	4	0.98 ± 0.39***	33.33 ± 19.34***	87.49
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.42 ± 0.29***	11.57 ± 7.64***	95.65
NS	0.2 ml	15	0	7.24 ± 0.50	266.5 ± 22.52	-
n-Butanol fraction (ARRF3)	80 mg/kg	7	0	0.25 ± 0.25***	7.28 ± 6.19***	97.12
	100 mg/kg	7	2	0.44 ± 0.27***	9.4 ± 5.81***	96.29
	120 mg/kg	7	5	0.4 ± 0.4***	10.5 ± 2.5***	95.85
	140 mg/kg	7	7	--	--	--
5-FU	20 mg/kg	7	0	0.38 ± 0.20***	11.85 ± 7.69***	95.32
NS	0.2 ml	15	0	7.55 ± 0.44	253.5 ± 22.65	--

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





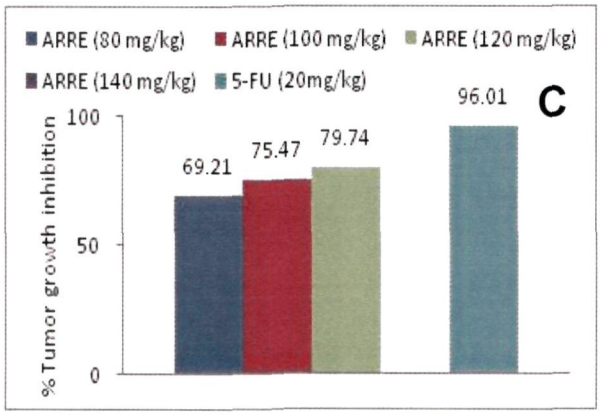
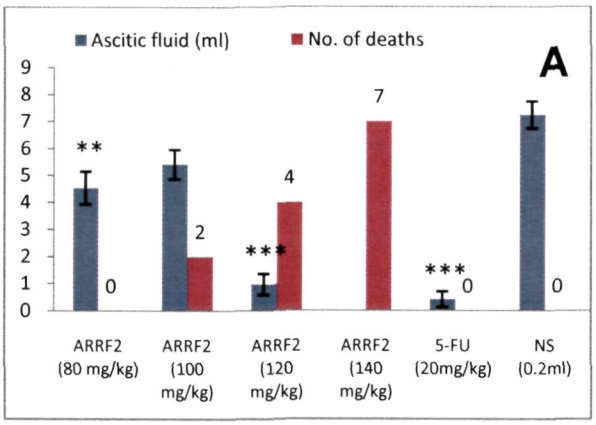
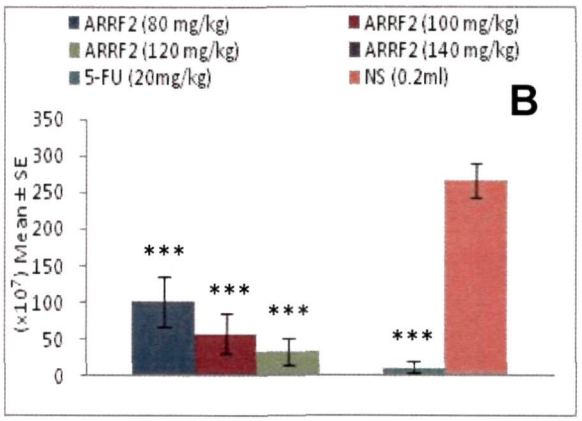


Fig. 4.29: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





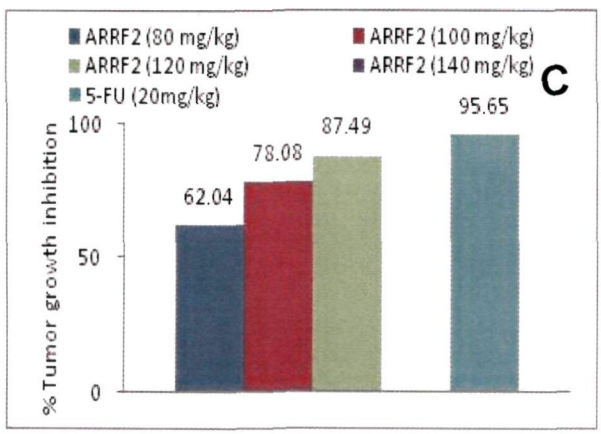


Fig. 4.30: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Methyl cholanthrene induced ascites whereas 5-FU produced 96.01 per cent tumor growth inhibition (Fig. 4.29-C). However, increasing doses of ARRE witnessed increasing toxicity to animals. While there were 1 and 5 deaths respectively in groups treated with 100 and 120 mg/kg, all the 7 animals in ARRE 140 mg/kg treated group died during the experiment.

More or less similar pattern of activity and toxicity was observed with ARRF2. As depicted in Fig. 4.30-A, volume of ascitic fluid declined significantly ($p<0.001$) with 80, 100 and 120 mg/kg of ARRF2 and 5-FU treatments as compared to NS treatment. On the other hand, all animals died with 140 mg/kg of ARRF2. 2 and 4 deaths were seen with 100 and 120 mg/kg treatments also. Number of tumor cells too declined significantly ($p<0.001$) with ARRF2 80, 100 and 120 mg/kg treatments ($101.14 \pm 33.68 \times 10^7$, $58.4 \pm 26.64 \times 10^7$ and $33.33 \pm 19.34 \times 10^7$, respectively) as compared to control animals (Fig. 4.30-B). These treatments exhibited 62.04, 78.08 and 87.49 per cent tumor growth inhibition in ARRF2 80, 100 and 120 mg/kg treated groups whereas 5-FU yielded 95.65 per cent anticancer activity (Fig. 4.30-C).

Perusal of data presented in Table 4.18 and Fig. 4.31 reveals ARRF3 to be more superior in terms of anticancer activity and toxicity. Bare minimum amounts of ascitic fluid were recorded in animals treated with 80, 100 and 120 mg/kg of ARRF3 and 5-FU (0.25 ± 0.25, 0.44 ± 0.27, 0.4 ± 0.4 and 0.38 ± 0.20 ml, respectively) which were significantly lesser ($p<0.001$) than in NS treated animals (7.55 ± 0.44). There were 2 and 5 deaths in 100 and 120 mg/kg treated groups respectively and in group treated with 140 mg/kg, all animals died during the experiment. Number of tumor cells also declined correspondingly ($7.28 \pm 6.19 \times 10^7$, $9.4 \pm 5.81 \times 10^7$, $10.5 \pm 2.5 \times 10^7$ and $11.85 \pm 7.69 \times 10^7$, respectively) (Fig. 4.31-B). Net effect was 97.12, 96.29 and 95.85 per cent tumor growth inhibition with ARRF3 80, 100 and 120 mg/kg doses which is as good as the activity of positive control (95.32 per cent) (Fig. 4.31-C).

Overall, per cent growth inhibition of Methyl cholanthrene induced ascites in groups treated with 80, 100, 120 mg/kg doses of ARRF3 was quite higher than the similar doses of ARRF2 and ARRF2 yielded higher growth inhibition than similar doses of ARRE.

4.3.4 Ehrlich tumor (solid)

Four treatment groups of animals bearing Ehrlich tumor (solid) were treated with 80, 100, 120 and 140 mg/kg doses of ARRE. Positive control group received 22 mg/kg of 5-Fluorouracil (5-FU) and the tumor bearing control group was administered 0.2 ml normal saline (NS). Tumor weights in different groups taken on days 5, 9 and 13 are presented in Table 4.19 and Fig. 4.32. Statistical analysis of tumor weights observed on day 13 showed that 5-FU and ARRE 100, 120 and 140 mg/kg significantly ($p<0.001$) reduced tumor growth as compared to NS treated group. In ARRE 80 mg/kg treated group also, day 13 tumor weight was significantly ($p<0.05$) lower than NS treated group. Based on day 13 tumor weights, per cent growth inhibition of Ehrlich tumor (solid) in 5-FU treated group was 58.59 and in other groups treated with ARRE 80, 100, 120 and 140 mg/kg, it was 18.36, 32.01, 47.58 and 54.21, respectively (Fig. 4.32-B).

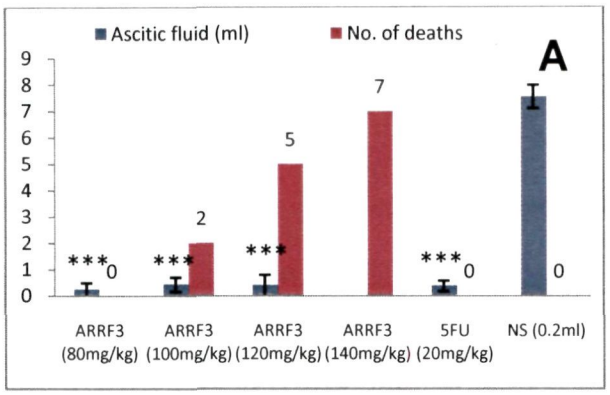
Treatment with similar doses of ARRF2 resulted in slightly better control of tumor growth. As can be seen in Fig. 4.33-A, there was significant ($p<0.001$) decline in tumor weights on day 13 in 5-FU and ARRF2 100 and 120 mg/kg treated groups as compared to NS treated group. Group treated with ARRF2 80 and 140 mg/kg also had significantly ($p<0.01$) lower tumor weights. However, 120 and 140 mg/kg doses were associated with 2 and 4 deaths, respectively. Growth inhibition of Ehrlich tumor (solid) was observed to be 56.72 per cent in 5-FU treated group whereas ARRF2 80, 100, 120 and 140 mg/kg treatments yielded 26.94, 35.55, 43.66 and 57.67 per cent anticancer activity, respectively (Fig. 4.33-B).

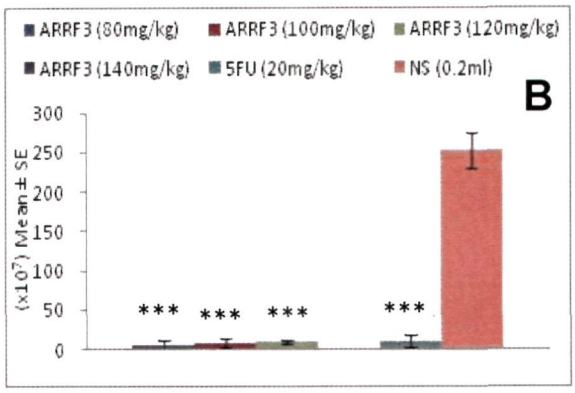
Anticancer activity of ARRF3, studied on similar lines against Ehrlich tumor (solid) was found to be better than the positive control (5-FU) used in the study (Table 4.19 and Fig. 4.34-B). It was however accompanied by 1 and 5 deaths with 100 and 120 mg/kg doses. Further increase in dose to 140 mg/kg proved highly toxic to animals with death of all animals in the group. Like 5-FU, day 13 tumor weights had reduced significantly ($p<0.001$) with ARRF3 80, 100 and 120 mg/kg. These doses of ARRF3 exhibited 61.34, 65.00 and 60.49 per cent anticancer activity against Ehrlich tumor (solid) respectively, while the anticancer activity of 5-FU was observed to be 57.67 per cent against the same model of murine cancer (Fig. 4.34-B).

Table 4.19: Anticancer activity of *Asparagus racemosus* roots against murine Ehrlich tumor (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (ARRE)	80 mg/kg	0/7	712.57 ± 86.33	1023.57 ± 85.08	1493.57 ± 89.98*	18.36
	100 mg/kg	0/7	583.28 ± 67.05	661.28 ± 59.15	1243.71 ± 95.74***	32.01
	120 mg/kg	0/7	401.42 ± 55.12	636.28 ± 71.50	958.85 ± 78.31***	47.58
	140 mg/kg	1/7	373.57 ± 52.29	546.42 ± 72.83	835.33 ± 84.77***	54.21
5-FU	22 mg/kg	0/7	353.71 ± 53.54	519.28 ± 88.15	757.57 ± 71.74***	58.59
NS	0.2 ml	0/15	887.6 ± 51.25	1312.2 ± 62.94	1829.5 ± 78.32	-
Chloroform fraction (ARRF2)	80 mg/kg	0/7	598.85 ± 65.46	806.28 ± 7020	1221.85 ± 91.23**	26.94
	100 mg/kg	0/7	513.57 ± 67.65	754.42 ± 67.91	1077.85 ± 61.49***	35.55
	120 mg/kg	2/7	448.85 ± 59.26	624.42 ± 60.46	941.6 ± 108.00***	43.66
	140 mg/kg	4/7	326.57 ± 53.39	496.85 ± 61.36	708.0 ± 230.38**	57.67
5-FU	22 mg/kg	0/7	341.85 ± 68.95	508.28 ± 78.37	723.85 ± 85.81***	56.72
NS	0.2 ml	0/15	821.3 ± 62.47	1206.2 ± 68.76	1672.6 ± 73.63	-
n-Butanol fraction (ARRF3)	80 mg/kg	0/7	334.14 ± 53.45	466.28 ± 77.72	665.71 ± 76.35***	61.34
	100 mg/kg	1/7	291.85 ± 47.85	363.14 ± 52.84	602.83 ± 82.28***	65.00
	120 mg/kg	5/7	327.14 ± 53.74	466.28 ± 77.72	680.5 ± 168.5***	60.49
	140 mg/kg	7/7	--	--	--	--
5-FU	22 mg/kg	0/7	371.85 ± 57.90	523.28 ± 67.23	781.85 ± 93.91***	57.67
NS	0.2 ml	0/15	835.9 ± 74.17	1222.4 ± 75.09	1722.4 ± 80.14	--

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





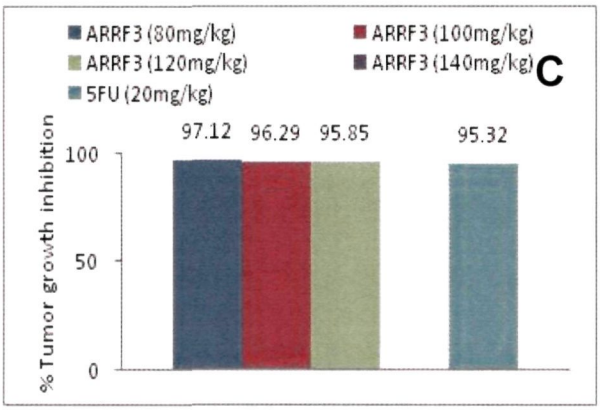
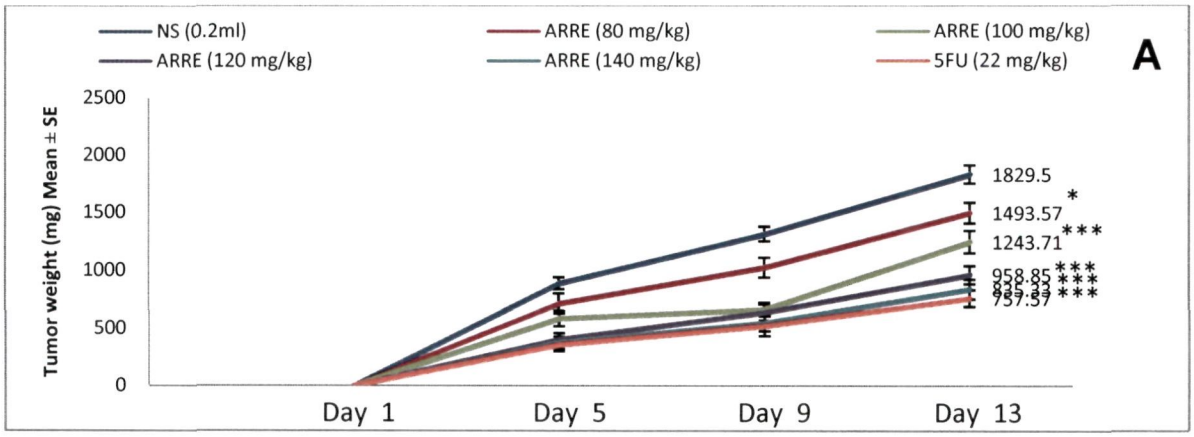


Fig. 4.31: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots on (ARRF3) Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid and the number of deaths in treated mice. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



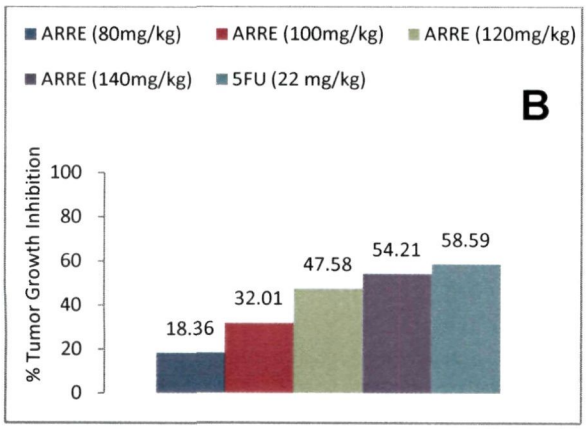
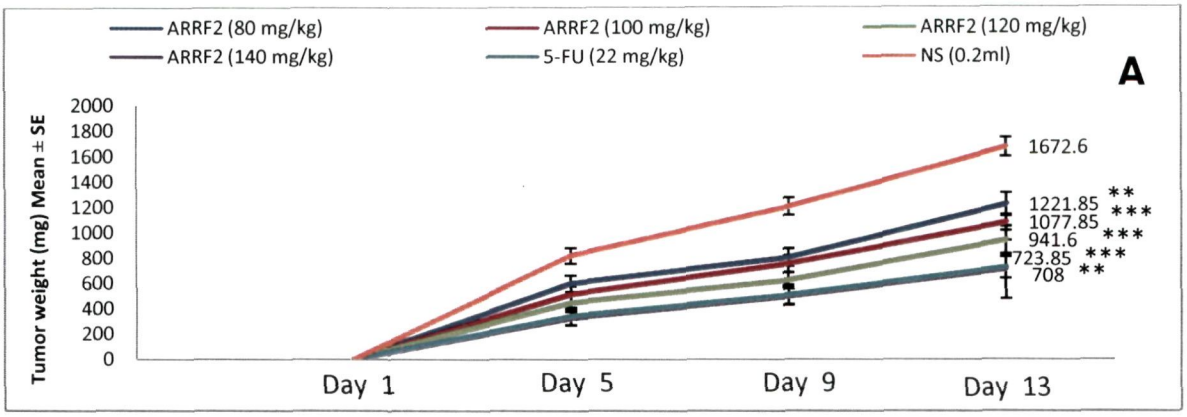


Fig. 4.32: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on murine Ehrlich tumor (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



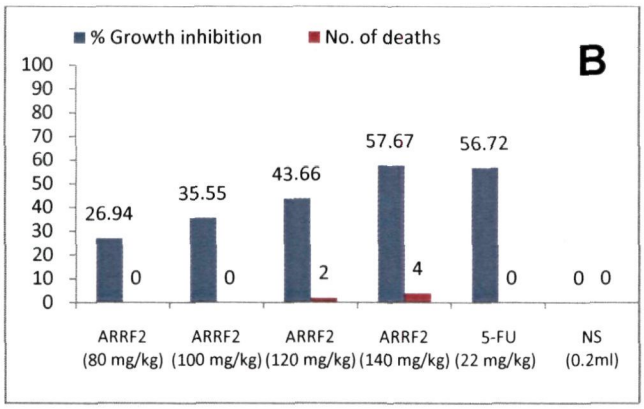
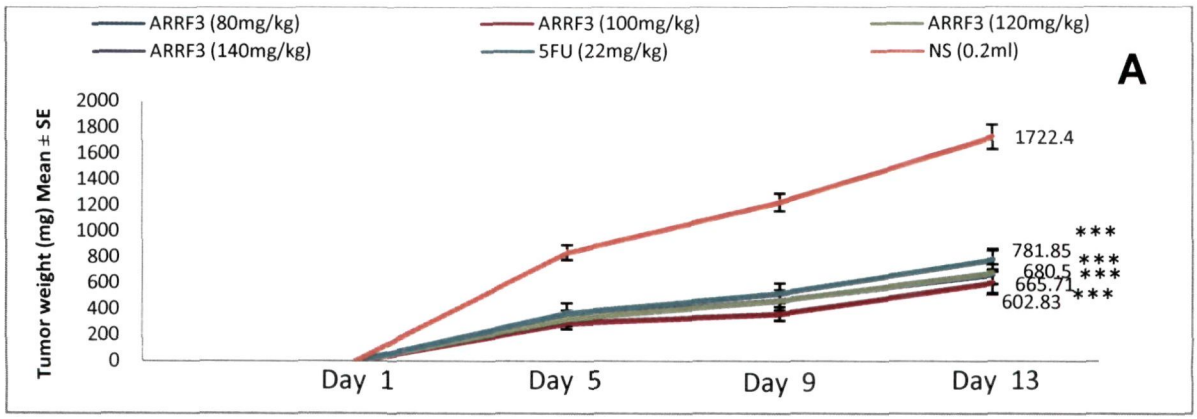


Fig. 4.33: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on murine Ehrlich tumor (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



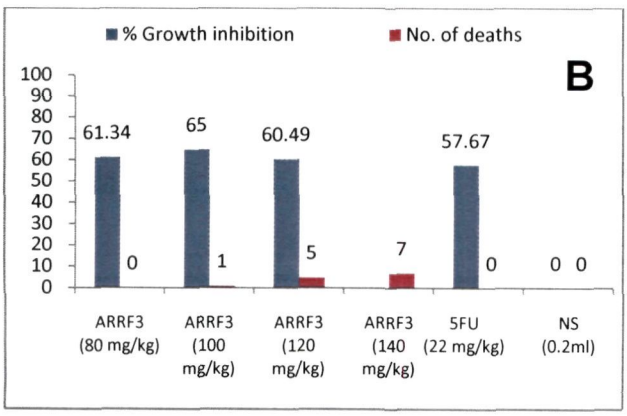


Fig. 4.34: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on murine Ehrlich tumor (solid) in mice. A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

It can thus be deduced that while overall anticancer activity of ARRF3 was quite higher than ARRF2 and ARRF2 was more efficacious than ARRE, higher anticancer activity was associated with higher toxicity also.

4.3.5 Sarcoma-180 (solid)

Anticancer activity of ARRE studied at 80, 100, 120 and 140 mg/kg dose levels against Sarcoma-180 (solid) as presented in Table 4.20 and Fig. 4.35 revealed that 120 and 140 mg/kg doses significantly ($p<0.001$) inhibited the tumor growth like 5-FU. 100 mg/kg treated group also showed significantly lower ($p<0.01$) tumor weight as compared to NS treated group on day 13. Anticancer activity of ARRE in terms of growth inhibition of Sarcoma-180 (solid) was observed to be 10.46, 21.16, 29.03 and 45.53 per cent respectively, with 80, 100, 120 and 140 mg/kg doses of ARRE. 5-FU inhibited the tumor growth by 57.34 per cent (Fig. 4.35-B). However, the anticancer activity observed with 140 mg/kg dose was accompanied by 4 deaths also.

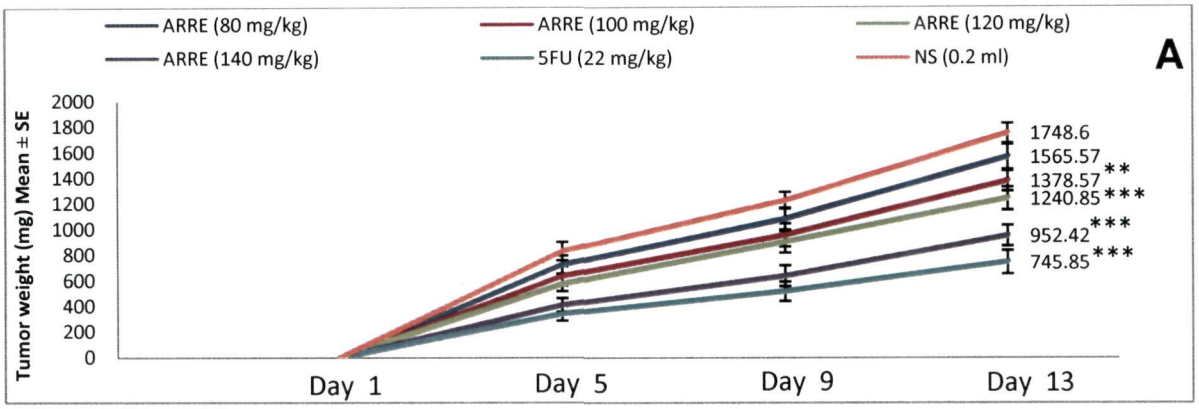
ARRF2 exhibited better anticancer activity than ARRE at similar dose levels but was associated with little more toxicity also. Lower doses (80 and 100 mg/kg) were well tolerated by animals but yielded only moderate growth inhibition (22.33 and 33.60 per cent) of Sarcoma-180 (solid) which happened to be statistically significant ($p<0.01$ and $p<0.001$, respectively). Higher doses (120 and 140 mg/kg) significantly ($p<0.001$) reduced tumor weights (by 43.01 and 47.09 per cent, respectively) but were bit intolerable as revealed by 3 and 5 deaths, respectively (Fig. 4.36-B). In 5-FU treated group, tumor growth was inhibited to the extent of 55.75 per cent.

Anticancer activity of ARRF3 at 80, 100 and 120 mg/kg doses against Sarcoma-180 (solid) was observed to be comparable to 5-FU. With these doses day 13 tumor weights had declined significantly ($p<0.001$) as compared to control group (Fig. 4.37-A), exhibiting 55.06, 53.07 and 57.26 per cent tumor growth inhibition. However, a dose of 140 mg/kg of ARRF3 was not at all tolerated by animals. 100 and 120 mg/kg doses were also bit intolerable as revealed by 2 and 4 deaths with these doses (Fig. 4.37-B). Overall, ARRF3 was found to possess higher anticancer activity than ARRF2 and ARRF2 was more efficacious than ARRE. Nonetheless, higher anticancer activity was associated with higher levels of toxicity too.

Table 4.20: Anticancer activity of *Asparagus racemosus* roots against murine Sarcoma-180 (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (ARRE)	80 mg/kg	0/7	727.28 ± 71.26	1083.71 ± 84.89	1565.57 ± 95.82	10.46
	100 mg/kg	0/7	637.57 ± 74.74	958.42 ± 90.34	1378.57 ± 79.49**	21.16
	120 mg/kg	0/7	578.57 ± 58.60	904.28 ± 82.89	1240.85 ± 90.70***	29.03
	140 mg/kg	4/7	412.14 ± 54.11	640.42 ± 80.70	952.42 ± 79.72***	45.53
5-FU	22 mg/kg	0/7	343.85 ± 55.03	519.42 ± 75.78	745.85 ± 91.33***	57.34
NS	0.2 ml	0/15	831.6 ± 71.94	1227.4 ± 65.94	1748.6 ± 76.97	-
Chloroform fraction (ARRF2)	80 mg/kg	0/7	584.0 ± 74.49	888.42 ± 91.61	1302.85 ± 97.44**	22.33
	100 mg/kg	0/7	508.42 ± 71.90	772.71 ± 90.26	1113.85 ± 90.12***	33.60
	120 mg/kg	3/7	414.57 ± 61.59	652.28 ± 88.66	956.0 ± 134.03***	43.01
	140 mg/kg	5/7	398.57 ± 58.28	622.28 ± 84.22	887.5 ± 63.5***	47.09
5-FU	22 mg/kg	0/7	347.42 ± 57.02	527.71 ± 86.11	742.14 ± 101.78***	55.75
NS	0.2 ml	0/15	791.85 ± 66.82	1212.85 ± 97.14	1677.5 ± 72.06	-
n-Butanol fraction (ARRF3)	80 mg/kg	0/7	361.14 ± 63.96	559.71 ± 82.70	800.71 ± 95.18***	55.06
	100 mg/kg	2/7	372.42 ± 61.97	581.85 ± 84.34	836.14 ± 85.91***	53.07
	120 mg/kg	4/7	341.28 ± 63.77	504.42 ± 84.58	761.57 ± 93.29***	57.26
	140 mg/kg	7/7	--	--	--	--
5-FU	22 mg/kg	0/7	351.14 ± 52.07	532.85 ± 76.14	779.57 ± 94.78***	56.25
NS	0.2 ml	0/15	847.42 ± 72.74	1272.42 ± 96.47	1782.0 ± 69.53	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.



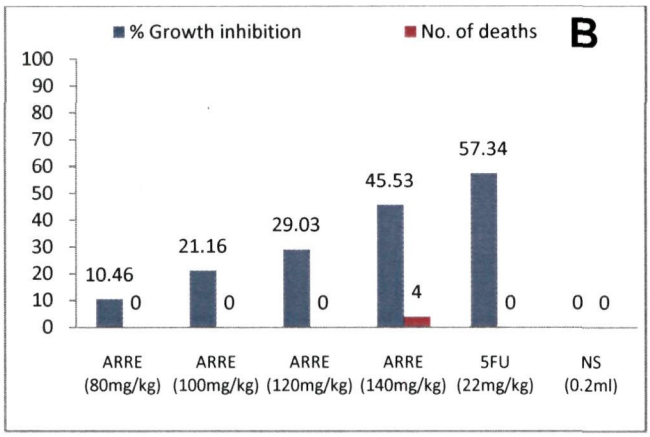
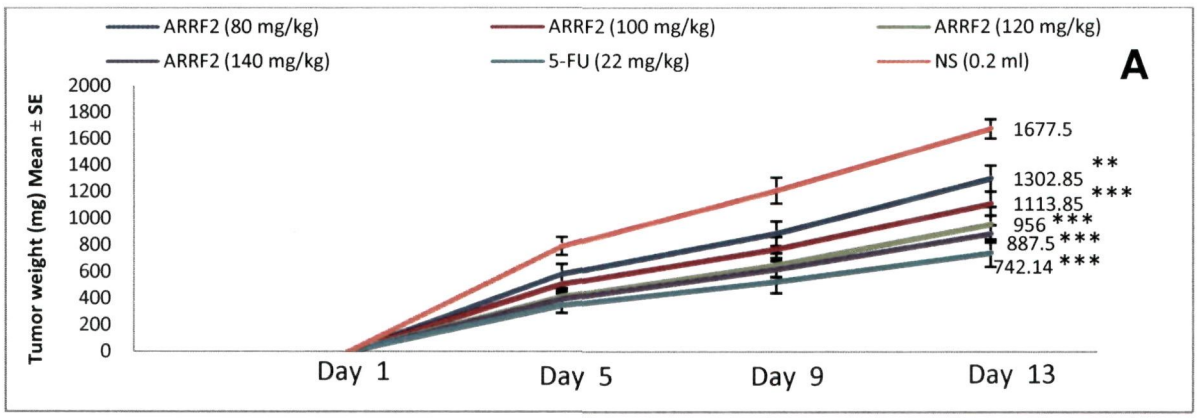


Fig. 4.35: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



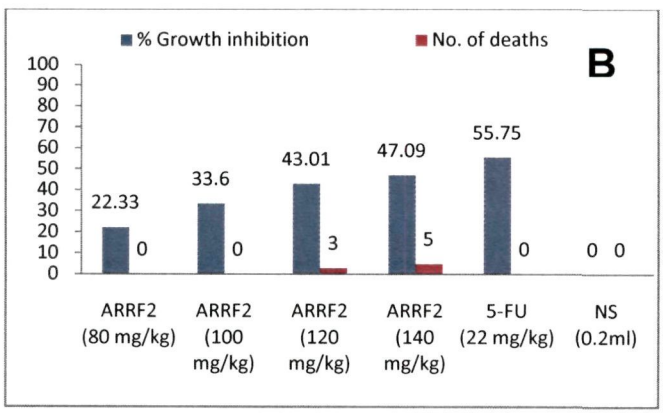


Fig. 4.36: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.3.6 L1210 Lymphoid leukemia

Anticancer activity of ARRE, ARRF2 and ARRF3 studied at 80, 100, 120 and 140 mg/kg in CD2F1 mice transplanted with L1210 Lymphoid leukemia cells is depicted in Table 4.21 and Fig. 4.38, 4.39 and 4.40. Mean survival time (MST) in NS treated group was 7.33 days and in positive control group it was 16.75 days (Fig. 4.38-A). Animals treated with ARRE 80, 100, 120 and 140 mg/kg had an MST of 110.77, 104.50, 104.22 and 95.49 per cent of tumor bearing control, respectively. Net result of treatment with 80, 100 and 120 mg/kg of ARRE was 10.77, 4.50 and 4.22 per cent increase in life span of treated animals over tumor bearing control animals. Higher dose of 140 mg/kg in fact shortened the life span of animals thereby showing negative increase in life span (Fig. 4.38-C). 5-FU treated group however showed 128.51 per cent increase in life span.

As depicted in Fig. 4.39, all groups of animals treated with 80, 100, 120 and 140 mg/kg of ARRF2 had an MST less than that of NS treated group (7.33 days), and therefore yielded negative increase in life span (-2.32, -5.73, -6.96 and -12.56, respectively). 5-FU treated group however showed 90.99 per cent increase in life span over tumor bearing control animals (Fig. 4.39-C). It can be observed that the MST of animals bearing L1210 Lymphoid leukemia shortened progressively with increasing doses of ARRF2 (Fig. 4.39-B).

Anticancer activity of ARRF3 studied on similar lines against L1210 Lymphoid leukemia is depicted in Fig. 4.40. Among all doses of ARRF3, only the dose of 80 mg/kg exhibited 33.0 per cent increase in life span over tumor bearing control animals, which constitutes moderate anticancer activity against L1210 Lymphoid leukemia. Other doses either showed negligible (9.0 per cent) or negative (-4.0 and -11.0 per cent) increase in life span. Nonetheless, 5-FU treated group had 90.99 per cent increase in life span over and above NS treated control group (Fig. 4.40-C) and it constituted significant anticancer activity against L1210 Lymphoid leukemia.

4.3.7 P388 Lymphocytic leukemia

Data on *in vivo* anticancer activity of ARRE studied in CD2F1 mice transplanted with P388 Lymphocytic leukemia cells at 80, 100, 120 and 140 mg/kg dose levels is depicted in Table 4.22 and Fig. 4.41.

Table 4.21: Anticancer activity of *Asparagus racemosus* roots against L1210 Lymphoid leukemia in CD2F1 mice

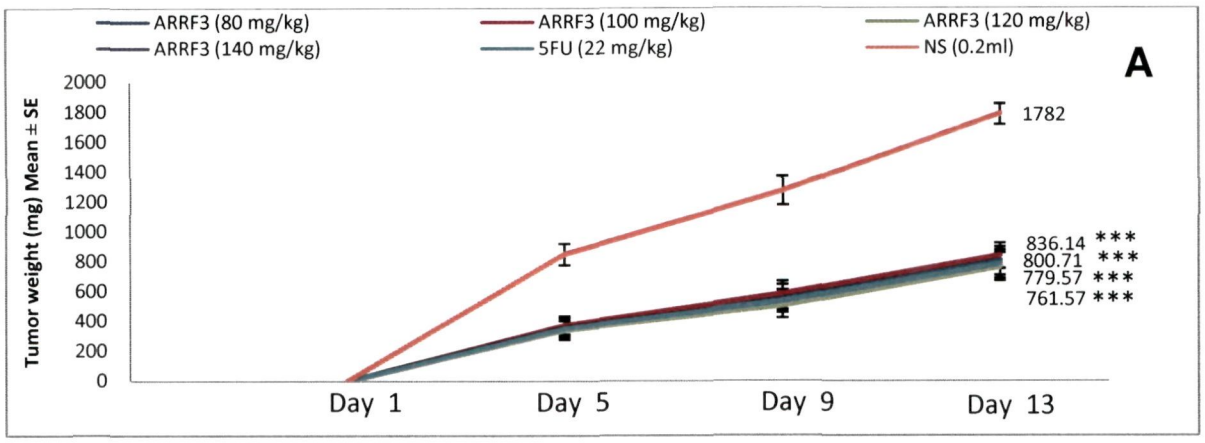
Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (ARRE)	80 mg/kg	6	8.12	110.77	10.77
	100 mg/kg	6	7.66	104.50	4.50
	120 mg/kg	6	7.64	104.22	4.22
	140 mg/kg	6	7.0	95.49	-4.51
5-FU	20 mg/kg	6	16.75	228.51	128.51
NS	0.2 ml	6	7.33	-	-
Chloroform fraction (ARRF2)	80 mg/kg	6	7.16	97.68	-2.32
	100 mg/kg	6	6.91	94.27	-5.73
	120 mg/kg	6	6.82	93.04	-6.96
	140 mg/kg	6	6.41	87.44	-12.56
5-FU	20 mg/kg	6	14.0	190.99	90.99
NS	0.2 ml	6	7.33	-	-
n-Butanol fraction (ARRF3)	80 mg/kg	6	9.75	133	33
	100 mg/kg	6	7.98	109	9
	120 mg/kg	6	7.03	96	-4
	140 mg/kg	6	6.53	89	-11
5-FU	20 mg/kg	6	14.0	190.99	90.99
NS	0.2 ml	6	7.33	-	-

5-FU: 5-fluorouracil; NS: Normal saline.

Table 4.22: Anticancer activity of *Asparagus racemosus* roots against P388 Lymphocytic leukemia in CD2F1 mice

Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (ARRE)	80 mg/kg	6	8.57	112	12
	100 mg/kg	6	9.42	123	23
	120 mg/kg	6	11.56	151	51
	140 mg/kg	6	14.0	182.76	82.76
5-FU	20 mg/kg	6	19.0	248.04	148.04
NS	0.2 ml	6	7.66	-	-
Chloroform fraction (ARRF2)	80 mg/kg	6	8.04	105	5
	100 mg/kg	6	9.42	122.98	22.98
	120 mg/kg	6	8.88	116	16
	140 mg/kg	6	10.33	134.85	34.85
5-FU	20 mg/kg	6	19.0	248.04	148.04
NS	0.2 ml	6	7.66	-	-
n-Butanol fraction (ARRF3)	80 mg/kg	6	11.47	168	68
	100 mg/kg	6	17.14	251.09	151.09
	120 mg/kg	6	8.81	129	29
	140 mg/kg	6	6.48	95	-5
5-FU	20 mg/kg	6	18.96	263	163
NS	0.2 ml	6	6.83	-	-

5-FU: 5-fluorouracil; NS: Normal saline.



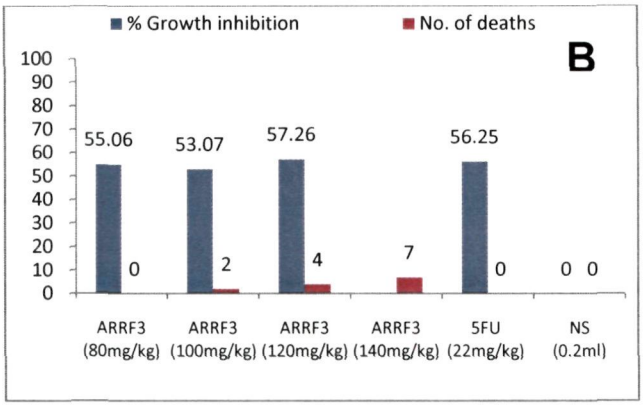
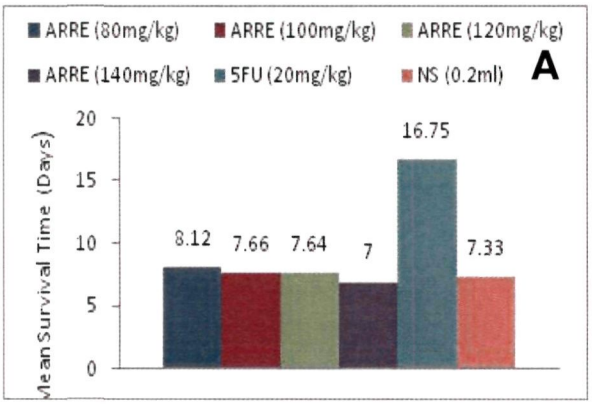
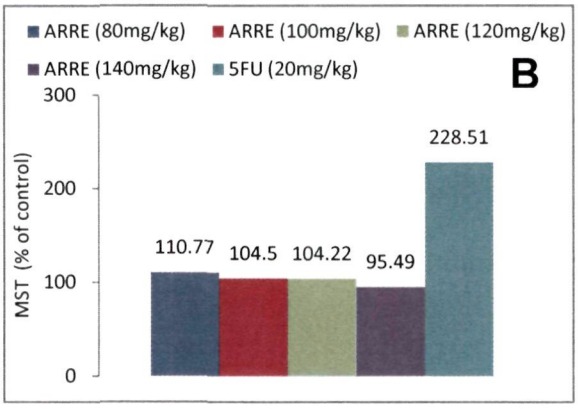


Fig. 4.37: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





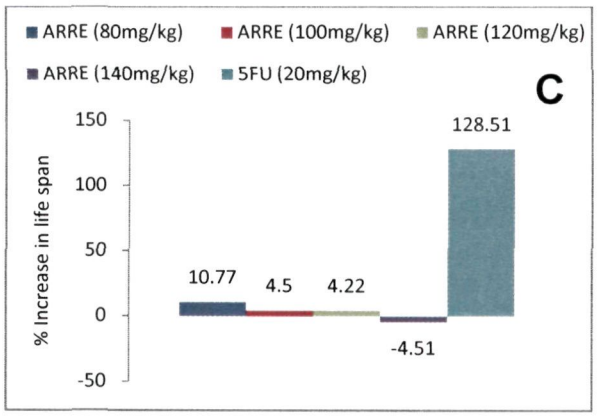
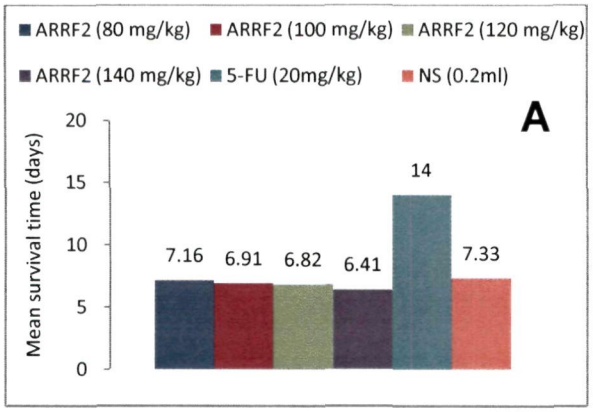
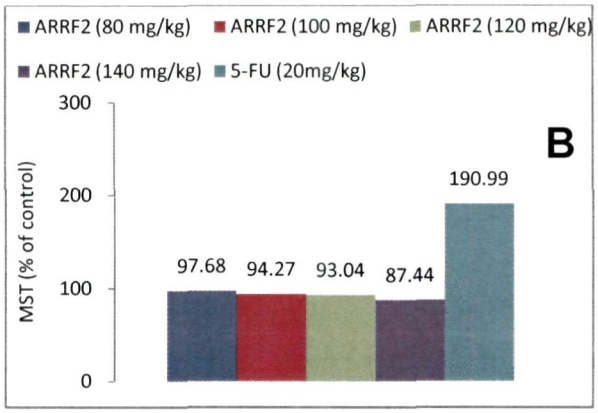


Fig. 4.38: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice.
5-FU: 5-fluorouracil. NS: Normal saline.





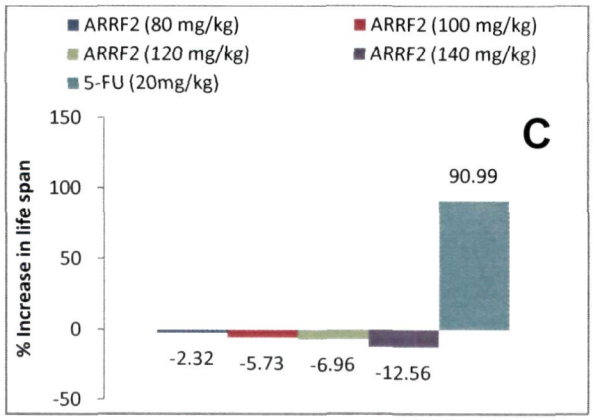
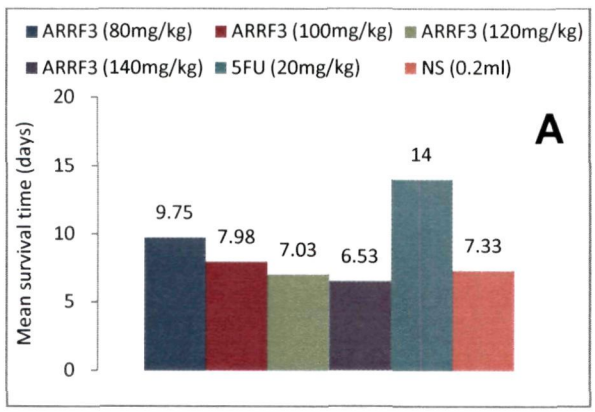
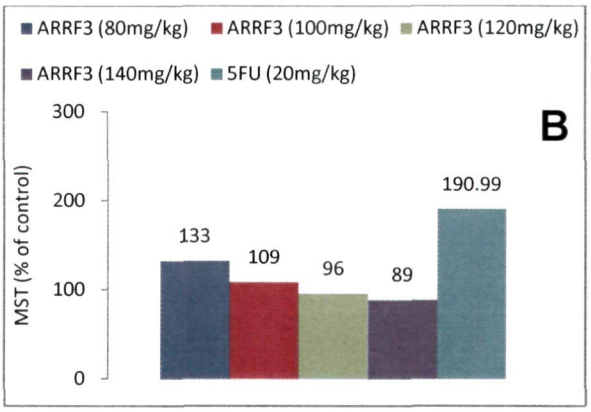


Fig. 4.39: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of treated mice. B: Mean survival time of treated mice expressed as per cent of tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.





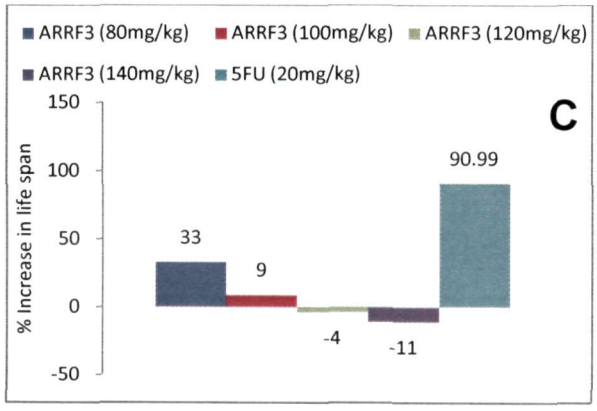
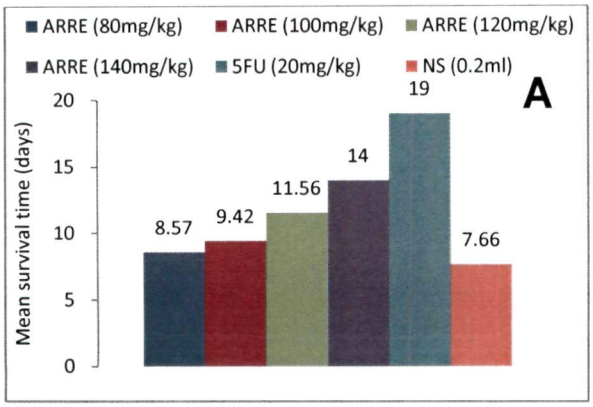


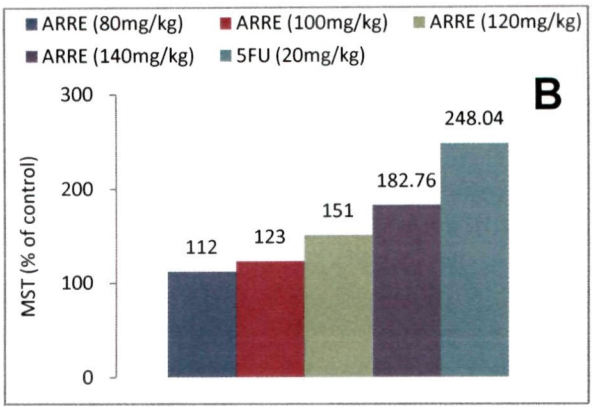
Fig. 4.40: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.

Mean survival time (MST) in NS treated group was 7.66 days and in positive control group it was 19.00 days. In groups treated with ARRE 80, 100, 120 and 140 mg/kg, MST was 112.0, 123.0, 151.0 and 182.76 per cent of tumor bearing control group, respectively. Thus, lower doses (80 and 100 mg/kg) were found to increase the life span of treated animals non-significantly whereas higher doses (120 and 140 mg/kg) exhibited significant increase the life span (51.0 and 82.76 per cent, respectively) over tumor bearing control animals (Fig. 4.41-C). 5-FU treatment however increased the life span of P388 Lymphocytic leukemia bearing animals by 148.04 per cent.

Highest dose of ARRF2 (140 mg/kg) increased the life span of treated animals moderately (34.85 per cent). Other doses of ARRF2 also increased the life span (by 5.0, 22.98 and 16.0 per cent) but such increase was not considered significant. Nonetheless, 5-FU treated group witnessed 148.04 per cent increase in the life span over P388 Lymphocytic leukemia bearing control animals (Fig. 4.42-C).

Anticancer activity ARRF3 studied on similar lines against P388 Lymphocytic leukemia is depicted in Table 4.22 and Fig. 4.43. In NS treated group, the MST was 6.83 days and in positive control group it was 18.96 days. Other groups treated with ARRF3 80, 100, 120 and 140 mg/kg witnessed an MST of 168.0, 251.09, 129.0 and 95.0 per cent of NS treated control group, respectively (Fig. 4.43-B). Thus, lower doses (80 and 100 mg/kg) of ARRF3 had significant effect on increasing the life span of P388 Lymphocytic leukemia bearing animals whereas higher doses (120 and 140 mg/kg) either did not significantly increase the life span or tended to be toxic to P388 Lymphocytic leukemia bearing animals. 5-FU at 20 mg/kg increased the life span of treated animals by 163.0 per cent over tumor bearing control animals (Fig. 4.43-C). It was thus evident that *Asparagus racemosus* roots possess definite potential of anticancer activity against P388 Lymphocytic leukemia.





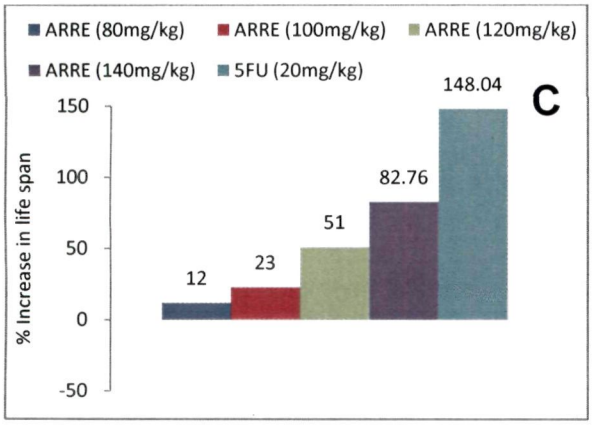
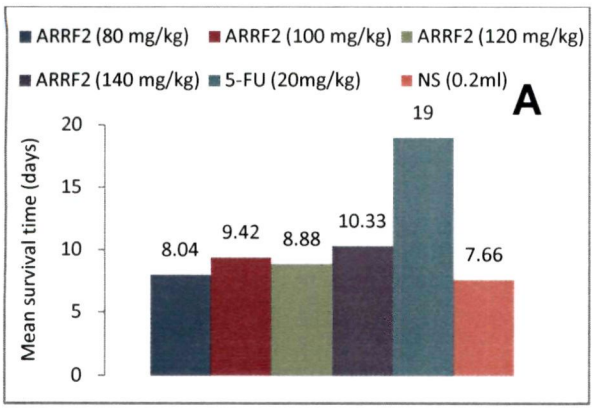
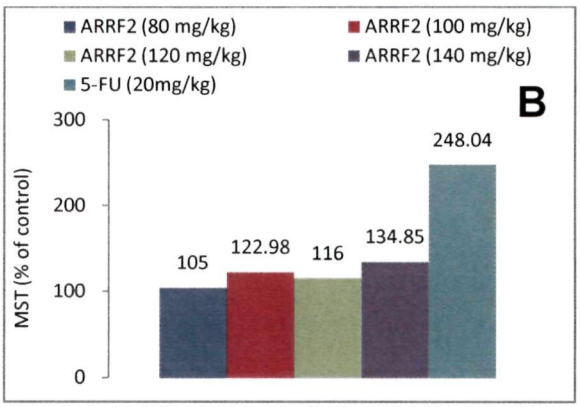


Fig. 4.41: Effect of different doses of alcoholic extract of *Asparagus racemosus* roots (ARRE) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.





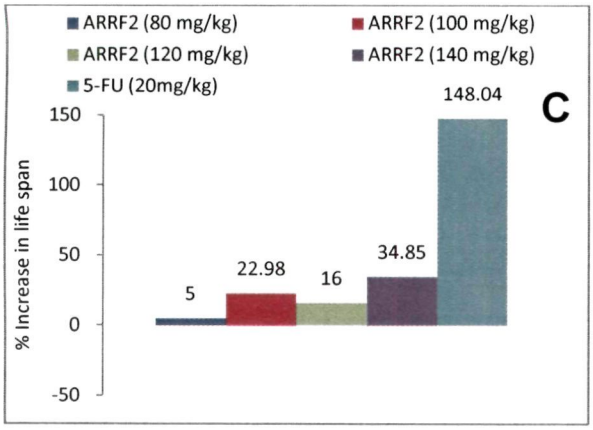
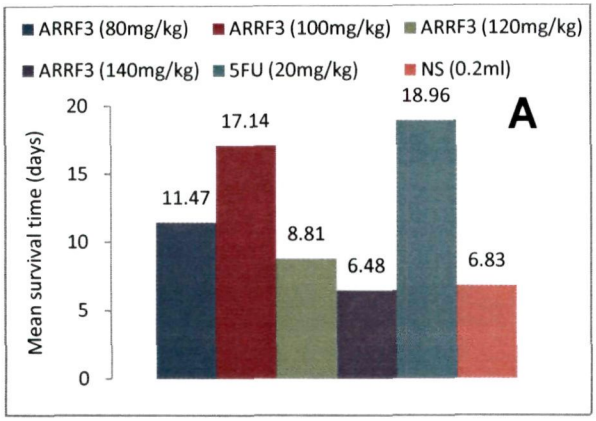
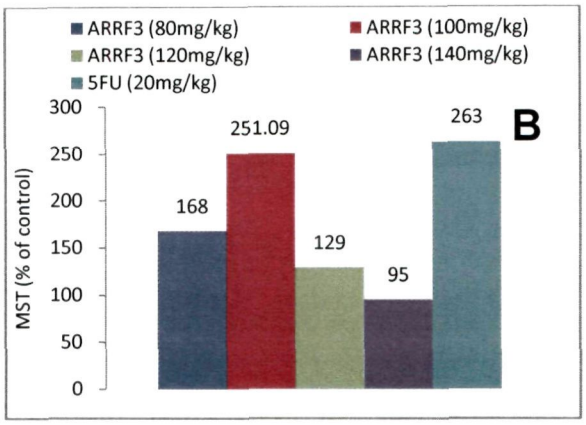


Fig. 4.42: Effect of different doses of chloroform fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF2) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.





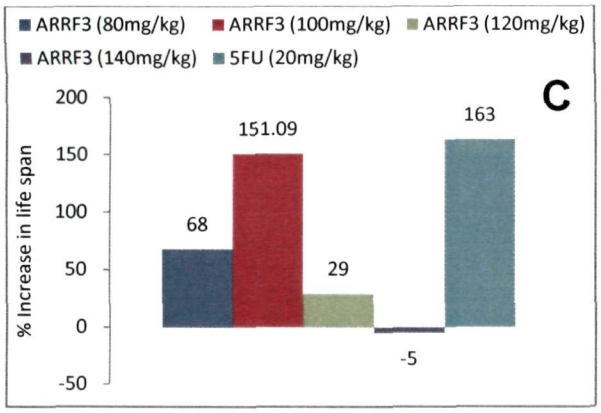
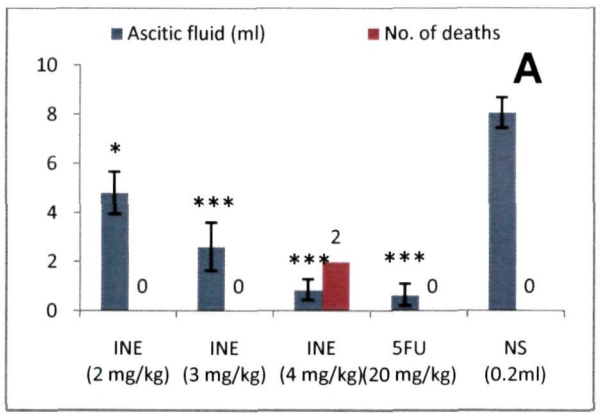
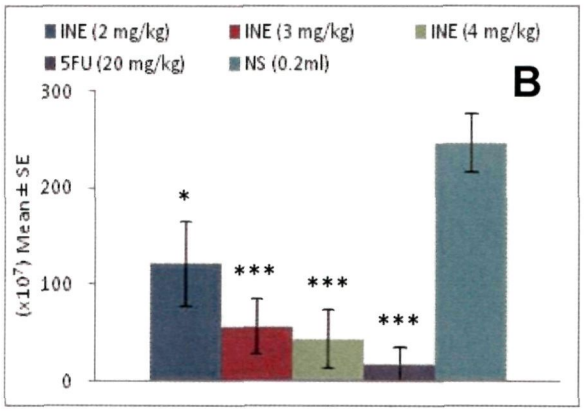


Fig. 4.43: Effect of different doses of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice. 5-FU: 5-fluorouracil. NS: Normal saline.





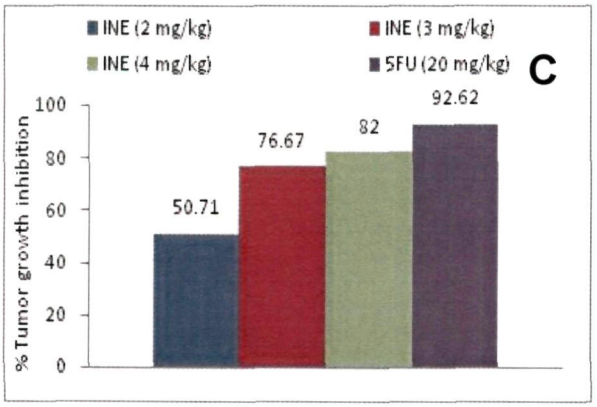


Fig. 4.44: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.4 *In vivo* anticancer activity of alcoholic extract and its n-hexane fraction from *Ipomoea nil* aerial parts against murine cancer models

4.4.1 Ehrlich ascites carcinoma (EAC)

After induction of Ehrlich Ascites Carcinoma in non-inbred Swiss mice, the animals were assigned to different groups. Four treatment groups were treated with 2, 3 and 4 mg/kg respectively of alcoholic extract of *Ipomoea nil* aerial parts (INE) intraperitoneally (i.p.). Fifth group was treated with 20 mg/kg of 5-Fluorouracil (5-FU) i.p. and it served as positive control. The last group (tumor bearing control) was not administered any drug except 0.2 ml normal saline (NS). Data obtained on *in vivo* anticancer activity of INE against Ehrlich Ascites Carcinoma is presented in Table 4.23 and Fig. 4.44.

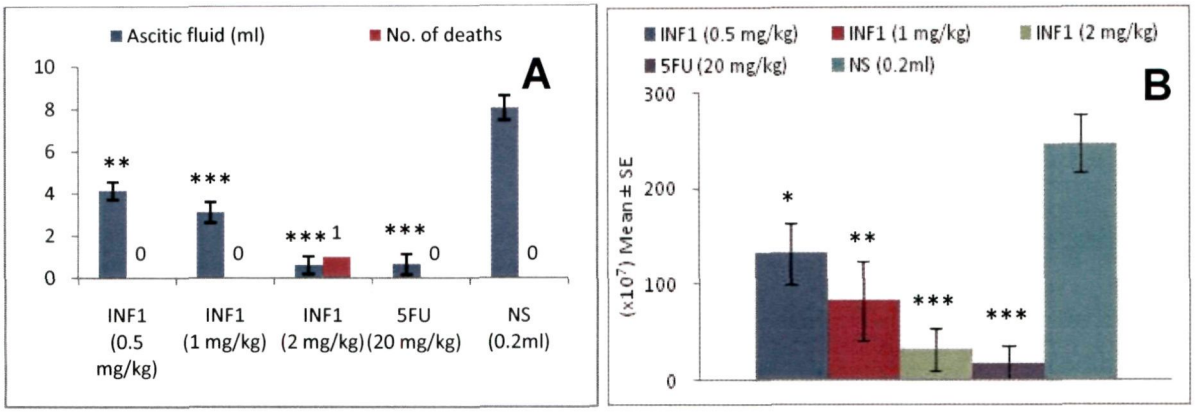
The average volume of ascitic fluid in NS treated group was 8.06 ± 0.62 ml and in 5-FU treated positive control group it was significantly ($p<0.001$) lower (0.65 ± 0.45 ml). Animals treated with INE 2, 3 and 4 mg/kg recorded reduction in the volume of ascitic fluid in dose-dependent manner (4.81 ± 1.09, 2.62 ± 0.88 and 0.86 ± 0.57 ml, respectively). Such reduction was statistically significant ($p<0.001$) in groups treated with 3 and 4 mg/kg of INE (Fig. 4.44-A). Exactly same pattern was evident for the number of tumor cells (Fig. 4.44-B). Total number of tumor cells in NS treated group was 246.1 ± 30.10 x 10^7 whereas in 5-FU treated group it was significantly ($p<0.001$) lower (18.14 ± 18.14 x 10^7). In treated groups absolute number of tumor cells with INE 2, 3 and 4 mg/kg doses was found to be 121.28 ± 44.28 x 10^7, 56.57 ± 28.55 x 10^7 and 44.2 ± 30.50 x 10^7, respectively. Statistically, there was significant ($p<0.001$) reduction in the number of tumor cells in animals treated with INE 3 and 4 mg/kg. This resulted in 50.71, 76.67 and, 82.0 per cent inhibition in the growth of Ehrlich Ascites Carcinoma with INE 2, 3 and 4 mg/kg respectively, whereas 5-FU treated group showed 92.62 per cent tumor growth inhibition. Anticancer activity of INE at 4 mg/kg was comparable to 5-FU but was associated with 2 deaths (4.44-C).

In vivo anticancer activity of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) against Ehrlich ascites carcinoma was studied at 0.5, 1 and 2 mg/kg dose levels. Statistical analysis of data presented in Fig. 4.45-A showed that there was

Table 4.23: Anticancer activity of *Ipomoea nil* aerial parts against murine Ehrlich ascites carcinoma

Treatments	Dose	Animals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (INE)	2 mg/kg	7	0	4.81 ± 1.09*	121.28 ± 44.28*	50.71
	3 mg/kg	7	0	2.62 ± 0.88***	56.57 ± 28.55***	76.67
	4 mg/kg	7	2	0.86 ± 0.57***	44.2 ± 30.50***	82
n-Hexane fraction (INF1)	0.5 mg/kg	7	0	4.14 ± 0.86**	131.85 ± 32.0*	46.42
	1 mg/kg	7	0	3.12 ± 0.99***	82.85 ± 41.23**	66.33
	2 mg/kg	7	1	0.61 ± 0.43***	31.83 ± 21.52***	87.06
5-FU	20 mg/kg	7	0	0.65 ± 0.45***	18.14 ± 18.14***	92.62
NS	0.2 ml	15	0	8.06 ± 0.62	246.1 ± 30.10	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.



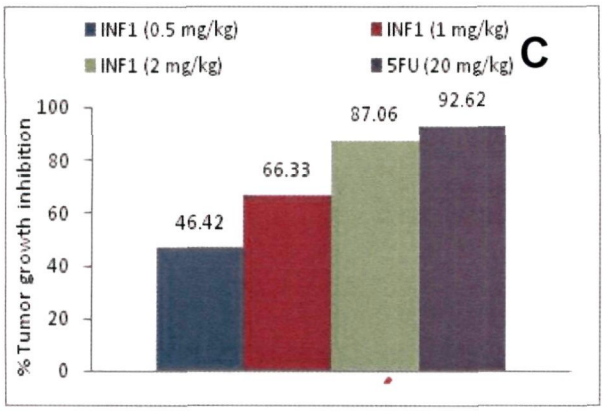
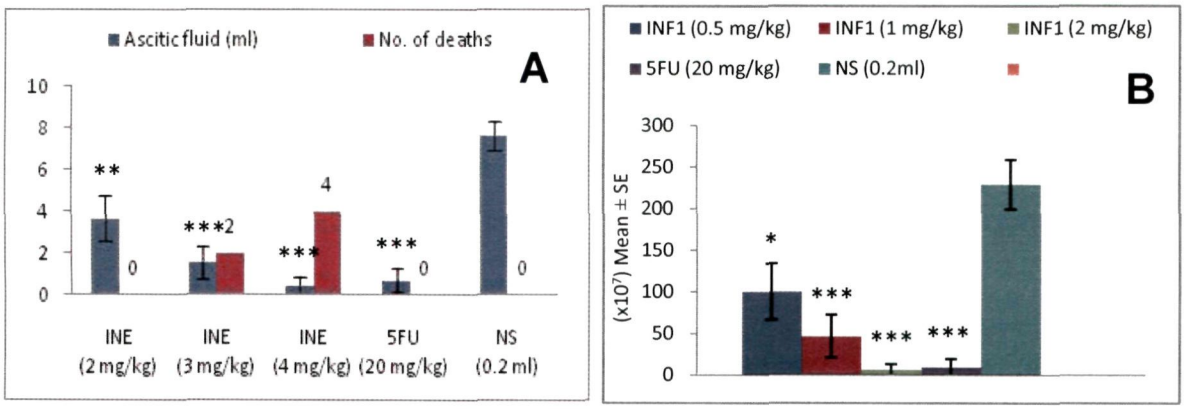


Fig. 4.45: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on murine Ehrlich ascites carcinoma. A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



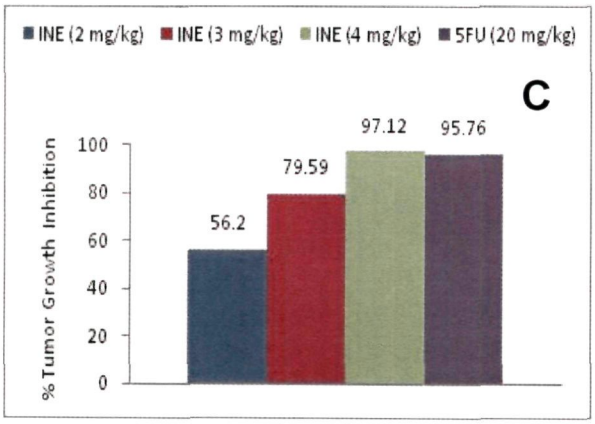


Fig. 4.46: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

significant ($p<0.001$) reduction in the volumes of ascitic fluid in groups treated with INF1 1 and 2 mg/kg (3.12 ± 0.99 and 0.61 ± 0.43 ml, respectively). Likewise, the number of tumor cells in animals treated with INF1 0.5, 1 and 2 mg/kg (131.85 ± 32.0 x 10^7, 82.85 ± 41.23 x 10^7 and 31.83 ± 21.52 x 10^7, respectively) were also found to be significantly ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) less than that of NS treated control animals (Fig. 4.45-B).

Tumor growth inhibition in groups treated with INF1 0.5, 1 and 2 mg/kg was 46.42, 66.33 and 87.06 per cent, respectively (Fig. 4.45-C). It can be seen that although doses of INF1 were less than half of INE, its anticancer activity was comparable to 2, 3 and 4 mg/kg doses of INE.

4.4.2 Sarcoma-180 (ascites)

In vivo anticancer activity of INE was also assessed against Sarcoma-180 (ascites) at 2, 3 and 4 mg/kg dose levels and the data is presented in Table 4.24 and Fig. 4.46. The volume of ascitic fluid in 5-FU treated group (0.71 ± 0.56 ml) showed significant ($p<0.001$) decline as compared to NS treated group (7.58 ± 0.69 ml). Dose –dependent decline in the volume of ascitic fluid was also recorded in animals treated with INE 2, 3 and 4 mg/kg (3.65 ± 1.10, 1.58 ± 0.79 and 0.43 ± 0.43 ml, respectively) that proved to be statistically significant (Fig. 4.46-A).

Total number of tumor cells too exhibited dose-dependent decline from 229.3 ± 29.60 x 10^7 in NS treated group to 100.42 ± 33.80 x 10^7, 46.8 ± 25.80 x 10^7 and 6.66 ± 6.66 x 10^7 in INE 2, 3 and 4 mg/kg treated groups, respectively (Fig. 4.46-B). Corresponding growth inhibition of Sarcoma-180 (ascites) was observed to be 56.20, 79.59 and 97.12 per cent whereas 5-FU treatment inhibited the tumor growth by 95.76 per cent (Fig. 4.46-C). However, increasing doses of INE (3 and 4 mg/kg) witnessed proportionate increase in toxicity with 2 and 4 deaths, respectively.

In vivo anticancer activity of INF1 against Sarcoma-180 (ascites) was studied at 0.5, 1 and 2 mg/kg dose levels. Perusal of data presented in Table 4.24 and Fig. 4.47 reveals similar pattern of anticancer activity exhibited by INF1 with more or less similar

Table 4.24: Anticancer activity of *Ipomoea nil* aerial parts against murine Sarcoma-180 (ascites)

Treatments	Dose	Aanimals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (INE)	2 mg/kg	7	0	3.65 ± 1.10**	100.42 ± 33.80*	56.20
	3 mg/kg	7	2	1.58 ± 0.79***	46.8 ± 25.80***	79.59
	4 mg/kg	7	4	0.43 ± 0.43***	6.66 ± 6.66***	97.12
n-Hexane fraction (INF1)	0.5 mg/kg	7	0	3.7 ± 0.90*	111.14 ± 51.53*	51.53
	1 mg/kg	7	0	1.51 ± 1.02***	41.42 ± 27.33***	81.93
	2 mg/kg	7	2	0.82 ± 0.56***	18.2 ± 18.2***	92.06
5-FU	20 mg/kg	7	0	0.71 ± 0.56***	9.71 ± 9.71***	95.76
NS	0.2 ml	15	0	7.58 ± 0.69	229.3 ± 29.60	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.

inhibition in the growth of Sarcoma-180 (ascites) as that of INE at more than double the doses. Another striking feature of INF1 was the reduced level of toxicity even at highest dose of 2 mg/kg which witnessed only two deaths during the course of experiment.

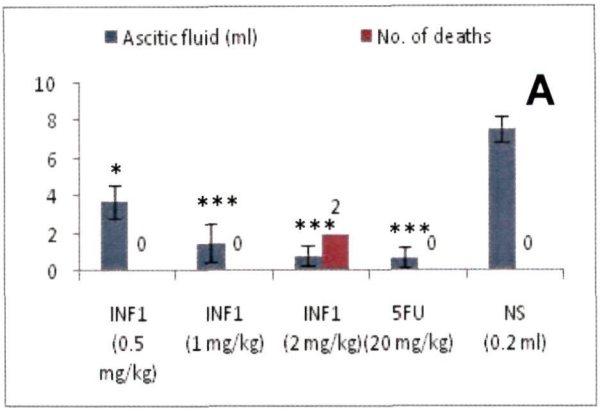
4.4.3 Methyl cholanthrene induced ascites

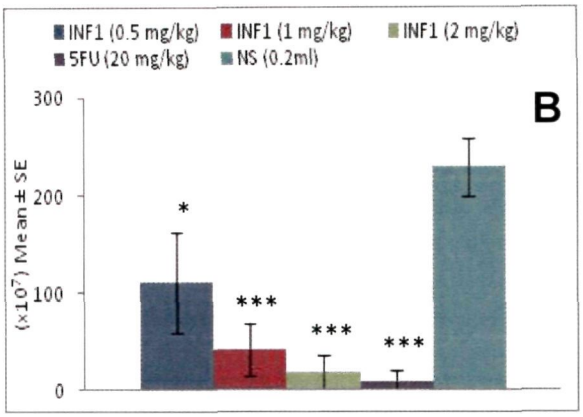
Table 4.25 and Fig. 4.48 explain the anticancer activity of INE against Methyl cholanthrene induced ascites studied at 2, 3 and 4 mg/kg doses. The volume of ascitic fluid in 5-FU treated positive control group (0.7 ± 0.49 ml) showed significant ($p<0.001$) decline from 8.29 ± 0.58 ml observed in NS treated control group. INE 2, 3 and 4 mg/kg treated animals also had significantly ($p<0.001$) reduced volumes of ascitic fluid (2.55 ± 0.42, 0.7 ± 0.49 and 0.65 ± 0.42 ml, respectively) (Fig. 4.48-A). Total number of tumor cells too showed significant ($p<0.001$) reduction in INE treated groups as compared to NS treated animals (Fig. 4.48-B). Effectiveness of INE against Methyl cholanthrene induced ascites as illustrated in Fig. 4.48-C was 71.23, 92.06 and 97.19 per cent with 2, 3 and 4 mg/kg doses respectively, whereas in 5-FU treated group, the tumor growth inhibition was 93.36 per cent. Higher levels of effectiveness (92.06 and 97.19 per cent) observed with 3 and 4 mg/kg of INE were accompanied by 1 and 3 deaths, respectively.

In vivo anticancer activity of INF1 against Methyl cholanthrene induced ascites studied in the same experiment at 0.5, 1 and 2 mg/kg dose levels also showed significantly reduced volumes of ascitic fluid (5.1 ± 0.87, 2.04 ± 0.83 and 0.68 ± 0.56 ml, respectively) (Fig. 4.49-A). Likewise, the number of tumor cells (101.28 ± 50.08, 60.85 ± 26.09 x 10^7 and 15.4 ± 15.4 x 10^7, respectively) in treated groups declined significantly as compared to NS treated control animals (Fig. 4.49-B). Corresponding tumor growth inhibition was 60.77, 76.43 and 94.03 per cent with 0.5, 1 and 2 mg/kg doses of INF1 (Fig. 4.49-C). However, higher anticancer activity observed with 2 mg/kg of INF1 witnessed 2 deaths of experimental mice.

4.4.4 Ehrlich tumor (solid)

Three groups of non-inbred Swiss mice transplanted with EAC cells in the right thigh muscles were treated with 2, 3 and 4 mg/kg of alcoholic extract of *Ipomoea nil* aerial parts (INE) intraperitoneally (i.p.). Positive control group was treated with 22 mg/kg of





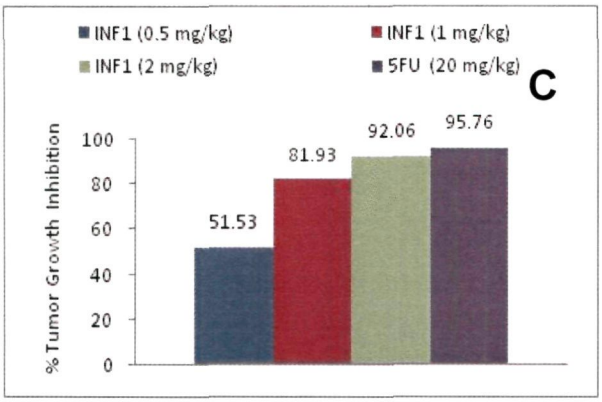
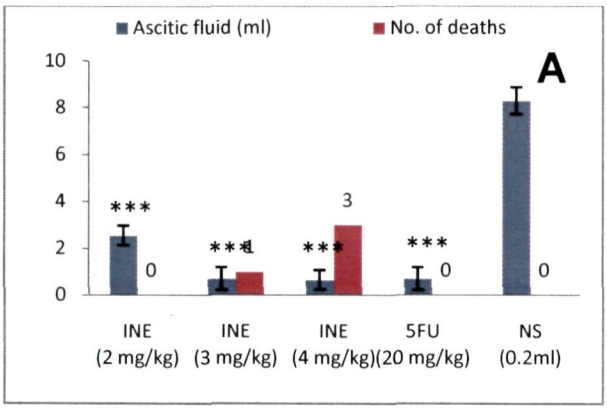
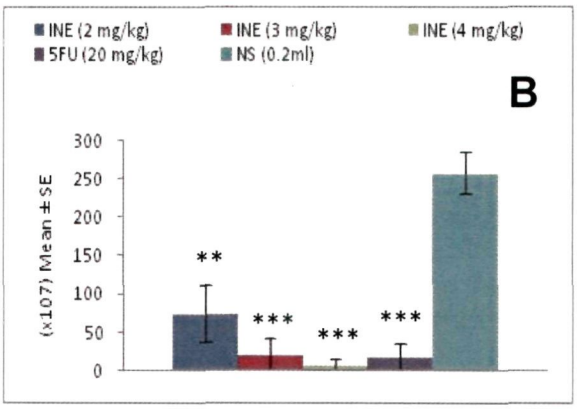


Fig. 4.47: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on murine Sarcoma-180 (ascites). A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth. 5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





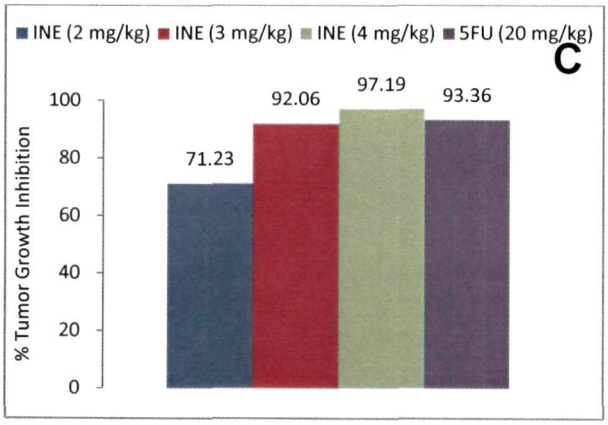
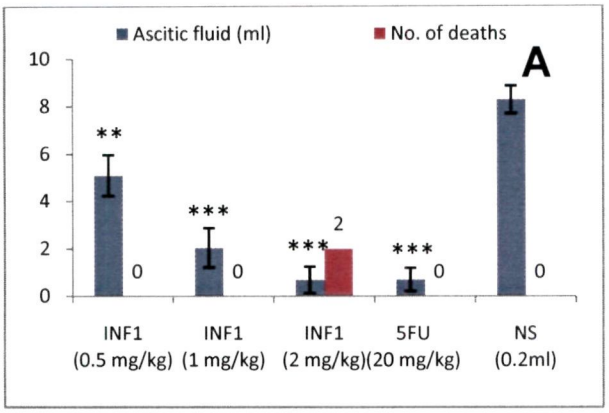


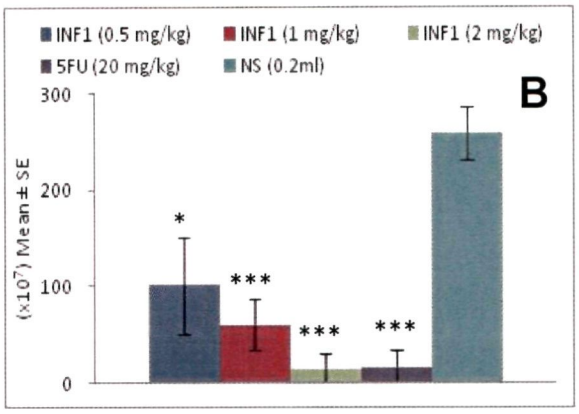
Fig. 4.48: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control *$p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 4.25: Anticancer activity of *Ipomoea nil* aerial parts against Methyl cholanthrene induced ascites in mice

Treatments	Dose	Aanimals	Deaths	Volume of ascitic fluid (ml)	No. of tumor Cells (x 10^7)	Growth inhibition (%)
Alcoholic extract (INE)	2 mg/kg	7	0	2.55 ± 0.42***	74.28 ± 37.14**	71.23
	3 mg/kg	7	1	0.7 ± 0.49***	20.5 ± 20.5***	92.06
	4 mg/kg	7	3	0.65 ± 0.42***	7.25 ± 7.25***	97.19
n-Hexane fraction (INF1)	0.5 mg/kg	7	0	5.1 ± 0.87**	101.28 ± 50.08*	60.77
	1 mg/kg	7	0	2.04 ± 0.83***	60.85 ± 26.09***	76.43
	2 mg/kg	7	2	0.68 ± 0.56***	15.4 ± 15.4***	94.03
5-FU	20 mg/kg	7	0	0.7 ± 0.49***	17.14 ± 17.14***	93.36
NS	0.2 ml	15	0	8.29 ± 0.58	258.2 ± 27.29	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.





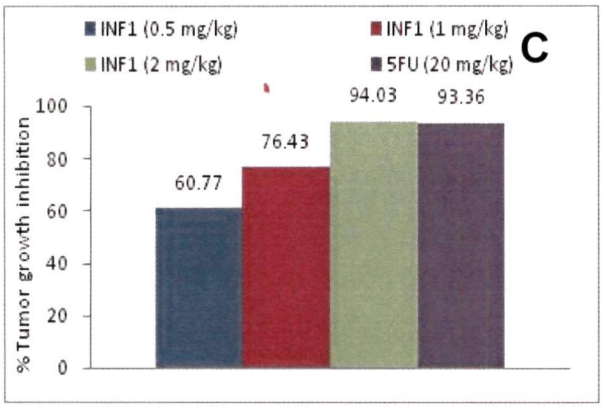
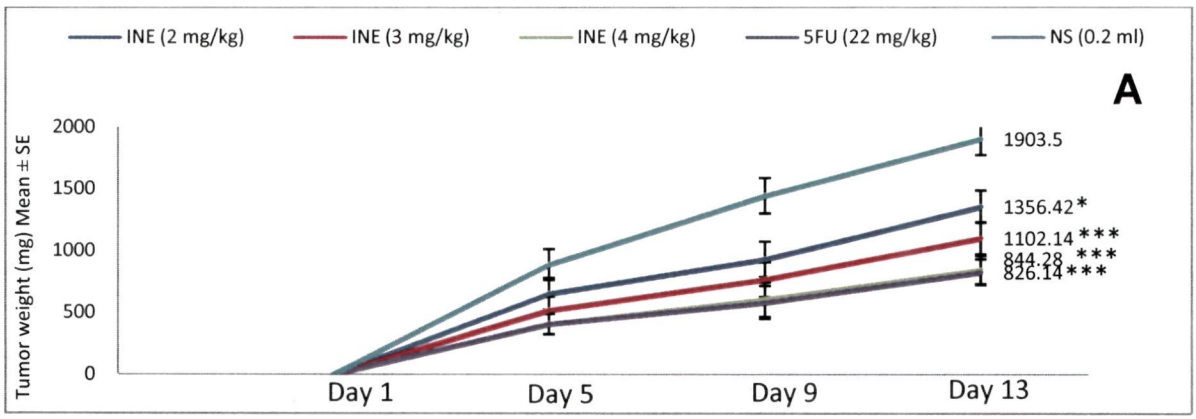


Fig. 4.49: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on Methyl cholanthrene induced ascites in mice. A: Effect on the volume of ascitic fluid and No. of deaths. B: Effect on total number of tumor cells in the peritoneal cavity of mice. C: Per cent inhibition of tumor growth.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



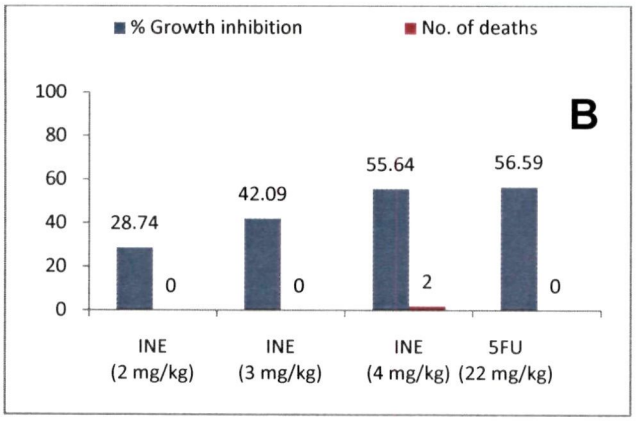


Fig. 4.50: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on murine Ehrlich tumor (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

5-Fluorouracil (5-FU) i.p. and the tumor bearing control group was administered 0.2 ml normal saline (NS). Data on tumor growth observed in different groups on days 5, 9 and 13 is presented in Table 4.26 and Fig. 4.50-A. Statistical analysis of tumor weights observed on day 13 showed significant ($p<0.001$) reduction in tumor growth in 5-FU and INE 3 and 4 mg/kg treated animals as compared to NS treated group. However, 2 deaths were noticed in 4 mg/kg treated group. In other words, growth of Ehrlich tumor (solid) was inhibited by 28.74, 42.09 and 55.64 per cent in groups treated with INE 2, 3 and 4 mg/kg, respectively (Fig. 4.50-B). Likewise, 5-FU treated group showed 56.59 per cent growth inhibition of Ehrlich tumor (solid).

n-Hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) exhibited same pattern of tumor growth and more or less similar anticancer activity against Ehrlich tumor (solid) at 0.5, 1 and 2 mg/kg dose levels (Fig. 4.51-A). Only one death was observed in group treated with highest dose of INF1 (2 mg/kg). This proved the presence of higher anticancer activity and lesser toxicity in INF1 as compared to INE. Anticancer activity of 5-FU (56.59 per cent) was comparable with highest dose of INF1.

4.4.5 Sarcoma-180 (solid)

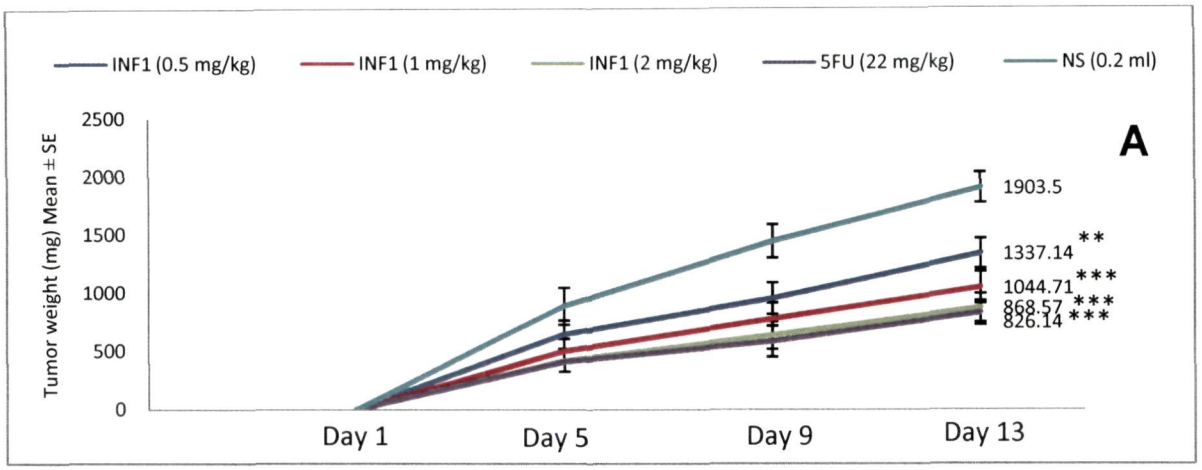
Anticancer activity of INE against Sarcoma-180 (solid) was similarly studied at 2, 3 and 4 mg/kg dose levels and the data obtained is illustrated in Table 4.27 and Fig. 4.52-A. Statistical analysis of tumor weights observed on day 13 in different groups showed that the positive control group and INE 3 and 4 mg/kg treated groups had significantly ($p<0.001$) lower tumor weights than NS treated group. Growth inhibition of Sarcoma-180 (solid) with 2, 3 and 4 mg/kg doses of INE was 15.19, 49.59 and 53.50 per cent, respectively (Fig. 4.52-B) whereas 5-FU at 22 mg/kg dose reduced tumor weight by 55.15 per cent. However, 4 mg/kg dose of INE recorded 2 deaths of experimental mice.

As depicted in Fig. 4.53-A, all doses (0.5, 1 and 2 mg/kg) of INF1 exhibited significantly ($p<0.001$) reduced growth of Sarcoma-180 (solid) on day 13. Growth inhibition of Sarcoma-180 (solid) calculated on the basis of day 13 tumor weights in groups treated with 0.5, 1 and 2 mg/kg doses of INF1 was 30.99, 47.65 and 59.48 per cent, respectively (Fig. 4.53-B). This proved the presence of significantly higher anticancer activity in

Table 4.26: Anticancer activity of *Ipomoea nil* (aerial parts) against murine Ehrlich tumor (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (INE)	2 mg/kg	0/7	651.14 ± 126.15	931.0 ± 143.63	1356.42 ± 130.40*	28.74
	3 mg/kg	0/7	519.57 ± 108.88	766.42 ± 142.44	1102.14 ± 129.08***	42.09
	4 mg/kg	2/7	406.28 ± 82.97	603.85 ± 138.07	844.28 ± 115.05***	55.64
n-Hexane fraction (INF1)	0.5 mg/kg	0/7	642.28 ± 121.74	952.0 ±1 35.95	1337.14 ± 127.62**	29.75
	1 mg/kg	0/7	501.42 ± 104.19	776.42 ± 138.18	1044.71 ± 136.47***	45.11
	2 mg/kg	1/7	416.42 ± 89.64	634.0 ± 118.07	868.57 ± 122.57***	54.36
5-FU	22 mg/kg	0/7	409.28 ± 84.19	582.0 ± 131.80	826.14 ± 103.92***	56.59
NS	0.2 ml	0/15	886.85 ± 159.48	1444.71 ± 143.28	1903.5 ± 131.61	--

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.



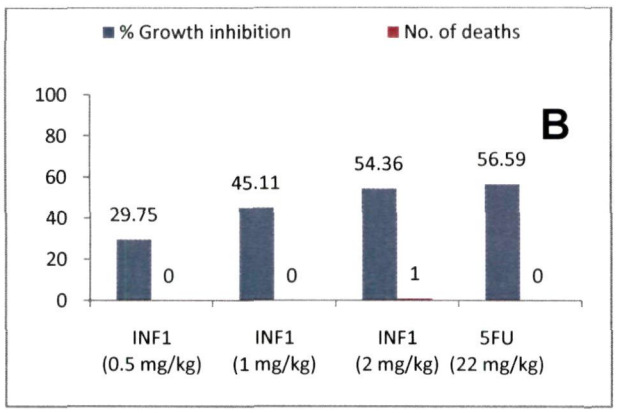
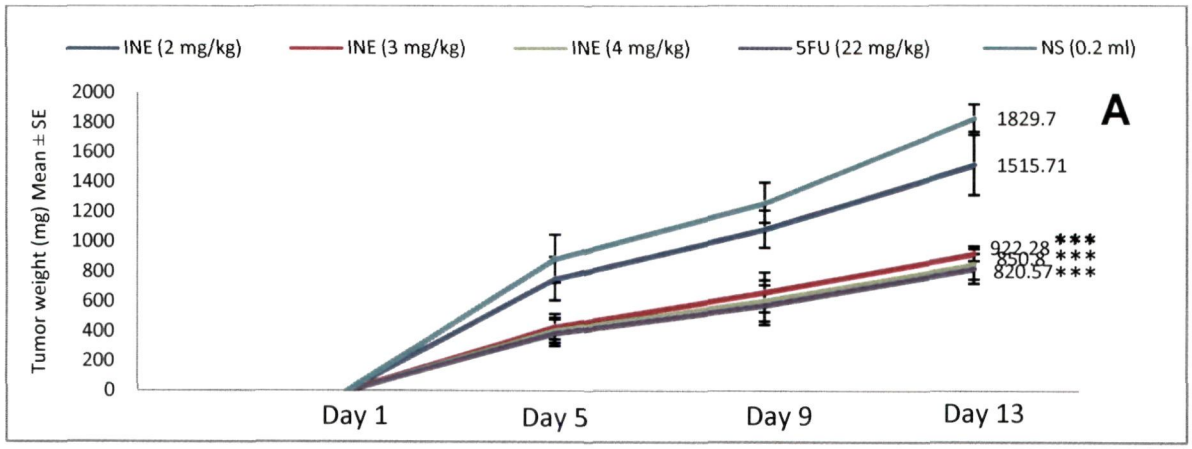


Fig. 4.51: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on murine Ehrlich tumor (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$



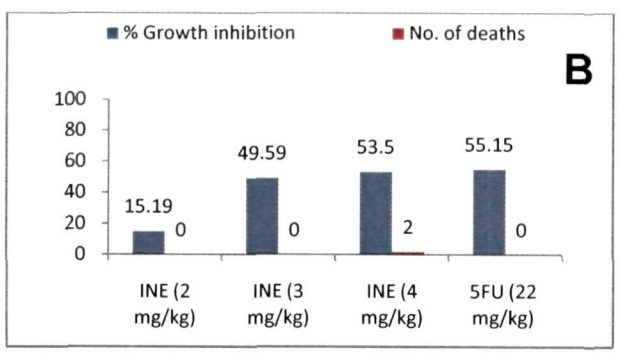


Fig. 4.52: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and the number of deaths in treated mice.
5-FU: 5-fluorouracil. NS: Normal saline. Treatment vs. Control * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 4.27: Anticancer activity of *Ipomoea nil* (aerial parts) against murine Sarcoma-180 (solid)

Treatments	Dose	Deaths	Tumor Weight (mg)			Growth inhibition (%)
			Day 5	Day 9	Day 13	
Alcoholic extract (INE)	2 mg/kg	0/7	754.85 ± 146.31	1086.14 ± 125.02	1515.71 ± 201.43	15.19
	3 mg/kg	0/7	433.28 ± 84.84	663.14 ± 133.68	922.28 ± 49.59***	49.59
	4 mg/kg	2/7	408.0 ± 83.24	606.0 ± 137.54	850.8 ± 101.46***	53.50
n-Hexane fraction (INF1)	0.5 mg/kg	0/7	608.14 ± 146.02	878.14 ± 160.07	1262.57 ± 99.22***	30.99
	1 mg/kg	0/7	448.14 ± 86.36	651.57 ± 161.82	957.71 ± 112.81***	47.65
	2 mg/kg	3/7	356.71 ± 93.28	522.0 ± 112.17	740.5 ± 158.66***	59.48
5-FU	22 mg/kg	0/7	392.42 ± 88.66	576.71 ± 132.53	820.57 ± 101.05***	55.15
NS	0.2 ml	0/15	888.28 ± 160.22	1262.42 ± 134.08	1829.7 ± 91.06	-

*, **, *** Significantly different as compared to control values at 5% ($p<0.05$), 1% ($p<0.01$) and 0.1% ($p<0.001$) levels of significance, respectively.
5-FU: 5-fluorouracil; NS: Normal saline.

INF1 than INE. At the same time, highest dose of INF1 (2 mg/kg) proved bit intolerable as it witnessed 3 deaths of experimental mice.

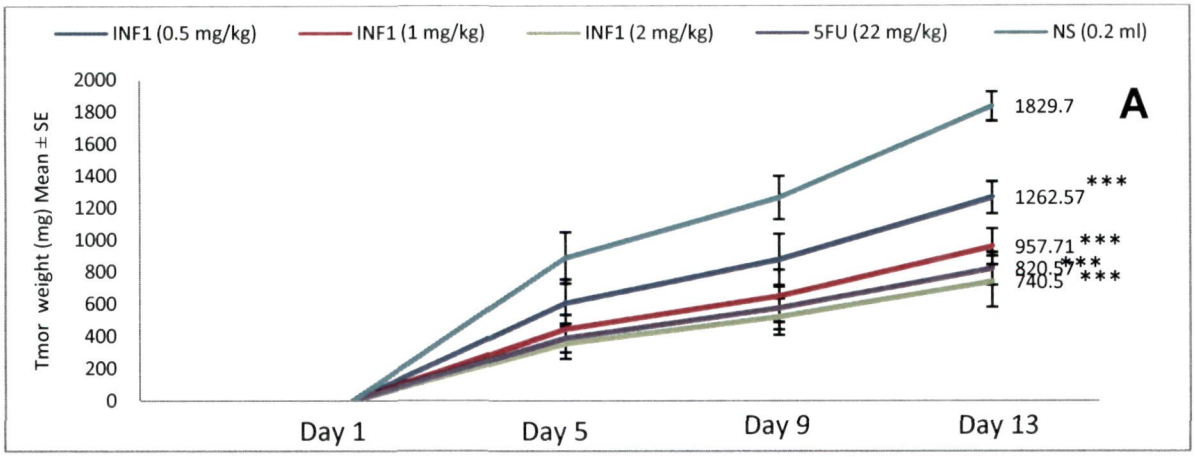
4.4.6 L1210 Lymphoid leukemia

Anticancer activity of INE studied at 2, 3 and 4 mg/kg in CD2F1 mice transplanted with L1210 Lymphoid leukemia cells is depicted in Table 4.28 and Fig. 4.54. Mean survival time (MST) in NS treated group was 7.91 days and in positive control group it was 14.21 days (Fig. 4.54-A). Animals treated with INE 2, 3 and 4 mg/kg had an MST of 115.17, 103.79 and 96.83 per cent of tumor bearing control, respectively. Net result of treatment with 2, 3 and 4 mg/kg of INE was 15.17, 3.79 and -3.17 per cent increase in life span of treated animals over tumor bearing control animals. It is thus evident that while lower dose of INE did not produce significant increase in life span of treated animals, increasing doses of INE progressively shortened the life span of animals leading to negative increase in life span at the highest dose of 4 mg/kg (Fig. 4.54-C).

Anticancer activity of INF1 against L1210 Lymphoid leukemia studied at 0.5, 1 and 2 mg/kg dose levels in the same experiment showed exactly same pattern as that of INE (Fig. 4.55-A). In this case also, lower dose (0.5 mg/kg) did not produce significant increase in life span of treated animals and increasing doses of INF1 progressively shortened the life span of animals leading to negative increase in life span at the highest dose of 2 mg/kg (Fig. 4.55-C). Positive control group however showed 79.64 per cent increase in life span of L1210 Lymphoid leukemia bearing animals.

4.4.7 P388 Lymphocytic leukemia

Data on *in vivo* anticancer activity of INE studied in CD2F1 mice transplanted with P388 Lymphocytic leukemia cells at 2, 3 and 4 mg/kg dose levels is depicted in Table 4.29 and Fig. 4.56. Mean survival time (MST) in NS treated group was 8.8 days and in positive control group it was 15.5 days. In groups treated with INE 2, 3 and 4 mg/kg, MST was 93.75, 87.0 and 79.54 per cent of tumor bearing control group, respectively. Thus, rather than increasing the life span of treated animals, all doses of INE in fact shortened the life span and produced negative increase in the life span (Fig. 4.56-C). 5-FU treatment however increased the life span of P388 Lymphocytic leukemia bearing animals by 76.13 per cent.



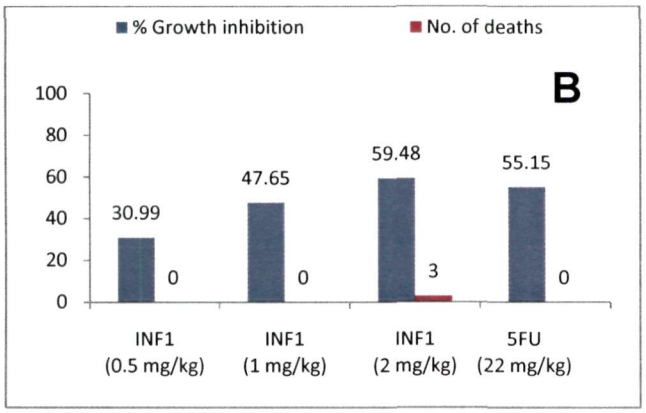
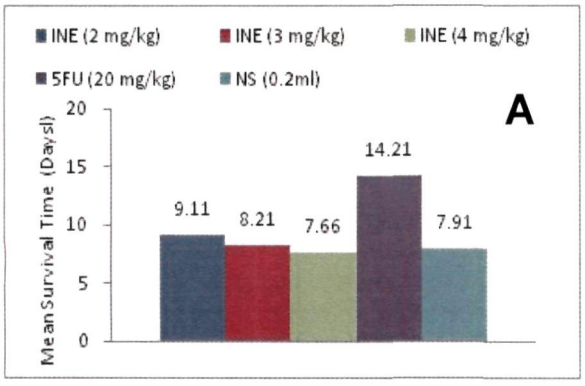
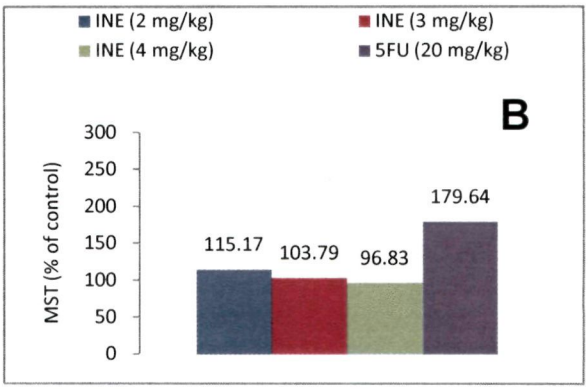


Fig. 4.53: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on murine Sarcoma-180 (solid). A: Effect on the weight of tumor measured on days 1, 5, 9 and 13. B: Per cent inhibition of solid tumor growth and No. of deaths.
5-FU: 5-fluorouracil. NS: Normal saline.
Treatment vs. control * $p<0.05$, ** $p<0.01$, *** $p<0.001$





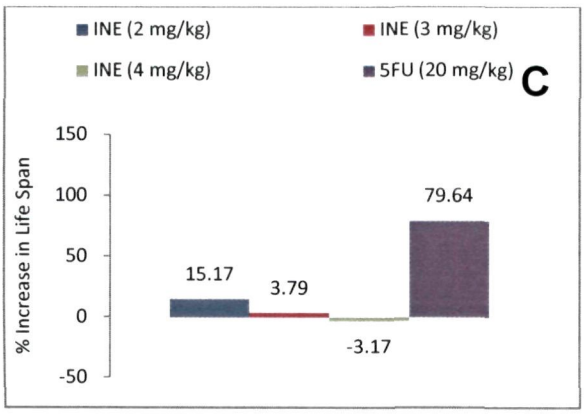


Fig. 4.54: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice.. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice.
5-FU: 5-fluorouracil. NS: Normal saline.

Table 4.28: Anticancer activity of *Ipomoea nil* aerial parts against L1210 Lymphoid leukemia in CD2F1 mice

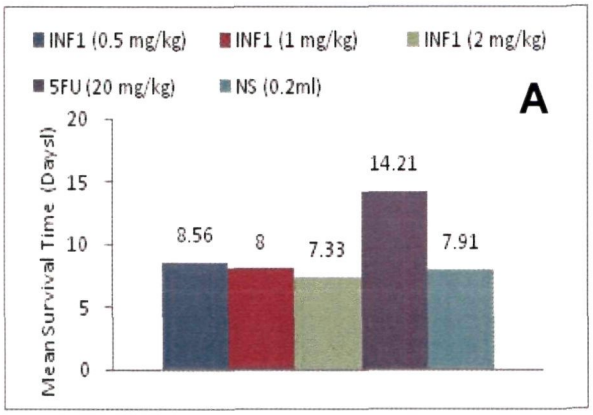
Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (INE)	2 mg/kg	6	9.11	115.17	15.17
	3 mg/kg	6	8.21	103.79	3.79
	4 mg/kg	6	7.66	96.83	-3.17
n-Hexane fraction (INF1)	0.5mg/kg	6	8.56	108.21	8.21
	1 mg/kg	6	8.00	101.13	1.13
	2 mg/kg	6	7.33	92.66	-7.34
5-FU	20 mg/kg	6	14.21	179.64	79.64
NS	0.2 ml	6	7.91	-	-

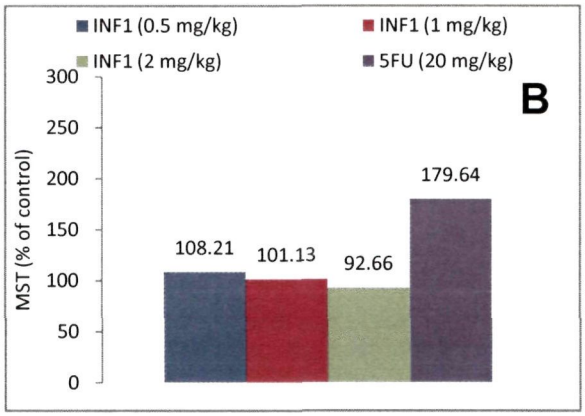
5-FU: 5-fluorouracil; NS: Normal saline.

Table 4.29: Anticancer activity of *Ipomoea nil* aerial parts against P388 Lymphocytic leukemia in CD2F1 mice

Treatments	Dose	Animals	Mean Survival Time (days)	% T/C	% Increase in Life Span
Alcoholic extract (INE)	2 mg/kg	6	8.25	93.75	-6.25
	3 mg/kg	6	7.66	87	-13
	4 mg/kg	6	7.0	79.54	-20.46
n-Hexane fraction (INF1)	0.5mg/kg	6	9.66	109.77	9.77
	1 mg/kg	6	7.0	79.56	-20.44
	2 mg/kg	6	6.25	71.02	-28.98
5-FU	20 mg/kg	6	15.5	176.13	76.13
NS	0.2 ml	6	8.8	-	-

5-FU: 5-fluorouracil; NS: Normal saline.





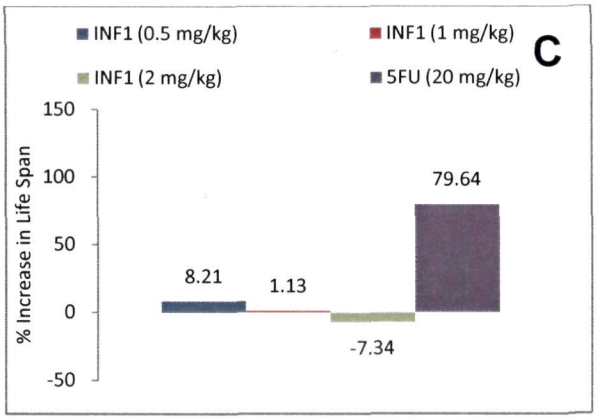
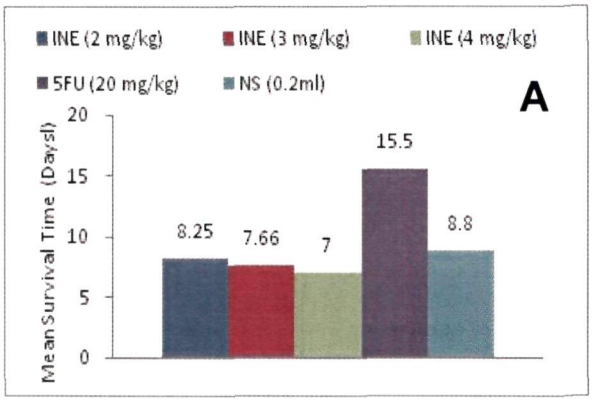
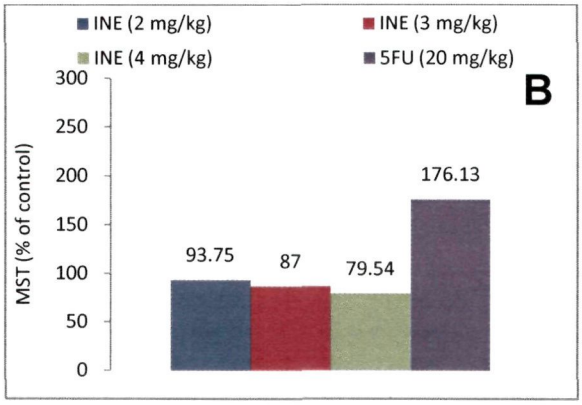


Fig. 4.55: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on L1210 Lymphoid leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice.
5-FU: 5-fluorouracil. NS: Normal saline.





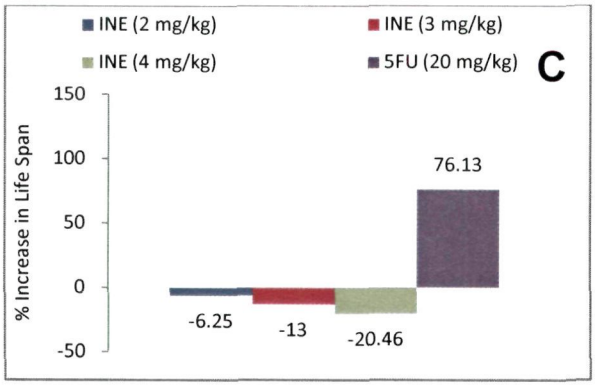


Fig. 4.56: Effect of different doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice.
5-FU: 5-fluorouracil. NS: Normal saline.

INF1 was similarly studied for its anticancer activity against P388 Lymphocytic leukemia at 0.5, 1 and 2 mg/kg dose levels in the same experiment. As depicted in Fig. 4.57-A, lower dose (0.5 mg/kg) produced only marginal increase in life span of treated animals (9.77 per cent) over tumor bearing control animals. But substantial toxicity was noticed with increasing doses of INF1, thereby shortening the life span of treated animals and producing negative increase in life span as compared to NS treated control group bearing P388 Lymphocytic leukemia (Fig. 4.57-C).

4.5 Comparative efficacy of different fractions

Studies on *in vivo* anticancer activity of alcoholic extracts and their promising fractions from *Azadirachta indica* leaves, *Asparagus racemosus* roots and *Ipomoea nil* aerial parts against various models of murine cancers proved the enrichment of anticancer activity in fractions isolated from corresponding extracts. For further clarification, anticancer activity of chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2), n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) and n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) at various dose levels against all *in vivo* models used in the study is presented in Table 4.30. As per the protocols described by Geran *et al.*, (1972), more than 50 per cent anticancer activity is termed significant. Thus, only against Ehrlich ascites carcinoma, AILF2 had more than 50 per cent anticancer activity at a dose of 250 mg/kg. Against all other models at all other doses, it showed less than 50 per cent anticancer activity. ARRF3 exhibited more than 50 per cent anticancer activity against all the models at 80, 100 and 120 mg/kg dose levels except L1210 and P388 leukemia models. Its 140 mg/kg dose proved to be lethal to animals in all experimental models. Further, it exhibited no significant anticancer activity against L1210 lymphoid leukemia at any of the dose levels studied. However, against P388 lymphocytic leukemia it showed significant increase in life span of treated animals at 80 and 100 mg/kg dose levels. Other two doses of ARRF3 showed no significant increase in life span.

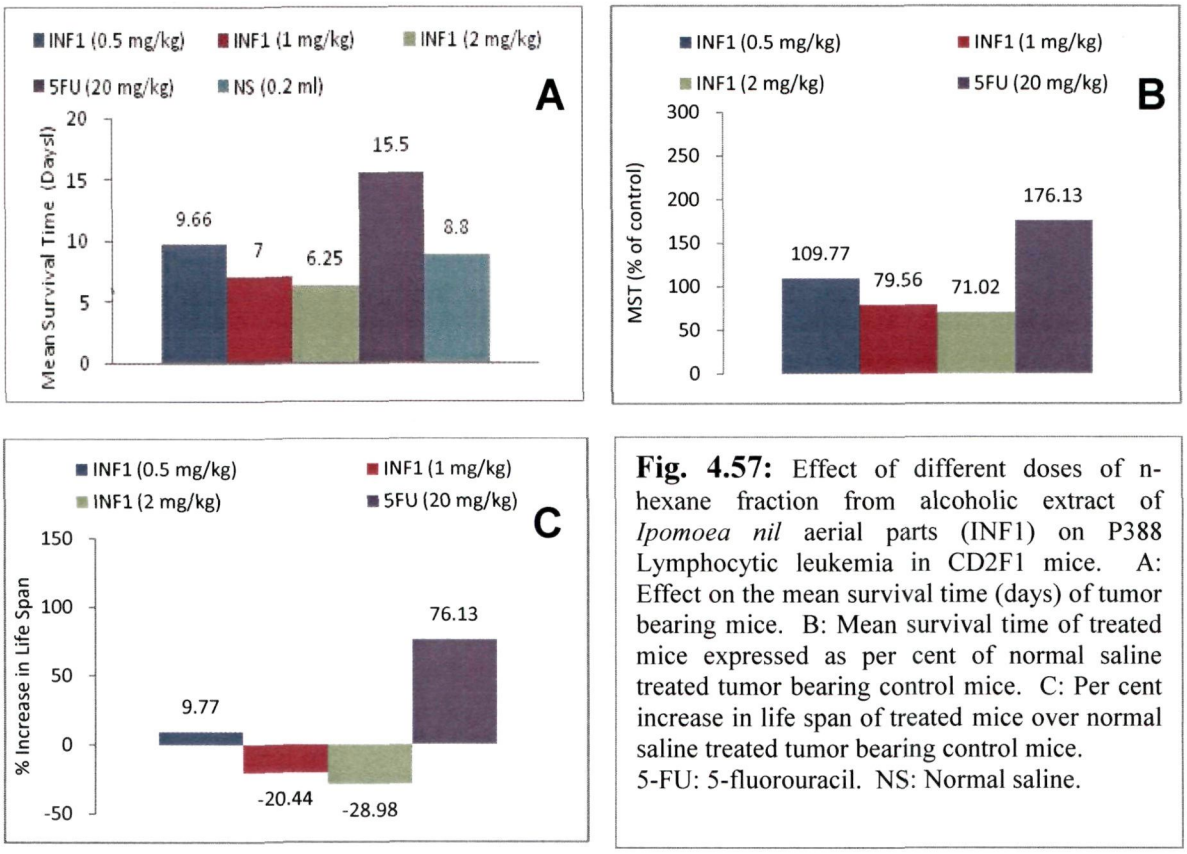


Fig. 4.57: Effect of different doses of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) on P388 Lymphocytic leukemia in CD2F1 mice. A: Effect on the mean survival time (days) of tumor bearing mice. B: Mean survival time of treated mice expressed as per cent of normal saline treated tumor bearing control mice. C: Per cent increase in life span of treated mice over normal saline treated tumor bearing control mice.
5-FU: 5-fluorouracil. NS: Normal saline.

INF1 at 2 mg/kg dose exhibited more than 50 per cent anticancer activity against all *in vivo* models except L1210 and P388 leukemia models, against which all doses of INF1 either proved toxic or had no significant effect on increasing the life span of treated animals. Its 0.5 and 1 mg/kg doses were also effective in showing more than 50 per cent anticancer activity against Sarcoma-180 (ascites) and Methyl cholanthrene induced ascites (Table 4.30).

Table 4.30: Comparative efficacy of AILF2, ARRF3 and INF1 against different murine cancer models

Tumor Models	Chloroform fraction from alcoholic extract of *Azadirachta indica* leaves (AILF2)				n-Butanol fraction from alcoholic extract of *Asparagus racemosus roots* (ARRF3)				n-Hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1)		
	100 mg/kg	150 mg/kg	200 mg/kg	250 mg/kg	80 mg/kg	100 mg/kg	120 mg/kg	140 mg/kg	0.5 mg/kg	1 mg/kg	2 mg/kg
	PER CENT GROWTH INHIBITION										
Ehrlich Ascites Carcinoma	31.51	36.61	37.33	50.36	89.00	94.13	91.36	Toxic	46.42	66.33	87.06
Sarcoma-180 (ascites)	1.05	18.31	28.39	33.39	82.78	88.19	89.58	Toxic	51.53	81.93	92.06
Methyl cholanthrene induced ascites	10.95	15.99	16.64	18.54	97.12	96.29	95.85	Toxic	60.77	76.43	94.03
Ehrlich Tumor (solid)	4.28	10.65	28.74	40.05	61.34	65.0	60.49	Toxic	29.75	45.11	54.36
Sarcoma-180 (solid)	-5.38	-3.48	9.06	21.71	55.06	53.07	57.26	Toxic	30.99	47.65	59.48
	PER CENT INCREASE IN LIFE SPAN										
L1210 Lymphoid leukemia	9.14	17.32	-4.51	-13.65	33	9	-4	-11	8.21	1.13	-7.34
P388 Lymphocytic leukemia	2.19	5.41	6.14	-3.81	68	151.09	29	-5	9.77	-20.44	-28.98

More than 50 per cent anticancer activity was considered significant and is shown in red font.

4.6 Toxicity: myelosuppression

All the isolates evaluated for *in vivo* anticancer activity were also studied for their toxic effects with reference to myelosuppression in Sarcoma-180 (solid) bearing mice. Doses yielding best anticancer activity were administered from day 1-9 and femoral marrow cellularity of treated and control animals was determined on day 13. Results are presented in Table 4.31. Number of nucleated cells per femur was found to be 11.64 ± 0.82 x 10^6 in normal untreated (without tumor) animals and 9.25 ± 1.91 x 10^6 in normal saline treated tumor bearing animals resulting in 20.53 per cent myelosuppression. 5-FU treated animals had 6.47 ± 2.25 x 10^6 cells per femur which were significantly ($p<0.05$) less than that of normal untreated animals. Likewise, animals treated with 250 mg/kg of AILE and AILF2 showed 8.41 ± 1.06 x 10^6 and 9.41 ± 0.65 x 10^6 cells per femur, respectively which were also significantly ($p<0.05$) less than that of normal untreated animals. Similarly, 8.93 ± 1.65 x 10^6, 9.93 ± 1.62 x 10^6 and 9.88 ± 1.65 x 10^6 cells per femur were recorded in groups treated with 120, 100 and 80 mg/kg of ARRE, ARRF2 and ARRF3, respectively. Myelosuppression in these groups was found to be 23.28, 14.89 and 15.12 per cent, respectively. However, groups treated with INE (3 mg/kg) and INF1 (2 mg/kg) showed 5.61 ± 1.61 x 10^6 and 6.06 ± 1.28 x 10^6 cells per femur which were again significantly ($p<0.01$) less than normal untreated animals. Myelosuppression in these groups was 51.80 and 47.93 per cent, respectively.

4.7 Mechanistic (Apoptotic) studies

Careful perusal of Table 4.30 reveals that AILF2 had less than 50 per cent anticancer activity against all the models at all the doses studied except Ehrlich ascites carcinoma against which it exhibited 50.36 per cent anticancer activity. Even this magnitude of activity does not bear much significance in anticancer chemotherapy. It was therefore considered that AILF2 does not hold much promise towards the development of novel anticancer therapeutics. Hence it was dropped at this stage and was not studied further. ARRF3 and INF1 exhibited consistently good and much more than 50 per cent anticancer activity against most of the models. It seems to be likely that novel anticancer chemotherapeutic agent(s) may emerge from them in future. It was therefore pertinent to study the mechanism of cancer cell death induced by them. With this view, following

Table 4.31: Effect of different extracts and their fractions on femoral marrow cellularity of Sarcoma-180 (solid) bearing mice

Treatments	Dose	No. of cells per femur x 10^6 (Mean ± SE)	Myelosuppression (%)
AILE	250 mg/kg	8.41 ± 1.06*	27.74
AILF2	250 mg/kg	9.41 ± 0.65*	19.15
ARRE	120 mg/kg	8.93 ± 1.65	23.28
ARRF2	100 mg/kg	9.93 ± 1.62	14.89
ARRF3	80 mg/kg	9.88 ± 1.65	15.12
INE	3 mg/kg	5.61 ± 1.61**	51.80
INF1	2 mg/kg	6.06 ± 1.28**	47.93
5-FU	20 mg/kg	6.47 ± 2.25*	44.41
NS	0.2 ml	9.25 ± 1.91	20.53
Normal untreated	-	11.64 ± 0.82	-

Femoral cellularity values are Mean ± SE obtained from seven mice bearing Sarcoma-180 (solid) unless otherwise stated.

*, ** Significantly different as compared to normal untreated control values at 5% ($p<0.05$) and 1% ($p<0.01$) levels of significance, respectively.

5-FU: 5-fluorouracil; NS: Normal saline.

studies were conducted to ascertain the mechanism behind their chemotherapeutic potential.

4.7.1 Cell cycle analysis of HL-60 cells

HL-60 cells treated with 50 µg/ml concentrations of ARRE, ARRF2 and ARRF3 were incubated for 24 h. Cell cycle analysis in terms of propidium iodide uptake by treated cells was performed by flow cytometry. 6 per cent of untreated cells were found to be in sub-G_1 phase whereas 18, 19 and 30 per cent of cells treated with cells 50 µg/ml of ARRE, ARRF2 and ARRF3, respectively were in sub-G_1 phase (Fig. 4.58). Further, G_2/M phase was not affected which indicated that the samples did not produce mitotic block or cause delay in cell cycle.

In case of INF1, HL-60 cells treated with 3 and 5 µg/ml concentrations of INF1 were incubated for 24 h. Cell cycle analysis by flow cytometry demonstrated 14 and 22 per cent of cells treated with 3 and 5 µg/ml of INF1 respectively, in sub-G_1 phase (Fig. 4.59) whereas 4 per cent of untreated cells were found to be in sub-G_1 phase of the cell cycle. In this case also, G_2/M phase was not affected which indicated that INF1 did not produce mitotic block or cause delay in cell cycle.

4.7.2 Loss of mitochondrial membrane potential

HL-60 cells treated with 50 µg/ml concentration of ARRE, ARRF2 and ARRF3 were incubated for 24 h. Fraction of cells losing mitochondrial membrane potential was determined in terms of Rhodamine 123 uptake by mitochondria of treated cells by flow cytometry. In untreated cells, there was 2 per cent loss of mitochondrial membrane potential whereas in cells treated with 50 µg/ml of ARRE, ARRF2 and ARRF, there was 15, 30 and 39 per cent loss of mitochondrial membrane potential, respectively (Fig. 4.60). In case of INF1, HL-60 cells were treated with 3 and 5 µg/ml concentrations of INF1 and incubated for 24 h. In untreated cells, loss of mitochondrial membrane potential determined in terms of Rhodamine 123 uptake by flow cytometry was found to be 6 per cent (Fig. 4.61) whereas in cells treated with 3 and 5 µg/ml of INF1 there was 20 and 31 per cent loss of mitochondrial membrane potential.

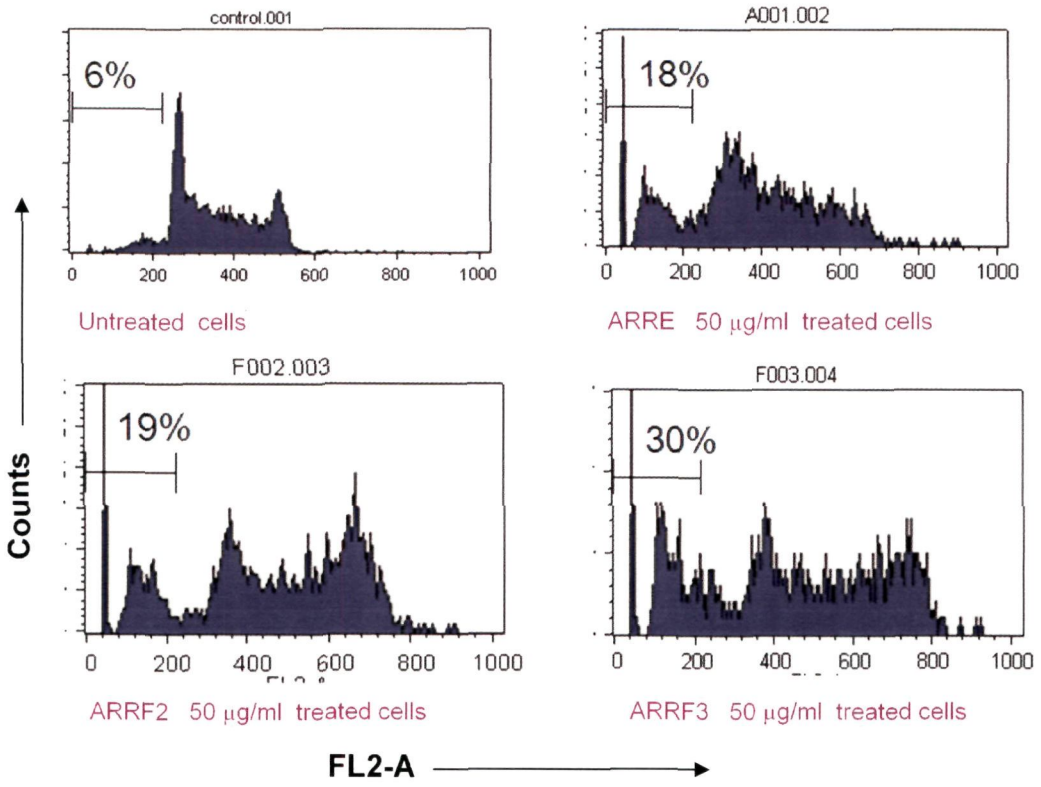


Fig. 4.58: Cell cycle analysis of HL-60 cells treated with ARRE, ARRF2 and ARRF3. Hypo-diploid (sub-G_1, DNA < 2n) fraction of cell population is shown in %age. Data are representative of one of three similar experiments.

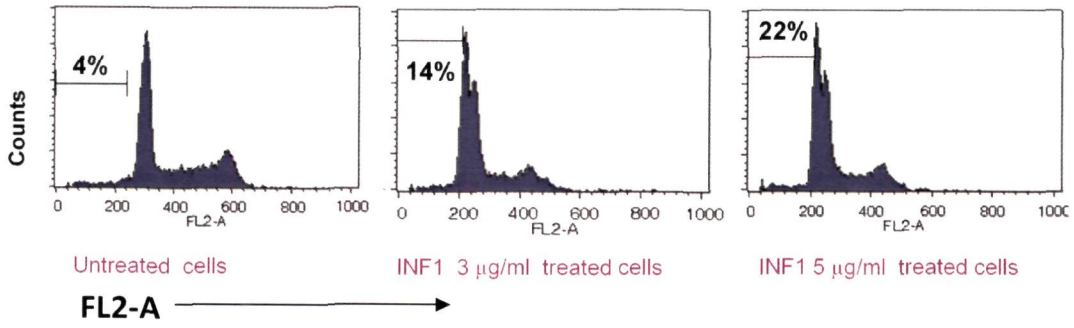


Fig. 4.59: Cell cycle analysis of HL-60 cells treated with different concentrations of INF1. Hypo-diploid (sub-G_1, DNA < 2n) fraction of cell population is shown in %age. Data are representative of one of three similar experiments.

4.7.3 Scanning Electron Microscopy of HL-60 cells

HL-60 cells were seeded in complete growth medium and treated with 25 µg/ml of n-butanol fraction from the alcoholic extract of *Asparagus racemosus* roots (ARRF3) and 10 µg/ml of n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) for 5 h. and processed for scanning electron microscopy (SEM). Untreated HL-60 cells showed rough surface due to the presence of microvilli (Fig. 4.62-A&B). Cells treated with ARRF3 (25 µg/ml) (Fig. 4.62-C&D) and INF1 (10 µg/ml) (Fig. 4.62-E&F) demonstrated reduction in size with smoothening of cell surface, blebbing of plasma membrane and apoptotic bodies.

4.7.4 Scanning Electron Microscopy of EAC cells

Ehrlich ascites carcinoma (EAC) bearing mice were treated with 0.2 ml of normal saline, 80 mg/kg of ARRF3 and 2 mg/kg of INF1 from day 1-9. On day 12, cells collected from the ascitic fluid of treated animals were processed for scanning electron microscopy. Tumor cells from normal saline treated animals were found to be spherical in shape and having microvilli on surface with a few filopods (Fig. 4.63-A,B,C&D). In EAC cells from ARRF3 80 mg/kg treated animals (Fig. 4.63-E,F,G&H) and INF1 2 mg/kg treated animals (Fig. 4.63-I,J,K&L) there was cell shrinkage, smoothening of cell surface, blebbing of the plasma membrane and formation of apoptotic bodies.

4.7.5 Light Microscopy (histology) of Sarcoma-180 (solid) tissue

After induction of Sarcoma-180 (solid) in BALB/c mice on day 0, animals were treated with 0.2 ml of normal saline, 80 mg/kg of ARRF3 and 2 mg/kg of INF1 from day 1-9. On day 13, small pieces of tumor tissue were dissected out and processed for histological examination. Semi-thin sections stained with toluidine blue and observed under light microscope revealed high nucleo-cytoplasmic ratio in tumor cells from normal saline treated animals (Fig. 4.64-A). Tumor cells from animals treated with ARRF3 (80 mg/kg) and INF1 (2 mg/kg) showed shrinkage of cells, condensation of chromatin, cytoplasmic condensation, vacuolization, fragmentation of nucleus and formation of apoptotic bodies (Fig. 4.64-B&C).

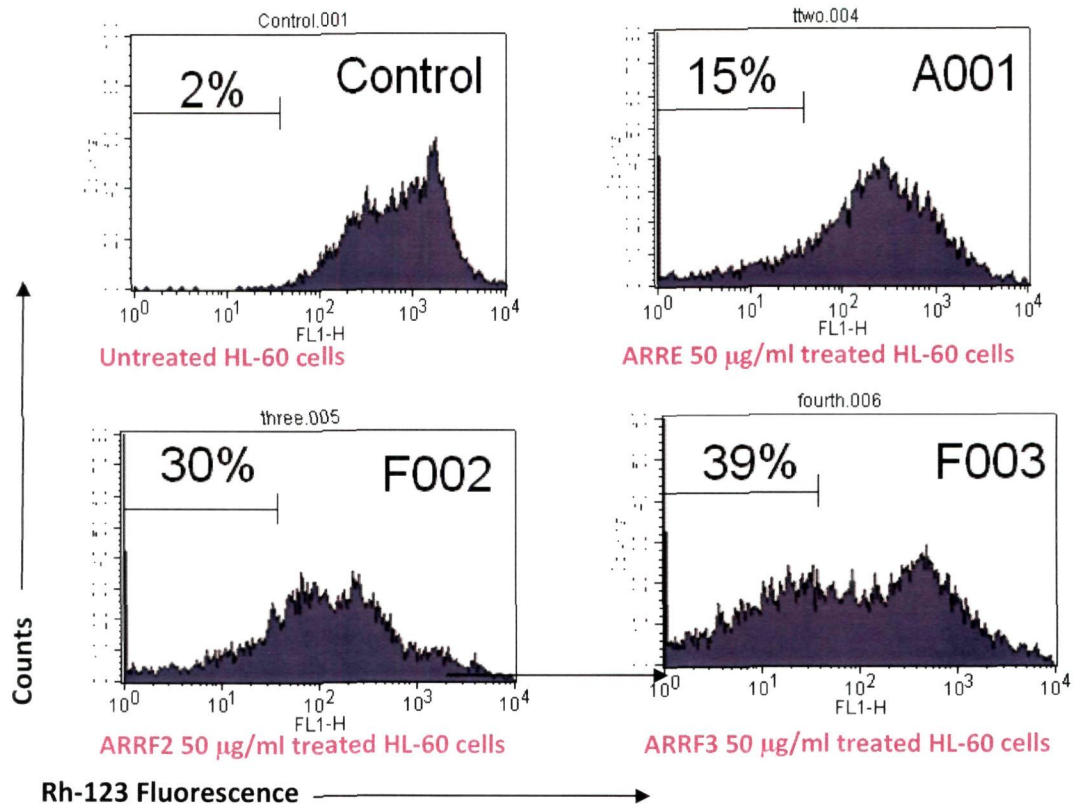


Fig. 4.60: ARRE, ARRF2 and ARRF3 induced loss of mitochondrial membrane potential in HL-60 cells. Fraction of cell population losing mitochondrial membrane potential is shown in %age. Data are representative of one of three similar experiments.

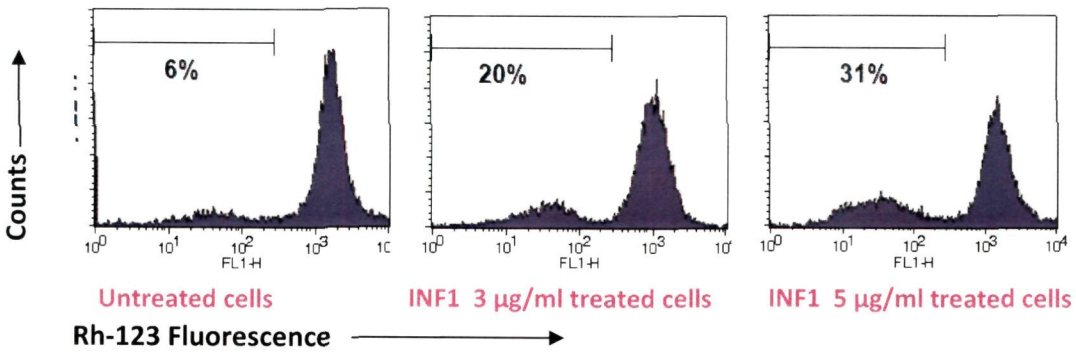


Fig. 4.61: Loss of mitochondrial membrane potential in HL-60 cells treated with different concentrations of INF1. Fraction of cell population losing mitochondrial membrane potential is shown in %age. Data are representative of one of three similar experiments.

Fig. 4.62: Scanning Electron Microscopy of HL-60 cells.

A and B: Untreated Control HL-60 cells showing rough surface and microvilli.

C and D: HL-60 cells treated with ARRF3 (25 μg/ml) for 5 h.

E and F: HL-60 cells treated with INF1 (10 μg/ml) for 5 h.

Treated cells show reduction in cell size, smoothening of cell surface, blebbing of the plasma membrane and apoptosis (↑).

Magnification: A, C & E 2000X
B, D & F 4000X.

Fig. 4.63: Scanning Electron Microscopy of Ehrlich ascites carcinoma cells.

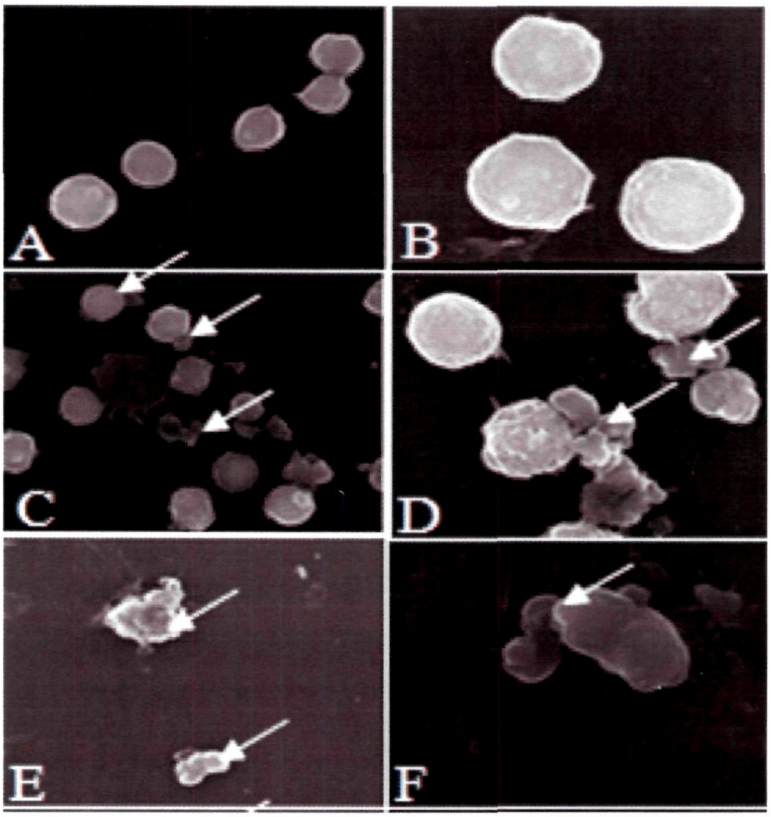
A, B, C & D: Ascites cells from normal saline treated control animals. Cells are spherical in shape and have microvilli on surface (↑) with a few filopods.

E, F, G & H: EAC cells from ARRF3 (80 mg/kg) treated animals and

I, J, K & L: EAC cells from INF1 (2 mg/kg) treated animals.

Treatments have caused cell shrinkage, smoothening of cell surface, blebbing of the plasma membrane and apoptosis (asterisk).

Magnification: A, E & I 2000X
B, F & J 5000X
C, D, G, H, K & L 8000X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C
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E
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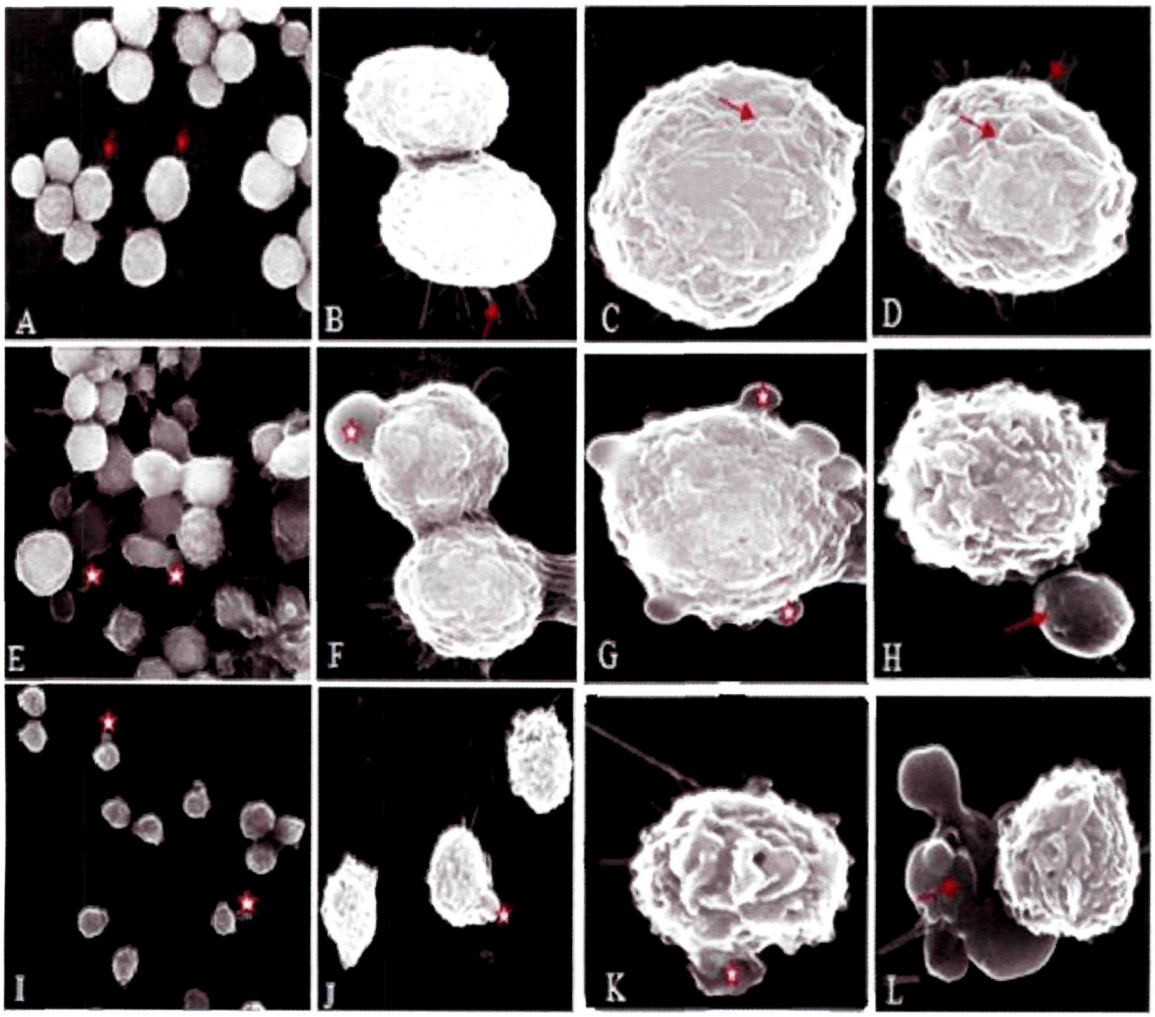
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4.7.6 Transmission Electron Microscopy (TEM) of Sarcoma-180 (solid) tissue

Small pieces of tumor tissue dissected out from Sarcoma-180 (solid) bearing animals were processed for TEM studies also. Ultrathin sections obtained with ultramicrotome were stained with 2 per cent aqueous uranyl acetate and lead citrate and examined with an electron microscope (JEOL, JEM-100CXII, Japan) at 60 KV. Ultra-structural details of tumor cells from normal saline treated animals revealed large nuclei of cells with uniformly distributed chromatin, healthy mitochondria, ribosomes, rough endoplasmic reticulum and a double layer of nuclear membrane (Fig. 4.65-A,B,C&D).

Tumor cells from ARRF3 (80 mg/kg) treated animals showed chromatin condensation and decrease in the size of nucleus with indistinct nuclear membrane (Fig. 4.66-A&B) indicating disintegration of nucleus and the cell proceeding towards apoptosis. Fig. 4.67-A,B,C&D show cells from INF1 (2 mg/kg) treated animal at various stages of apoptosis. Changes typical of apoptosis like marginalization of chromatin material, fragmentation of nucleus forming micronuclei, loss of mitochondrial cristae and disintegration of nuclear membrane were clearly visible.

Fig. 4.64: Histology of murine Sarcoma-180 (solid) tumor tissue.

A: Tumor tissue from normal saline treated control animal showing tumor cells with high nucleo-cytoplasmic ratio (↑).

B and C: Tumor tissue from ARRF3 (80 mg/kg) and INF1 (2 mg/kg) treated animals respectively, showing more apoptotic cells (arrow head) and vacuolization (V).

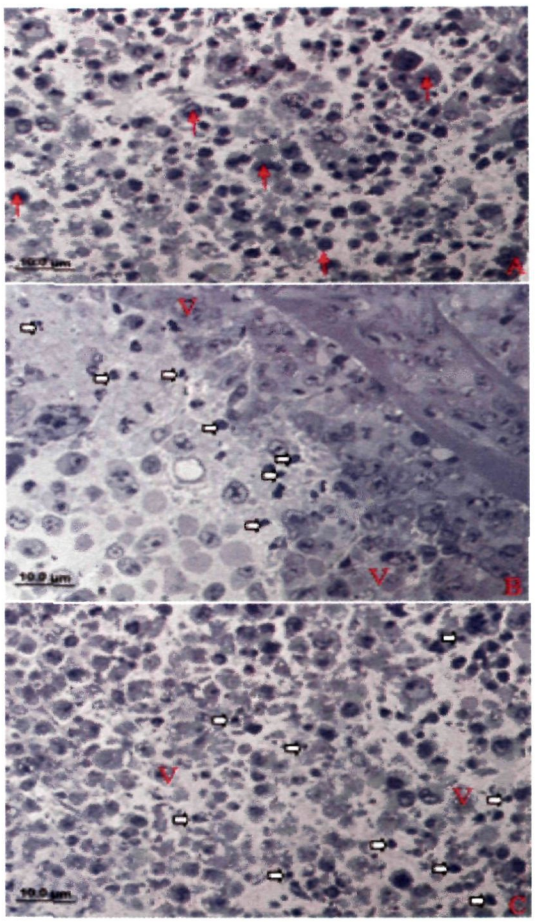
Magnification: 400 X, Bar 10 μM.

Fig. 4.65: Transmission Electron Microscopy of murine Sarcoma-180 (solid) tumor cells.

A-D: Tumor cells from normal saline treated animals showing ultrastructural details.

N-Nucleus showing uniformly distributed chromatin.
M-Mitochondria.
R- Ribosomes.
RER- Rough endoplasmic reticulum
(↑)- a double layer nuclear membrane.

Magnification: A-4800X, B-10000X, C-19000X and D-14000X.


V
V
V
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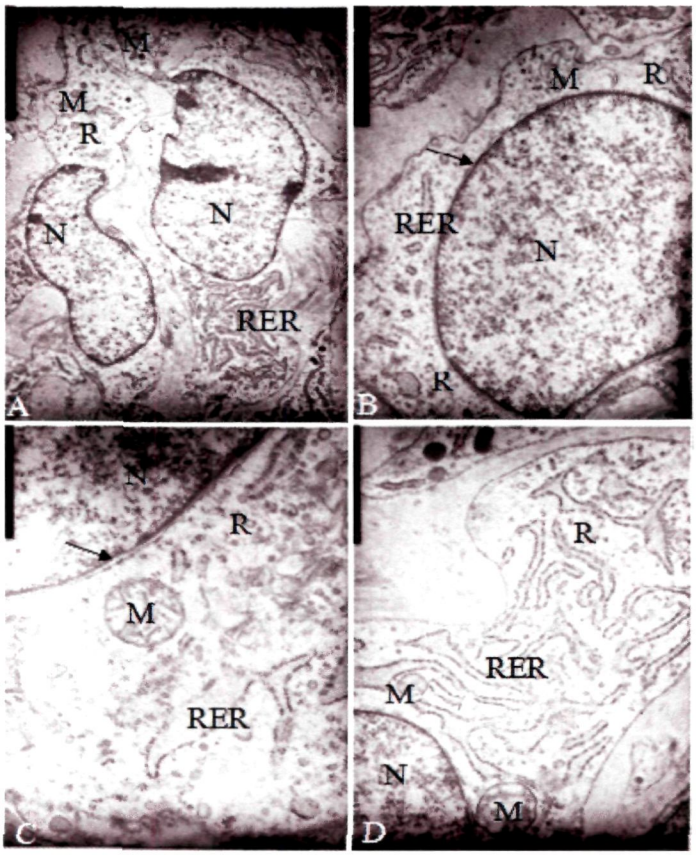
A
B
C


M
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R
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RER
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RER
M
N
M
D

Fig. 4.66: Transmission Electron Microscopy of Sarcoma-180 (solid) tumor cells showing effect of ARRF3 on the ultra structure.

A: Two cells from ARRF3 (80 mg/kg) treated animals showing chromatin condensation and decrease in size of the nucleus (N).

B: The magnified view of cell from ARRF3 (80 mg/kg) treated animal showing ultra structural details.

N-Nucleus showing chromatin condensation and decrease in size.
M-Mitochondria.
R- Ribosomes.
RER- Rough endoplasmic reticulum and
(↑): An indistinct nuclear membrane.

Magnification: A-5800X; B-19000X.

Fig. 4.67: Transmission Electron Microscopy of Sarcoma-180 (solid) tumor cells from INF1 (2 mg/kg) treated animal (A-D).

A: A cell showing marginalization of chromatin material (↑) and effect on cell organelles.

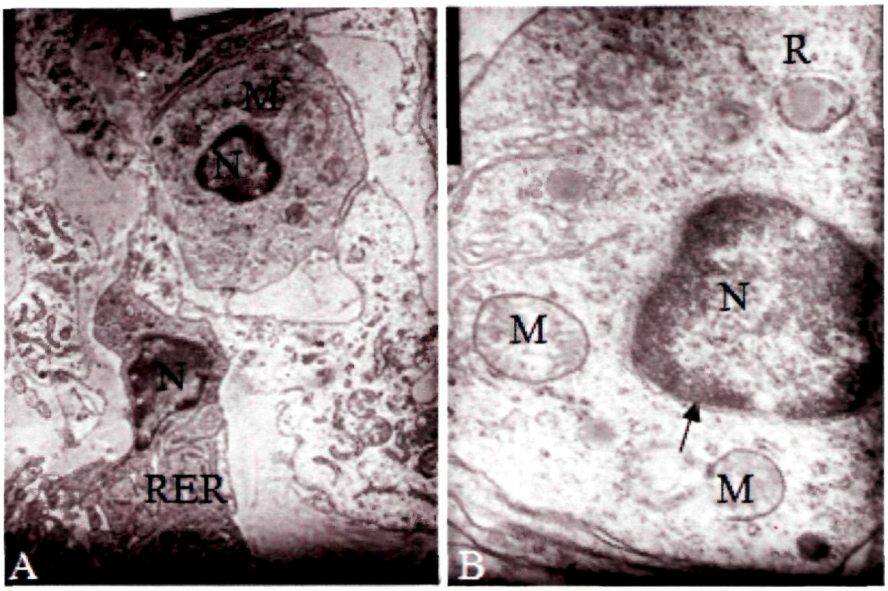
B: A cell showing fragmentation of nucleus forming micronuclei (Mn) and loss of cell organelles. The less affected cells are also seen.

C: The treated cell showing micronuclei and effect on cell organelles.

D: The magnified view of C above, showing effect on cell organelles.

N-Nucleus showing chromatin condensation.
M-Mitochondria.
R- Ribosomes.
RER- Rough endoplasmic reticulum.

Magnification: A-10000X, B-5800X, C-10000X and D-19000X.


M
N
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RER
A
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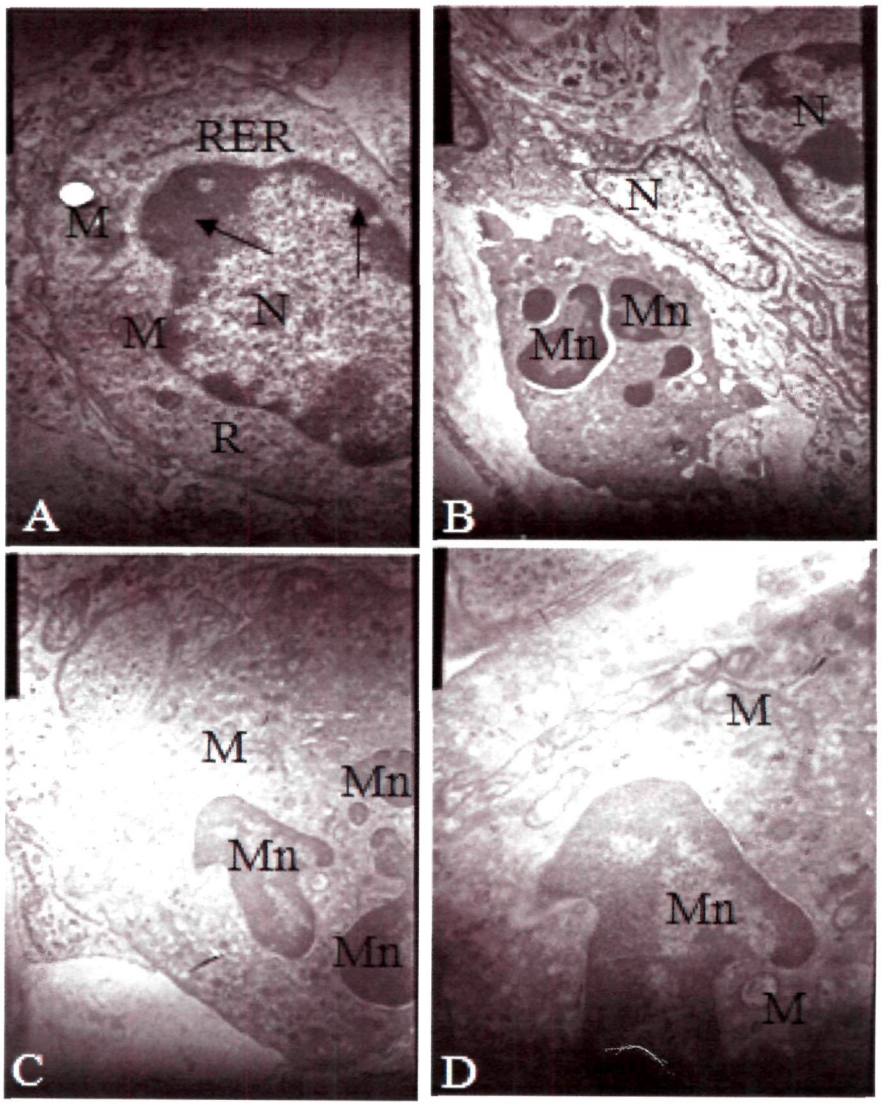
RER
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Chapter V

Discussion

CHAPTER - V

DISCUSSION

Natural products are the most consistent sources of drug leads. Therefore, the use of natural products has been the single most successful strategy for the discovery of new medicines used in anticancer therapy. Many naturally occurring plant-derived substances ingested in the human diet exhibit anticarcinogenic and antimutagenic effects (Farombi, 2004). Many authors have emphasized that the phytochemicals isolated from spices, such as pepper, are potent biological agents with interesting anticancer properties. The screening of natural products thus plays a considerable role in the discovery of new biologically active compounds and hence in the development of anticancer therapeutics. More and more people have therefore started searching for new antitumor agents from natural sources. In the present study, five plants (*Azadirachta indica* A. Juss., *Asparagus racemosus* Willd., *Ipomoea nil* L. Roth, *Costus speciosus* Koen ex. Retz. and *Butea monosperma* (Lam.) Taub.) were identified to be studied for their anticancer activity as many of them are relatively unexplored for this activity.

Anticancer activity of *Azadirachta indica* leaves

Azadirachta indica is a widely prevalent and esteemed wonder tree, mainly cultivated in Indian subcontinent (Schmutterer, 1995). Every part of this tree has been used in traditional Indian medicine for household remedy, against various human diseases (Chatterjee and Pakrashi, 1994). Although Neem has been extensively used in 'ayurveda' (a scripture on traditional medicine used in ancient India), its recognition was chiefly

limited within rural Indian population (Satyavati *et al.*, 1976). Curiosity and interest on this medicinal plant has been growing around the globe and US National Academy of Science in its 1992 report considered Neem tree for solving global health problems. Several pharmacological activities and medicinal applications of various parts of neem tree are known. Biological activities of Neem tree are reported where crude extracts and their different fractions from leaf, bark, root, seed and oil were used. These reports have exhibited a wide diversity in biological functions from a group of compounds present in the various parts of the plant (Biswas *et al.*, 2002).

In the present investigations, leaves of *Azadirachta indica* have been studied for anticancer activity against a panel of cancer cell lines *in vitro* and murine models of various cancers *in vivo*. Under *in vitro* studies, alcoholic extract of *Azadirachta indica* leaves exhibited growth inhibition of various cancer cell lines up to 44 per cent at 10 μg/ml whereas at 30 μg/ml concentration the cytotoxic activity ranged from 36-76 per cent with seven cell lines exhibiting more than 50 per cent growth inhibition. The extract was most active at 100 μg/ml with cytotoxic activity ranging from 57-94 per cent. All the cell lines exhibited more than 70 per cent growth inhibition at this concentration except two cell lines (MCF-7 and D17). Hydro-alcoholic and aqueous extracts did not show much promise as far as *in vitro* cytotoxicity is concerned. Polarity based fractionation of alcoholic extract yielded n-hexane, chloroform, n-burtanol and aqueous fractions out of which 30 μg/ml concentration of chloroform fraction exhibited cytotoxic activity in the range of 0-71 per cent against different cell lines with five cell lines showing more than 40 per cent growth inhibition (Table 4.2). This fraction at 50 μg/ml concentration was the most active exhibiting cytotoxicity in the range of 0-97 per cent against different cell lines with eight cell lines showing more than 50 per cent growth inhibition. It was particularly effective against colon (Colo-205, 502713) and prostate (Du-145) cancer cell lines. Thus, 50 μg/ml concentration of chloroform fraction showed cytotocic activity comparable to 100 μg/ml concentration of alcoholic extract. Little cytotoxic activity was noticed in n-butanol fraction also. However, n-hexane and aqueous fractions exhibited negligible cytotoxicity against all the cell lines studied. This indicates that the chemical constituents responsible for cytotoxic activity are medium polar in nature which are enriched in chloroform fraction.

Results of the cytotoxic activity of neem leaves observed in the present study are in agreement with Kumar *et al.*, (2006), who showed an ethanolic extract of neem leaves to

cause apoptotic death of prostate cancer cells (PC-3). Nimbolide, extracted from the flowers of the neem tree (*Azadirachta indica*) is reported to have antiproliferative activity against U937, HL-60, THP1 and B16 cancer cell lines (Roy *et al.*, 2007). Harish *et al.* (2009) also reported the treatment with nimbolide to result in dose- and time-dependent inhibition in growth of BeWo cells with IC_{50} values of 2.01 and 1.19 μM for 7 and 24 h respectively. It is common in plants that secondary metabolites present in one part may also be found in the other plant part and more so in leaves. Leaves of *Azadirachta indica* contain nimbolide also (Basak and Chakraborty, 1968). Therefore, it is expected that the chemical constituent responsible for cytotoxic activity observed in the present study and that of Kumar *et al.* (2006), Roy *et al.* (2007) and Harish *et al.* (2009) may be the same. The variation in cytotoxicity from cell line to cell line can be explained in the light of variation in their molecular characteristics, which is true for other cytotoxic agents also. Negligible cytotoxicity of aqueous extract and fraction against studied cell lines corresponds well with the findings of Baral and Chattopadhyay, (2004), who reported that the aqueous extract of neem leaves had no direct cytotoxic effect on Ehrlich carcinoma cells. Further, they did not find any significant difference in the growth of Ehrlich ascites carcinoma in swiss mice treated therapeutically with aqueous extract of neem leaves (1 unit/mice/week) for 4 weeks and PBS treated control group, implying thereby that the aqueous extract of *Azadirachta indica* leaves is devoid of anticancer activity.

Under *in vivo* studies, alcoholic extract of *Azadirachta indica* leaves (AILE) inhibited the growth of Ehrlich ascites carcinoma (EAC) by -1, 8.83, 34.33 and 55.56 per cent at 100, 150, 200 and 250 mg/kg dose levels, respectively whereas chloroform fraction from the alcoholic extract of *Azadirachta indica* leaves (AILF2) showed 31.51, 36.61, 37.33 and 50.36 per cent growth inhibition of EAC at similar doses, respectively. Similar were the findings in case of Ehrlich tumor (solid), wherein AILE at 100, 150, 200 and 250 mg/kg doses inhibited tumor growth by 0.22, 10.63, 20.92 and 37.41 per cent respectively (Fig. 4.15-B) and AILF2 by 4.28, 10.65, 28.74 and 40.05 per cent at similar doses, respectively (Fig. 4.16-B). These findings again prove that the anticancer activity was enriched in AILF2 as it yielded higher growth inhibition of EAC and ET (solid) than AILE at similar doses.

Haque *et al.* (2006) observed significant restriction in the growth of Ehrlich's carcinoma in swiss mice following prophylactic treatment with neem leaf preparation @ 1 unit once

weekly for four weeks which stimulated hematological system as evidenced by the increase in total count of RBC, WBC and platelets and hemoglobin percentage. However, there is no report on therapeutic efficacy of neem leaf extract or fraction. Perhaps this is the first report on therapeutic efficacy of alcoholic extract and its chloroform fraction from *Azadirachta indica* leaves against Ehrlich ascites carcinoma. Incidentally, Kumar *et al.* (2006) have also shown an ethanolic extract of neem leaves to cause apoptotic death of prostate cancer cells (PC-3) *in vitro*. Results of the present study thus not only prove the presence of therapeutic anticancer activity in alcoholic extract of *Azadirachta indica* leaves against Ehrlich ascites carcinoma but also point towards medium polar compounds to be responsible for it as revealed by enrichment of anticancer activity in chloroform fraction from alcoholic extract.

Against Sarcoma-180 (ascites) and Sarcoma-180 (solid), efficacy of AILE was found to be 0.96, -1.03, 11.46 and 23.85 per cent at 100, 150, 200 and 250 mg/kg doses (Fig. 4.11-C) and -6.13, -2.29, 7.38 and 16.95 per cent at similar doses, respectively (Fig. 4.17C). AILF2 yielded little higher growth inhibition against both the tumor systems, the respective figures being 1.05, 18.31, 28.39, 33.39 (Fig. 4.12-C) and -5.38, -3.48, 9.06 and 21.71 (Fig. 4.18-C). Statistically, these figures were not significantly different from control values. Literature is devoid of any report on therapeutic anticancer potential of alcoholic extract and its chloroform fraction from *Azadirachta indica* leaves against Sarcoma-180 tumor systems. Nonetheless, Fujiwara *et al.* (1982) demonstrated strong anti-tumor effect with complete regression of the tumors, with two water-soluble polysaccharides GIa and GIb isolated from the bark of *Melia azadirachta* administered in mice at a daily dose of 50 mg/kg after subcutaneous inoculation of Sarcoma-180 cells. The two studies are not comparable as the compounds reported by Fujiwara *et al.* (1982) were highly polar (water-soluble) and derived from the bark whereas medium polar compounds enriched in chloroform fraction derived from alcoholic extract of leaves were responsible for the present findings.

Arivazhagan *et al.* (2000) investigated the protective effects of neem leaves during *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine induced gastric carcinogenesis in rats. Chemopreventive potential of neem on 7,12-dimethylbenz[*a*]anthracene induced buccal pouch carcinogenesis in hamsters was also evaluated (Balasenthil *et al.*, 1999). Manikandan *et al.* (2008a) also observed that the administration of neem leaf fractions

reduced the incidence of DMBA-induced HBP carcinomas at a lower concentration compared to the crude extract. Results of all these investigations are in favour of the chemo-preventive function of neem, which may be mediated by modulation of lipid peroxidation, antioxidants and detoxification enzymes. However, no tumor killing (therapeutic) property of neem leaf has yet been described. In the present investigation also, *in vivo* anticancer activity of AILE and AILF2 against Methyl cholanthrene induced ascites was observed to be -13.91, -3.85, 5.64, 8.17 (Fig. 4.13) and 10.95, 15.99, 16.64, 18.54 per cent (Fig. 4.14) respectively, at 100, 150, 200 and 250 mg/kg. Statistically, these figures were not significantly different from control values implying thereby that the alcoholic extract and its chloroform fraction from *Azadirachta indica* leaves do not possess therapeutic potential against Methyl cholanthrene induced ascites.

Literature is scarce about *in vivo* anticancer potential of *Azadirachta indica* leaves against L1210 lymphoid leukemia and P388 lymphocytic leukemia. In the present investigation, increase in life span of L1210 lymphoid leukemia bearing animals treated with 100, 150, 200 and 250 mg/kg doses of AILE and AILF2 was found to be 1.50, 4.50, -4.51, -18.15 (Fig. 4.19) and 9.14, 17.32, -4.51, -13.65 per cent (Fig. 4.20), respectively. These values are much below the threshold stipulated for significant anticancer activity as per NCI protocols described by Geran *et al.* (1972). These findings indicate that while lower doses of AILE and AILF2 are devoid of significant anticancer activity, higher doses of both are toxic to animals. Similar results were obtained with AILE and AILF2 against P388 lymphocytic leukemia also. With 100, 150, 200 and 250 mg/kg doses of AILE and AILF2 increase in life span of P388 lymphocytic leukemia bearing animals was 3.13, 4.04, 14.88 and -1.7 (Fig. 4.21) and 2.19, 5.41, 6.14 and -3.81 per cent (Fig. 4.22), respectively. These values also are much below the threshold stipulated for significant anticancer activity as per NCI protocols described by Geran *et al.* (1972). However, it was noticed that the higher doses of AILE and AILF2 were not as toxic to P388 lymphocytic leukemia bearing animals as they were to L1210 lymphoid leukemia bearing animals.

Each and every test substance that is active *in vitro* does not necessarily show activity *in vivo*. In fact, many of the agents which are active *in vitro* either fail to show the activity *in vivo* or their activity potential does not communicate with their *in vitro* activity. It is quite obvious also because under *in vitro* test systems, the agent directly interacts with

cells whereas under *in vivo* test systems number of barriers are involved in the action of an agent. The barriers include bioavailability, biotranformations, pharmacokinetic profile, various transport mechanisms, efflux pumps and many more. It is thus observed that although AILE and its chloroform fraction (AILF2) were active against isolated cancer cell lines *in vitro*, under *in vivo* conditions their activity profile was not significant against most of the models used in the present study except against Ehrlich ascites carcinoma and Ehrlich tumor (solid) where they yielded statistically significant growth inhibition. Even the statistically significant anticancer activity exhibited by AILE and AILF2 against Ehrlich ascites carcinoma and Ehrlich tumor (solid) was much below the threshold stipulated for significant anticancer activity as per NCI protocols described by Geran *et al.,* (1972). Thus, it is concluded that AILE and AILF2 in their present forms do not possess significant anticancer potential. However, presence of some anticancer activity in AILE and its enrichment in AILF2 strongly demand further studies for the isolation of individual compounds present in AILF2 and their *in vivo* investigation with respect to bioavailability patterns, biotransformation, pharmacokinetic profile and anticancer activity against various cancers.

Anticancer activity of *Asparagus racemosus* roots

Asparagus racemosus Willd. is one of the important medicinal plants regarded as 'rasayana' (plant drugs promoting general well being by increasing cellular vitality and resistance) in the Ayurvedic system of medicine (Goyal *et al.,* 2003). It is an important medicinal plant of tropical and subtropical India. Its medicinal usage has been reported in the Indian and British Pharmacopoeias and in indigenous systems of medicine. The genus *Asparagus* includes about 300 species around the world. The genus is considered to be medicinally important because of the presence of steroidal saponins and sapogenins in various parts of the plant(s). Out of the 22 species of *Asparagus* recorded in India, *Asparagus racemosus* is the one most commonly used in traditional medicine. Beneficial effects of the roots of *A. recemosus* are suggested in nervous disorders, dyspepsia, diarrhoea, dysentery, tumors, inflammations, hyperdipsia, neuropathy, hepatopathy, cough, bronchitis, hyperacidity and certain infectious diseases (Sharma *et al.,* 2000). However, no scientific proof justifying all the above uses of the roots of *A. racemosus* is available so far. In the present investigation, roots of *Asparagus racemosus*

have been investigated for anticancer activity against a panel of cancer cell lines *in vitro* and murine models of various cancers *in vivo*.

The alcoholic extract of roots of *Asparagus racemosus* exhibited moderate (0-80 per cent) cytotoxicity against different cell lines at 10 μg/ml concentration and the maximum activity of 80 per cent growth inhibition was observed with A-549. At 30 μg/ml, the cytotoxicity ranged between 38-95 per cent and ten of the cell lines studied showed more than 50 per cent growth inhibition. 100 μg/ml concentration was the most active one showing 49-98 per cent cytotoxicity against different cell lines with nine of the twelve cell lines exhibiting more than 70 per cent growth inhibition (Table 4.3). Little less but significant cytotoxic activity was observed in hydro-alcoholic extract and negligible activity in aqueous extract. Polarity based fractionation of alcoholic extract yielded n-hexane, chloroform, n-burtanol and aqueous fractions out of which chloroform fraction at 30 μg/ml concentration, exhibited 24-74 per cent cytotoxicity and seven cell lines exhibited more than 40 per cent growth inhibition. At 50 μg/ml concentration, the cytotoxicity ranged between 36-99 per cent and the same seven cell lines exhibited more than 50 per cent growth inhibition. n-Butanol fraction was observed to be little more active (Table 4.4). At 30 μg/ml concentration it exhibited 16-81 per cent cytotoxic activity and nine cell lines exhibited more than 40 per cent growth inhibition and at 50 μg/ml concentration, eight cell lines showed more than 50 per cent growth inhibition. It was thus observed that the compounds with medium to high polarity enriched in chloroform and n-butanol fractions from alcoholic extract of *Asparagus racemosus* roots were responsible for cytotoxic activity. These findings are in close agreement with Shao *et al.* (1996) who reported that crude saponins were found in n-butanol fraction of methanolic extract of the shoots of asparagus and that they have antitumor activity. Saponins from other plant sources also are reported to possess cytotoxic activity against cancer cells. Sun *et al.* (2004) found that the saponins from *Tribulus terrestris* had potent inhibitory effect on liver cancer cell line BEL-7402. The crude saponin fraction from the roots of *Platycodon grandiflorum* exhibited significant inhibition on the proliferation of five kinds of cultured human tumor cell lines, viz., A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nervous system) and HCT-15 (colon), *in vitro* (Kim *et al.*, 2005). Very recently, steroid saponins of *Paris polyphylla* var. yunnanensis have also been found to be cytotoxic against lung adenocarcinoma cell line, *in vitro* (Yan *et al.*, 2009). It is thus possible that the cytotoxic principles enriched in n-

have been investigated for anticancer activity against a panel of cancer cell lines *in vitro* and murine models of various cancers *in vivo*.

The alcoholic extract of roots of *Asparagus racemosus* exhibited moderate (0-80 per cent) cytotoxicity against different cell lines at 10 μg/ml concentration and the maximum activity of 80 per cent growth inhibition was observed with A-549. At 30 μg/ml, the cytotoxicity ranged between 38-95 per cent and ten of the cell lines studied showed more than 50 per cent growth inhibition. 100 μg/ml concentration was the most active one showing 49-98 per cent cytotoxicity against different cell lines with nine of the twelve cell lines exhibiting more than 70 per cent growth inhibition (Table 4.3). Little less but significant cytotoxic activity was observed in hydro-alcoholic extract and negligible activity in aqueous extract. Polarity based fractionation of alcoholic extract yielded n-hexane, chloroform, n-burtanol and aqueous fractions out of which chloroform fraction at 30 μg/ml concentration, exhibited 24-74 per cent cytotoxicity and seven cell lines exhibited more than 40 per cent growth inhibition. At 50 μg/ml concentration, the cytotoxicity ranged between 36-99 per cent and the same seven cell lines exhibited more than 50 per cent growth inhibition. n-Butanol fraction was observed to be little more active (Table 4.4). At 30 μg/ml concentration it exhibited 16-81 per cent cytotoxic activity and nine cell lines exhibited more than 40 per cent growth inhibition and at 50 μg/ml concentration, eight cell lines showed more than 50 per cent growth inhibition. It was thus observed that the compounds with medium to high polarity enriched in chloroform and n-butanol fractions from alcoholic extract of *Asparagus racemosus* roots were responsible for cytotoxic activity. These findings are in close agreement with Shao *et al.,* (1996) who reported that crude saponins were found in n-butanol fraction of methanolic extract of the shoots of asparagus and that they have antitumor activity. Saponins from other plant sources also are reported to possess cytotoxic activity against cancer cells. Sun *et al.,* (2004) found that the saponins from *Tribulus terrestris* had potent inhibitory effect on liver cancer cell line BEL-7402. The crude saponin fraction from the roots of *Platycodon grandiflorum* exhibited significant inhibition on the proliferation of five kinds of cultured human tumor cell lines, viz., A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nervous system) and HCT-15 (colon), *in vitro* (Kim *et al.,* 2005). Very recently, steroid saponins of *Paris polyphylla* var. yunnanensis have also been found to be cytotoxic against lung adenocarcinoma cell line, *in vitro* (Yan *et al.,* 2009). It is thus possible that the cytotoxic

principles enriched in n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) of present studies may be saponins of *Asparagus racemosus.*

Alcoholic extract of *Asparagus racemosus* roots (ARRE) and its chloroform (ARRF2) and n-butanol (ARRF3) fractions were investigated for *in vivo* anticancer activity against EAC. ARRE at 80, 100, 120 and 140 mg/kg doses inhibited the tumor growth by 21.77, 33.05, 53.29 and 94.94 per cent, respectively (Fig. 4.23). Respective figures for ARRF2 and ARRF3 were 31.85, 39.06 and 84.66 per cent (Fig. 4.24) and 89.0, 94.13 and 91.36 per cent (Fig. 4.25) for the dose levels of 80, 100 and 120 mg/kg. While most of these values showed statistically significant anticancer activity, 140 mg/kg dose of ARRF2 and ARRF3 has been highly toxic to animals. Overall, per cent tumor growth inhibition in groups treated with 80, 100 and 120 mg/kg doses of ARRF3 was quite higher than similar doses of ARRF2 and ARRF2 has yielded higher growth inhibition than ARRE. The data obtained in *in vivo* studies support our own *in vitro* findings wherein n-butanol fraction proved more cytotoxic to cancer cells. This proves that the compounds with medium to high polarity enriched primarily in n-butanol fraction (ARRF3) and to a lesser extent in chloroform fraction (ARRF2) were responsible for the anticancer activity. These findings are again in close agreement with Shao *et al.*, (1996).

Similar pattern of anticancer activity with respect to ARRE, ARRF2 and ARRF3 was observed against Sarcoma-180 (ascites). At 80, 100, 120 and 140 mg/kg dose levels, ARRE inhibited the growth of Sarcoma-180 (ascites) by 21.26, 28.11, 43.41 and 76.73 per cent, respectively (Fig. 4.26). ARRF2 and ARRF3 exhibited even higher levels of growth inhibition. Respective figures in respect of ARRF2 and ARRF3 for 80, 100 and 120 mg/kg dose levels were 38.54, 61.24 and 79.94 per cent and 82.78, 88.19 and 89.58 per cent, respectively. While all of these values showed statistically significant anticancer activity, 140 mg/kg dose of ARRF2 and ARRF3 has been highly toxic to animals.

In case of Methyl cholanthrene induced ascites, ARRE, ARRF2 and ARRF3 yielded highly significant growth inhibition at 80, 100 and 120 mg/kg doses and 140 mg/kg dose of all three proved toxic to animals. The tumor growth inhibition by ARRE, ARRF2 and ARRF3 was in the range of 69.21-79.74, 62.04-87.49 and 95.85-97.12 per cent, respectively. These findings corroborate well with the findings of Rao (1981) who

reported that virgin female rats, normal or primed with 17-β-estradiol treatment, put on diets containing 0.25, 0.5, 1 or 2 per cent Asparagus root extract powder prior to their exposure to DMBA, showed a sharp decline in mammary tumor incidence. Findings of the present study are also in good agreement with those of Agrawal *et al.* (2008) who found that the hepatic tissues of Wistar rats treated with diethylnitrosamine (DEN) (200 mg/kg b wt, i.p.) once a week for 2 weeks, followed by treatment with DDT, a tumor promoter (0.05 per cent in diet) for 2 weeks, demonstrated the development of malignancy. Pretreatment of Wistar rats with the aqueous extract of the roots of *Asparagus racemosus* prevented the incidence of hepatocarcinogenesis. Results of the present study and the findings of Agrawal *et al.* (2008) thus prove that ARRE, ARRF2 and ARRF3 are effective also against chemical carcinogen induced tumorigenesis in addition to spontaneous tumors.

Anticancer activity of ARRE, ARRF2 and ARRF3 against Ehrlich tumor (solid) and Sarcoma-180 (solid) models has been quite impressive and in many instances more than that of the positive control used in the study. Against Ehrlich tumor (solid), ARRE, ARRF2 and ARRF3 yielded growth inhibition in the range of 18.36-54.21, 26.94-57.67 and 61.34-65.0 per cent at the dose levels of 80, 100 and 120 mg/kg. Likewise, against Sarcoma-180 (solid) respective growth inhibitions for ARRE, ARRF2 and ARRF3 were in the range of 10.46-45.53, 22.33-43.01 and 55.06-57.26 per cent at the same dose levels. A dose of 140 mg/kg proved highly toxic in both the cases. Consistently higher activity yielded by ARRF3 than ARRF2 and ARRE proves beyond doubt that the n-butanol fraction is enriched with anticancer principles.

In the present studies, no significant anticancer activity of ARRE, ARRF2 and ARRF3 was noticed against L1210 lymphoid leukemia. However, in P388 lympocytic leukemia bearing animals, ARRE at higher doses and ARRF3 at lower doses yielded significant increase in life span of treated animals.

Although no parallel studies on *Asparagus racemosus* roots are available in the literature, saponins present in *Asparagus racemosus* roots, expected to be enriched in ARRF3 may be responsible for the anticancer activity observed in the present investigation as proposed by Shao *et al.* (1996). Clinical studies have suggested that these health-promoting components affect immune system in ways that help to protect the human body against cancers and also lower cholesterol levels. Saponins decrease blood lipids,

lower cancer risks and lower blood glucose response (Shi *et al.*, 2004). Very recently, Yan *et al.* (2009) reported that the oral administration of steroid saponins of *Paris polyphylla* var. yunnanensis to T739 mice bearing LA795 lung adenocarcinoma significantly inhibited tumor growth. In the light of the highly significant anticancer activity observed in ARRF3 in present studies and the anticancer activity reported in saponins from other sources, it is pertinent to isolate individual compounds from ARRF3 and investigate them further with respect to their bioavailability, pharmacokinetic profile and anticancer activity against various other cancers which may lead to a new anticancer therapeutic. Synthetic analogues of the active principles with better anticancer activity and reduced toxicity can be of great value towards development of novel anticancer therapeutic.

Anticancer activity of *Ipomoea nil* aerial parts

Seeds and whole plant *Ipomoea nil* are sporadically reported to show antitumor and antifungal activities (Aswal *et al.*,1984; Saito *et al.*,1996; Koo *et al.*,1998). In the present investigation, aerial parts of *Ipomoea nil* have been systematically investigated in a well planned manner to explore its anticancer potential against a panel of cancer cell lines *in vitro* and murine models of various cancers *in vivo*.

The alcoholic extract of *Ipomoea nil* aerial parts showed an impressive cytotoxicity even at 10 μg/ml concentration, exhibiting 4-84 per cent growth inhibition of different cell lines with one of the cell line (HCT-15) showing 84 per cent growth inhibition. 30 μg/ml concentration was quite cytotoxic, inhibiting the growth of different cell lines from 42-94 per cent and preventing ten out of the twelve cell lines to grow beyond 50 per cent. At 100 μg/ml concentration, the alcoholic extract exhibited 83-100 per cent cytotoxic activity and all the twelve cancer cell lines studied showed more than 70 per cent growth inhibition (Table 4.5). Ko *et al.* (2004) also found 80 per cent ethanol extract of roots of *Pharbitis nil* to inhibit proliferation of AGS gastric cancer cell line. The cytotoxicity of hydro-alcoholic extract was little less than the alcoholic extract and the aqueous extract was found to be least cytotoxic.

Polarity based fractionation of alcoholic extract yielded n-hexane, chloroform, n-burtanol and aqueous fractions out of which n-hexane fraction emerged to be the most cytotoxic

one (Table 4.6). At all the three concentrations (10, 30 and 100 μg/ml) all the twelve cell lines studied showed promising growth inhibition. Chloroform fraction also exhibited good cytotoxicity but it was little less than the n-hexane fraction. n-Butanol fraction did not show good cytotoxicity whereas aqueous fraction was almost devoid of cytotoxic potential. Thus, n-hexane fraction at 50 μg/ml exhibited cytotoxic activity that was comparable to the activity of alcoholic extract at 100 μg/ml concentration. This indicates that there was enrichment of non-polar cytotoxic principles in n-hexane fraction. Findings of the present investigation confirm the findings of Aswal *et al.* (1984) who had reported the presence of cytotoxic activity in whole plant *Ipomoea nil* against human epidermoid carcinoma of nasopharynx in tissue culture. Our findings are also in agreement with those of Kim *et al.* (2009) who evaluated the compounds isolated from seeds of *Ipomoea nil* for their cytotoxic activities against four human cancer cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) using the SRB assay *in vitro*. Compounds Pharboside D and Pharboside E exhibited moderate cytotoxic activity against A549, SK-OV-3, SKMEL-2 and HCT15 cells. However, the compounds responsible for cytotoxic activity in our investigation may be different from Pharboside D and Pharboside E as they are isolated from seeds and possess only moderate cytotoxicity as reported by Kim *et al.* (2009) whereas compounds present in whole of aerial parts have yielded highly significant activity in our studies.

In vivo anticancer activity of alcoholic extract of *Ipomoea nil* (INE) and its n-hexane fraction (INF1) evaluated against Ehrlich ascites carcinoma (EAC) and Ehrlich tumor (solid) in the present investigation has been consistently impressive. INE at 2, 3 and 4 mg/kg doses showed 50.71, 76.67 & 82.0 and 28.74, 42.09 & 55.64 per cent growth inhibition against Ehrlich ascites carcinoma and Ehrlich tumor (solid), respectively. Activity of INF1 at 0.5, 1 and 2 mg/kg was 46.42, 66.33 & 87.06 and 29.75, 45.11 & 54.36 per cent against Ehrlich ascites carcinoma and Ehrlich tumor (solid), respectively. It is observed that (Fig. 4.45, 4.50, 4.46 and 4.51) INE and INF1 both exhibited tumor growth inhibition comparable to the positive control used in the study. It is further pointed out that INF1 showed anticancer activity similar to INE at half and less than half the doses of INE. This indicates that the principles responsible for anticancer activity observed in the present investigations are enriched in INF1. Handbook of Chinese Herbs and Formulas (1985) by Yeung Him-Che mentions the seeds of *Ipomoea nil* to possess

antitumor activity. Findings of the present investigation further prove that not only the seed but whole of aerial parts of the plant has promising anticancer activity.

Similar but more intense pattern of anticancer activity against Sarcoma-180 (ascites) and Sarcoma-180 (solid) was observed. INE at 2, 3 and 4 mg/kg doses exhibited 56.20, 79.59 & 97.12 and 15.19, 49.59 & 53.50 per cent growth inhibition against Sarcoma-180 (ascites) and Sarcoma-180 (solid), respectively (Fig. 4.46 & 4.52) whereas INF1 at 0.5, 1 and 2 mg/kg doses showed 51.53, 81.93, 92.06 and 30.99, 47.65, 59.48 per cent growth inhibition respectively, against the same models (Fig. 4.47 & 4.53). Against Methyl cholanthrene induced ascites also, INE and INF1 at aforementioned doses yielded quite impressive antitumor activity. 71.23, 92.06 & 97.19 and 60.77, 76.43 & 94.03 per cent inhibition in tumor growth was obtained with INE and INF1 at their respective doses (Fig. 4.48 & 4.49).

Literature reveals that different investigators studied roots (Ko *et al.,* 2004), seed (Kim *et al.,* 2009) and whole plant (Aswal *et al.,* 1984) of *Ipomoea nil* and reported the presence of anticancer activity in them. In the present investigation, aerial parts of the plant were used to prepare extracts and fractions and highly significant anticancer activity at very low doses against cancers of varied tissue origin including chemical carcinogen induced ascites, was observed. It is therefore likely that INF1 used in our study is enriched with anticancer compounds from seed, flowers, leaves, stem, etc.

Against L1210 lymphoid leukemia neither INE nor INF1 produced any significant increase in life span of treated animals. Similarly, against P388 lymphocytic leukemia also no significant increase in life span was observed with INE and INF1 treatments. Rather, higher doses of both showed deaths in treated animals due to toxicity. However, Aswal *et al.* (1984) found the whole plant *Ipomoea nil* to possess anticancer activity against P388 lymphocytic leukemia in mice. INE and INF1 used in our studies were prepared from aerial parts of the plant whereas Aswal *et al.* (1984) used whole plant including roots to study the activity. Roots of *Ipomoea nil* are also reported to contain apoptosis inducing principles (Ko *et al.,* 2004). It is therefore likely that the anticancer principles from roots of *Ipomoea nil* are lacking in INE and INF1 and the same might be responsible for anticancer potential observed by Aswal *et al.* (1984) against P388 lymphocytic leukemia.

Presence of β-sitosterol in *Ipomoea nil* has been acknowledged by many investigators (Jung da *et al.*, 2008; Kim *et al.*, 2009). β-sitosterol is mainly known and used for its cholesterol lowering property. But studies have shown that this phytochemical may have other health benefits like easing symptoms of benign prostatic enlargement, reducing risk of cancer and prevention of oxidative damage through its antioxidant activity. Epidemiological and experimental studies have suggested a protective role of β-sitosterol in the development of some types of cancer such as breast, colon and prostate cancer. *In-vivo* studies have shown that this phytochemical inhibited proliferation and induced apoptosis in human solid tumors such as colon and breast cancers. Likewise, presence of saponins (Pharbitosides) in seeds of *Ipomoea nil* and its cytotoxic activity against human cancer cell lines has been proved (Jung da *et al.*, 2008). Since INE and INF1 used in the present study were prepared from aerial parts of the plant, it is most likely that they may contain β-sitosterol, Pharbitosides and few other phytochemicals likely to possess anticancer properties. Therefore, the results of the present study seem to be the combined activity of many phytochemicals present in *Ipomoea nil* which has resulted in exceptionally high anticancer activity. It is therefore pertinent to isolate the active principles enriched in INF1 and develop them in the form of clinically useful novel anticancer therapeutics.

Anticancer activity of *Costus speciosus* aerial parts

Costus speciosus is widely cultivated as an ornamental plant, sometimes escaping from cultivation. In Ayurveda, rhizomes of *Costus speciosus* are ascribed to be bitter, astringent, acrid, cooling, aphrodisiac, purgative, antihelminthic, depurative, febrifuge, expectorant and tonic (Mandal *et al.*, 2007). However, aerial parts of the plant have not so far been studied for medicinal properties. Therefore, in the present studies, aerial parts of the plant have been investigated for anticancer activity against a panel of cancer cell lines *in vitro*.

Out of the three extracts (alcoholic, hydro-alcoholic and aqueous) prepared from the aerial parts of *Costus speciosus* none of the extracts exhibited any significant cytotoxic activity against any of the cell line at any of three concentrations (10, 30 and 100 μg/ml) studied. Incidentally, literature is also devoid of any report of similar studies on aerial

parts of the plant. It was therefore concluded that the aerial parts of *Costus speciosus* did not possess significant anticancer potential and was not studied further.

Anticancer activity of *Butea monosperma* flowers

Butea monosperma is a sacred tree, referred to as a treasurer of the Gods and is used in sacrifice related rituals. Butrin and isobutrin are the main constituents of flowers and have proven anti-hepatotoxic activity (Wagner *et al.*, 1986; Sehrawat *et al.*, 2006). In the present investigations, flowers of *Butea monosperma* have been investigated for anticancer activity against a panel of cancer cell lines from different tissues.

Alcoholic, hydro-alcoholic and aqueous extracts prepared from the flowers of *Butea monosperma* were subjected to *in vitro* cytotoxicity assays against different cancer cell lines at 10, 30 and 100 µg/ml concentrations. All the extracts exhibited negligible cytotoxic activity at all the concentrations studied. It was therefore considered that the flowers of *Butea monosperma* did not possess any promise as far as anticancer activity is concerned and no further studies were undertaken.

Mechanism of action of active isolates

In the present investigations, n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) and n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) emerged to be the most active isolates with definite potential for anticancer therapeutics. Many reports have revealed that induction of apoptosis has been the most desirable mode of action of anticancer agents (Sun *et al.*, 2003). Therefore, much of the contemporary research in the development of anticancer therapeutics from plants has been focused on investigating molecular mechanisms by which an agent induces cytotoxicity in cancer cells. Anticancer drugs causing cell death by inducing apoptosis are the drugs of choice. With this view, isolates with definite potential for anticancer therapeutics (ARRF3 and INF1) identified in the present investigations were investigated further for their ability to induce apoptosis in cancer cells employing light microscopy, scanning & transmission electron microscopy and flow-cytometry techniques.

Scanning electron microscopy of HL-60 cells treated with 25 µg/ml of ARRF3 and 10 µg/ml of INF1 and EAC cells collected from the ascitic fluid of animals treated with 80 mg/kg of ARRF3 and 2 mg/kg of INF1 revealed typical morphological changes suggestive of apoptosis such as cell shrinkage, smoothening of cell surface, blebbing of the plasma membrane and formation of apoptotic bodies (Fig. 4.62 & 4.63). Untreated HL-60 cells and tumor cells from control animals were found to be spherical in shape and having microvilli on surface with a few filopods. Likewise, light microscopy of tumor tissue from Sarcoma-180 (solid) bearing animals treated with 80 mg/kg of ARRF3 and 2 mg/kg of INF1 showed shrinkage of cells, condensation of chromatin, cytoplasmic condensation, fragmentation of nucleus, apoptotic bodies and vacuolization (Fig. 4.64). Tumor tissue from normal saline treated animals revealed high nucleo-cytoplasmic ratio in tumor cells. Transmission electron microscopy of Sarcoma-180 (solid) tumor cells revealed condensation and marginalization of chromatin, decrease in the size and fragmentation of nucleus and loss of mitochondrial cristae on treatment with 80 mg/kg of ARRF3 and 2 mg/kg of INF1. These changes in the morphology of tumor cells/tissue are in good agreement with the original morphological criteria of apoptosis described by Wyllie *et al.* (1980) and thus prove the apoptosis inducing ability of ARRF3 and INF1.

Many anticancer agents and DNA damaging agents arrest cell cycle at the G_0/G_1, S, G_2/M phase and then induce apoptosis. The effect of anti-proliferative agent on cell cycle progression appears to depend on the concentration of the compound and also on the duration of the treatment (Loplay, 1996). In cell cycle analysis, the DNA of the cancer cell population is intercalated with fluorescent dye (like propidium iodide) and analyzed for its distribution in cell cycle and ploidy level as it flows through the cytometer. This technique is finding wide application especially with regard to discovery of anticancer agents from medicinal plants (Engh, 1993). Loss of DNA is a typical feature of apoptotic cells that occurs as a result of diffusion of degraded DNA out of the cells after cleavage by endonucleases. Cells that have lost DNA take up less stain on staining with propidium iodide and appear left to the G_0/G_1 peak. In the present investigation, ARRE, ARRF2 and ARRF3 at 50 µg/ml concentration showed 18, 19 and 30 per cent of treated HL-60 cells in the sub G_1 peak whereas 6 per cent of untreated cells were in the sub G_1 peak (Fig 4.58). This not only proves the apoptosis inducing ability of ARRE, ARRF2 and ARRF3 but also proves that the apoptosis inducing principles are enriched in ARRF3 as compared to ARRE and ARRF2. As discussed earlier, saponins present in *Asparagus*

racemosus roots, enriched in ARRF3 are expected to be responsible for the anticancer activity observed in the present investigation. Therefore, from the point of view of apoptosis inducing ability of saponins, present studies corroborate well with that of Yan *et al.* (2009) who demonstrated the steroid saponins from *Paris polyphylla* var. yunnanensis to induce apoptosis in tumor cells by TUNEL assay. Sun *et al.* (2004) also reported increased sub-G_1 peak in liver cancer cell line BEL-7402 treated with saponins from *Tribulus terrestris.* Findings of the present study also correspond with the findings of Shao *et al.* (1996) who reported that the saponins from *Asparagus racemosus* at 6 and 50 µg/ml inhibited the synthesis of DNA, RNA and protein in HL-60 cells.

In case of INF1, 14 and 22 per cent of HL-60 cells treated with 3 and 5 µg/ml concentrations, respectively were observed in sub G_1 peak with 4 per cent of untreated cells in sub G_1 peak (Fig 4.59). This not only shows the apoptosis inducing ability of INF1 but also proves the concentration dependent increase in the population of cells undergoing apoptosis. These results are in good agreement with Ko *et al.* (2004) who reported that the treatment with 80 per cent ethanol extract of roots of *Pharbitis nil* induced apoptotic cell death of AGS gastric cancer cell line as revealed by FACS analysis and Annexin V staining.

Death receptor pathway and mitochondrial pathway are the two major modes of programmed cell death. Mitochondria play a pivotal role in intrinsic apoptotic pathway. Mitochondrial dysfunction within the apoptotic process is often associated with loss of mitochondrial transmembrane potential. In the present studies, HL-60 cells treated with 50 µg/ml of ARRE, ARRF2 and ARRF3, showed 15, 30 and 39 per cent loss of mitochondrial membrane potential, respectively (Fig. 4.60) while untreated cells exhibited 2 per cent loss of mitochondrial membrane potential, indicating the involvement of intrinsic pathway in apoptosis induced by ARRE, ARRF2 and ARRF3. Sun *et al.* (2004) also reported decreased expression of Bcl-2 in *Tribulus terrestris* saponin treated BEL-7402 cells and thus the involvement of mitochondrial pathway in causing apoptotic death of cells.

In case of INF1, cells treated with 3 and 5 µg/ml of INF1 showed 20 and 31 per cent loss of mitochondrial membrane potential while in untreated cells only 6 per cent of mitochondria exhibited loss of membrane potential (Fig. 4.61) which indicated the involvement of mitochondrial pathway in apoptotic cell death caused by INF1. These

results agree well with Ko *et al.* (2004) who found that the treatment with 80 per cent ethanol extract of roots of *Pharbitis nil* increased the expression of apoptosis related Bax and cleavage of active caspase-3 protein in AGS gastric cancer cell line. They also confirmed the translocation of Bax to mitochondria.

Results of the present investigation thus prove dose-dependent apoptosis inducing ability of ARRF3 and INF1 characterised by membrane blebbing, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, increase in sub G_1 population and loss of mitochondrial membrane potential. It can be concluded from these studies that both, ARRF3 and INF1 induce apoptosis through intrinsic or mitochondrial-dependent apoptotic pathway in HL-60 cells. Present studies confirm the pro-apoptotic nature of both the isolates prospecting them for developing into potential novel anticancer therapeutics.

Toxicity of active isolates

Myelosuppression is the most common dose-limiting toxicity of anti-tumor agents as they inhibit stem and progenitor cell proliferation. Treatment regimens depending on dose escalation have a devastating effect on bone marrow (BM) cells. Progenitor cells have shorter duplication times than stem cells and therefore, these are more sensitive to chemotherapy-induced damage. As neutrophil leukocytes have the shortest half-life in circulation, their continuous renewal is essential to prevent the sequel of chemotherapeutic agent-induced neutropenia, infection and death (Benko *et al.*, 2003).

In the present investigation, all the isolates were studied for their myelosuppressive effects in Sarcoma-180 (solid) bearing mice. Normal untreated animals were found to have $11.64 \pm 0.82 \times 10^6$ cells per femur whereas normal saline treated Sarcoma-180 (solid) bearing mice showed 20.53 per cent myelosuppression on day 13 (Table 4.31). These figures corroborate well with those observed by Ghosh *et al.* (1999), who reported 20–25 per cent decrease in marrow cellularity of Ehrlich ascites carcinoma (EAC), Sarcoma-180 (S-180) (ascites) and Dalton's ascitic lymphoma (DAL) bearing animals on day 9. Treatment with 5-FU (22 mg/kg) resulted in significantly less ($p<0.05$) number of cell per femur with 44.41 per cent myelosuppression. Myelosuppression of similar magnitude (41 per cent) has been reported by Benko *et al.* (2003) on single intraperitoneal injection of 5-Fluorouracil (100 mg/kg) in BDF1 female mice.

ARRE, ARRF2 and ARRF3 administered for nine consecutive days in the same tumor bearing animals at 120, 100 and 80 mg/kg dose levels yielded 8.93 ± 1.65 x 10^6, 9.93 ± 1.62 x 10^6 and 9.88 ± 1.65 x 10^6 cells per femur, respectively. Statistically, the numbers of cells per femur in treated animals were not significantly different from that of normal untreated animals (Table 4.31). However, treatments with INE (3 mg/kg) and INF1 (2 mg/kg) exhibited 51.80 and 47.93 per cent myelosuppression, respectively. Numbers of cells per femur in animals treated with INE and INF1 were significantly less ($p<0.01$) than normal untreated animals. AILE and AILF2 at 250 mg/kg also showed 27.74 and 19.15 per cent myelosuppression, respectively. No similar studies on roots of *Asparagus racemosus*, aerial parts of *Asparagus racemosus* and leaves of *Azadirachta india* could be traced in the available literature.

Overall, the *in vitro* cytotoxic activity of AILE and AILF2 corresponded with the *in vivo* anticancer activity of these isolates against murine cancer models. However, *in vivo* studies demonstrated their anticancer activity to be far less than significant. It is therefore concluded that AILE and AILF2 in their present form do not possess significant potential for their development as anticancer drugs. However, further studies on individual compounds present in AILF2 with respect to their bioavailability patterns, biotransformation, pharmacokinetic profile and anticancer activity against various cancers may yield better insight into the anticancer potential of *Azadirachta india* leaves and should be taken up.

The cytotoxic activity of ARRF2 and ARRF3 was far superior than ARRE and same was reflected in the *in vivo* anticancer activity of these agents. This was further proved by the higher percentage of HL-60 cells getting arrested in sub G_1 phase of the cell cycle on treatment with ARRF3. Electron microscopic studies also revealed changes typical of apoptosis in HL-60 cells treated with ARRF3 and EAC cells and solid Sarcoma-180 tissues from animals treated with ARRF3. Interestingly, ARRF3 did not produce significant myelosuppression in Sarcoma-180 (solid) bearing animals. ARRF3 thus seems to be a perfect candidate to be taken up for the isolation of active principles contained in it and to further develop the same into novel anticancer therapeutic(s).

Under *in vitro* studies, INE and INF1 both showed an impressive cytotoxicity against all the cell lines studied with enrichment of cytotoxic activity in INF1. *In vivo* studies

revealed the presence of significantly higher anticancer activity in INF1 at very minute doses which at times exceeded the positive control used in the study. This was further supported by the dose dependent increase in the arrest of HL-60 cells in sub G_1 phase of the cell cycle by INF1. Electron microscopic studies also supported the apoptosis inducing potential of INF1. However, toxic effects of INF1 evaluated in Sarcoma-180 (solid) bearing animals showed that INF1 produced severe myelosuppression in treated animals. Myelosuppression of similar magnitude is common with most of the anticancer drugs including the positive control used in the study. Thus, INF1 also seems to possess significantly higher potential of a novel anticancer therapeutic.

Thus, the study has lead us to the identification of n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) and n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) as isolates that possess significant potential for the treatment and management of cancer (with the myelosuppression risk associated with INF1) and proposes them for their further development as novel anticancer drugs that may prove to be clinically useful.

Chapter VI

Summary and Conclusions

CHAPTER - VI

SUMMARY AND CONCLUSIONS

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Defining feature of cancer is the rapid creation of abnormal cells that avoid natural process of cell death (apoptosis). The incidence of cancer rises dramatically with age. Among domestic animals, cancer is the leading cause of overall deaths in dogs, likely attributable to the lack of significant coronary disease in the species. The most common cancers in dogs are mammary tumors, skin cancer, oral cancer, lymphoma and hemangiosarcoma. The most common cancers in cats are lymphoma, skin/connective tissue and mammary tumors.

In human beings, cancer has been recognized as a major health problem globally, ranking second only to heart disease, both in more developed and developing countries. It is a more common phenomenon nowadays than previously, in large extent due to the growth of the world's population and the relatively advanced age to which people now live, since it is a disease that is more common in elderly ages than in younger ages. It is estimated that at present, about 26 million people are living with cancer worldwide and 13 per cent of all deaths are caused by cancer. That's more than the percentage of deaths caused by HIV/AIDS, tuberculosis and malaria put together. Cancer is thus a public health problem of serious concern worldwide. It could be expected that by 2030 there will be approximately 26.4 million incident cases of cancer and 17.0 million cancer deaths a year.

The rising global cancer burden in people and animals makes the present day approaches to cure / control / manage cancer in patients quite far from adequate. The world is therefore in dire need of safer therapeutic agents that will control the growth of tumor without much harm to normal cells.

Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. They can be regarded as chemical libraries of structurally diverse compounds that constitute a promising approach in drug discovery. The discovery and development of efficacious anticancer agents, such as vinblastine and vincristine isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don, provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents. The fact that thus far only a relative handful of medicinal plants have been evaluated for their anti-cancer potential, holds promise for the identification of more anticancer agents from plants.

With this view, the present studies were planned to investigate the anticancer potential of following medicinal plants which are traditionally used by the folklore for various ailments including cancer.

- *Azadirachta indica* A. Juss. (Neem; family: *Balsaminaceae*)
- *Asparagus racemosus* Willd. (Shatavari; family: *Asparagaceae*)
- *Ipomoea nil* L. Roth (Japanese Morning Glory; family: *Convolvulaceae*)
- *Costus speciosus* Koen ex. Retz. (Crape ginger, family: *Costaceae*)
- *Butea monosperma* (Lam.) Taub. (Palas, family: *Fabaceae*)

The objectives of the present investigation were as follows:

1. To investigate *in vitro* cytotoxic potential of *Azadirachta indica* (leaves), *Butea monosperma* (flowers), *Asparagus racemosus* (roots), *Ipomoea nil* (aerial parts) and *Costus speciosus* (aerial parts) against ten cancer cell lines of human and/or animal origin and to identify the most promising plant extracts.
2. To fractionate the promising plant extracts to identify active isolate(s) and to enrich the cytotoxic activity.
3. To determine *in vivo* anticancer activity of the active isolate(s) in various animal models in order to evaluate their therapeutic potential.

4. To evaluate the active isolate(s) for their myelosuppressive effects in tumor bearing animals.
5. To determine the apoptotic potential of the active isolate(s) using light microscopy, electron microscopy and flow-cytometric techniques.

The leaves of *Azadirachta indica,* roots of *Asparagus racemosus,* flowers of *Butea monosperma* and aerial parts *of Ipomoea nil* and *Costus speciosus* were collected from Jammu and its surrounding areas after authentication by the taxonomist at the site of collection. Collected plant parts were chopped, dried under shade and extracted with 95 per cent ethanol, 50 per cent ethanol and water using repeated solvent extraction procedure to prepare alcoholic, hydro-alcoholic and aqueous extracts. All the extracts were evaluated for their cytotoxic potential against a panel of twelve cancer cell lines of different tissue origin (viz., Colo-205, 502713, HCT-15 and SW-620 from colon, SK-N-SH from CNS, Du-145 from prostate, A-549 from lungs, MCF-7 from breast, OVCAR-5 from ovary, HEP-2 from liver, IMR-32 from neuroblastoma and D17 from bone) at three different concentrations (10, 30 and 100 μg/ml) using Sulphorhodamine B assay. More than 30, 50 and 70 per cent growth inhibition of cancer cells in culture at 10, 30 and 100 μg/ml concentration of extracts respectively, was considered promising cytotoxic activity. Promising extracts identified on the basis of *in vitro* cytotoxicity were further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and all of them were subjected to cytotoxicity evaluation against the same panel of twelve cancer cell lines at 10, 30 and 50 μg/ml concentrations. The growth inhibition of 30, 40 and 50 per cent at these concentrations respectively, was considered promising cytotoxic activity. Extracts and fractions showing promising cytotoxic activity against more than 50 per cent of the cell lines studied were taken up for further investigation with respect to their *in vivo* anticancer activity against murine tumor models.

Out of three extracts of leaves of *Azadirachta indica* tested, only the alcoholic extract showed promising cytotoxicity in more than 50 percent of the cell lines studied at 30 and 100 μg/ml concentrations. It was further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions and all the fractions were again evaluated for their cytotoxic activity against the same panel of twelve cancer cell lines at 10, 30 and 50 μg/ml concentrations. Out of these four fractions, only the chloroform fraction showed promising cytotoxic activity against eight cell lines at 50 μg/ml concentration. Other

fractions had either poor or negligible cytotoxic activity against most of the cell lines. Therefore, the alcoholic extract of *Azadirachta indica* leaves and its chloroform fraction were considered to be of promise and selected for further investigations *in vivo* against murine cancer models.

The alcoholic extract of *Asparagus racemosus* roots exhibited promising cytototoxicity against five, ten and nine cell lines at 10, 30 and 100 μg/ml concentrations, respectively. Hydro-alcoholic extract also showed good cytotoxic activity but it was less active than that of alcoholic extract. Aqueous extract however showed poor activity against all the cell lines studied. This study revealed that out of the three extracts, most of the cytotoxic activity was confined in alcoholic extract of *Asparagus racemosus* roots and therefore, it was studied further by fractionating it into n-hexane, chloroform, n-butanol and aqueous fractions. Out of the four fractions from alcoholic extract of *Asparagus racemosus* roots, n-butanol fraction was found to be the most promising and enriched with excellent cytotoxicity. Chloroform fraction also possessed good cytotoxic activity against little less number of cell lines than that of n-butanol fraction. Therefore, the alcoholic extract of *Asparagus racemosus* roots and its chloroform and n-butanol fractions were selected for further *in vivo* investigations against murine cancer models.

An impressive cytotoxicity was exhibited by alcoholic extract of *Ipomoea nil* aerial parts. It promisingly inhibited the growth of seven and ten cell lines at 10 and 30μg/ml concentrations, respectively. At 100 μg/ml, the extract exhibited promising cytotoxic activity against all the twelve cancer cell lines studied. Hydro-alcoholic and aqueous extracts had moderate and poor activities, respectively. Therefore, the alcoholic extract was considered to hold definite promise and was further fractionated into n-hexane, chloroform, n-butanol and aqueous fractions. Out of these fractions, n-hexane fraction emerged to be the most cytotoxic one, which significantly inhibited the growth of all the studied cell lines at all the three (10, 30 and 50 μg/ml) concentrations. Chloroform fraction also exhibited good cytotoxicity but it was little less than the n-hexane fraction. n-Butanol and aqueous fractions however did not show good cytotoxicity. Thus, n-hexane fraction was found to hold significant promise as far as anticancer activity is concerned. Therefore, the alcoholic extract of *Ipomoea nil* aerial parts and its n-hexane fraction were selected for further *in vivo* studies against murine cancer models.

None of the extracts prepared from aerial parts of *Costus speciosus* exhibited promising cytotoxic activity at any of the concentrations studied. Likewise, negligible cytotoxic activity was exhibited by all the extracts of *Butea monosperma* flowers at the studied concentrations. It was therefore considered that the aerial parts of *Costus speciosus* and flowers of *Butea monosperma* are without any promise as far as anticancer activity is concerned and so they were not investigated further.

Based on the cytotoxic activity, alcoholic extract of *Azadirachta indica* leaves and its chloroform fraction, alcoholic extract of *Asparagus racemosus* roots and its chloroform and n-butanol fractions and alcoholic extract of *Ipomoea nil* aerial parts and its n-hexane fraction were selected for further investigation with respect to their *in vivo* anticancer activity against seven murine cancer modes viz., Ehrlich ascites carcinoma, Sarcoma-180 (ascites), Methyl cholanthrene induced ascites, Ehrlich tumor (solid), Sarcoma-180 (solid), L1210 Lymphoid leukemia and P388 Lymphocytic leukemia.

Anticancer activity of alcoholic extract of *Azadirachta indica* leaves (AILE) and its chloroform fraction (AILF2) was evaluated at 100, 150, 200 and 250 mg/kg (i.p.) dose levels against all the above models. While AILE showed statistically significant growth inhibition (34.33 and 55.56 per cent) of Ehrlich ascites carcinoma (EAC) only at 200 and 250 mg/kg doses respectively, AILF2 showed significant growth inhibition of EAC at all the four dose levels (31.51, 36.61, 37.33 and 50.36 per cent, respectively). In case of Sarcoma-180 (ascites), growth inhibition in groups treated with 100, 150, 200 and 250 mg/kg doses of AILF2 was little higher than similar doses of AILE. However, the growth inhibition by both (AILE & AILF2) was not significant statistically. Methyl cholanthrene induced ascites was inhibited to the extent of -13.91, -3.85, 5.64 and 8.17 per cent by 100, 150, 200 and 250 mg/kg doses of AILE respectively, whereas respective figures for AILF2 were 10.95, 15.99, 16.64 and 18.54 per cent. Thus, increased activity was observed with AILF2 over AILE at similar doses but it was statistically non-significant.

Only the 250 mg/kg dose of AILE exhibited significant anticancer activity against Ehrlich tumor (solid) whereas AILF2 significantly inhibited the growth of this tumor at 200 and 250 mg/kg doses (28.74 and 40.05 per cent, respectively). While no significant anticancer activity was exhibited by any of the doses of AILE against Sarcoma-180

(solid), only marginal anticancer activity was displayed by AILF2 that too, at highest dose of 250 mg/kg.

Anticancer activity of AILE and AILF2 against L1210 Lymphoid leukemia and P388 Lymphocytic leukemia was studied in terms of per cent increase in the life span of treated animals over tumor bearing control animals. None of the doses of AILE and AILF2 displayed any significant increase in the life span of treated animals. On the contrary, higher dose (250 mg/kg) of both caused significant toxicity to L1210 Lymphoid leukemia bearing animals.

Alcoholic extract of *Asparagus racemosus* roots (ARRE) and its chloroform (ARRF2) and n-butanol (ARRF3) fractions were evaluated for their *in vivo* anticancer activity at 80, 100, 120 and 140 mg/kg dose levels against all the models. ARRE at 120 and 140 mg/kg doses showed significantly higher growth inhibition of Ehrlich ascites carcinoma (EAC). ARRF2 displayed higher levels of anticancer activity against EAC than ARRE at all doses and ARRF3 showed significant tumor growth inhibition at all doses. However, a dose of 140 mg/kg of ARRF2 and ARRF3 proved toxic to animals. Exactly similar pattern of activity was displayed by ARRE, ARRF2 and ARRF3 in case of Sarcoma-180 (ascites) also. However, Methyl cholanthrene induced ascites appeared more sensitive to ARRE, ARRF2 and ARRF3 treatments as all doses of these isolates produced significant tumor growth inhibition accompanied by toxicity at 140 mg/kg dose.

In case of Ehrlich tumor (solid), all doses of ARRE, ARRF2 and ARRF3 exhibited significant growth inhibition except 140 mg/kg dose of ARRF3 which proved toxic to animals bearing this tumor. By and large, similar was the activity of these isolates against Sarcoma-180 (solid) too. However, none of these agents produced any significant increase in the life span of L1210 Lymphoid leukemia bearing animals. On the contrary, higher doses of ARRF2 and ARRF3 shortened the life span of treated animals thereby indicating toxicity. Nonetheless, higher doses (120 and 140 mg/kg) of ARRE exhibited significant increase in the life span (51.0 and 82.76 per cent, respectively) of P388 Lymphocytic leukemia bearing animals as also the lower doses (80 and 100 mg/kg) of ARRF3 (68.0 and 151.09 per cent, respectively).

2, 3 and 4 mg/kg doses of alcoholic extract of *Ipomoea nil* aerial parts (INE) and 0.5, 1 and 2 mg/kg doses of its n-hexane fraction (INF1) were used to investigate their anticancer potential against all murine tumor models. All doses of INE and INF1 produced significant and dose dependent inhibition in the growth of EAC. Exactly similar patterns of activity were found in case of Sarcoma-180 too, except that the highest doses of INE and INF1 both tended to be toxic. Methyl cholanthrene induced ascites was also inhibited significantly and dose dependently by all doses of INE and INF. In case of solid tumors (Ehrlich and Sarcoma-180) also, significantly high and dose dependent reduction in the tumor weights of treated animals was observed with all doses of INE and INF1. Tumor growth inhibition by highest doses of both was comparable to the anticancer efficacy of the positive control (5-FU) used in the study but was accompanied by little bit toxicity to animals.

Lower dose of INE and INF1 did not produce significant increase in the life span of animals bearing L1210 Lymphoid leukemia. Increasing doses of both progressively shortened the life span of animals thereby showing negative increase in life span. In case of P388 Lymphocytic leukemia, all doses of INE shortened the life span of tumor bearing animals. Substantial toxicity was noticed also with increasing doses of INF1.

Results of *in vivo* anticancer activity demonstrated that the n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots (ARRF3) and n-hexane fraction from alcoholic extract of *Ipomoea nil* aerial parts (INF1) were enriched with excellent anticancer activity and emerged to be the most active isolates with definite potential for new anticancer therapeutics. Therefore, these isolates were investigated further for their ability to induce apoptosis in cancer cells employing flow-cytometry, light microscopy and scanning and transmission electron microscopy techniques.

Cell cycle analysis of HL-60 cells treated with 50 µg/ml concentrations of ARRE, ARRF2 and ARRF3 showed 18, 19 and 30 per cent of cells in sub-G_1 phase. These treatments also resulted in 15, 30 and 39 per cent of cells losing mitochondrial membrane potential, respectively. Treatment with 3 and 5 µg/ml concentrations of INF1 resulted in 14 and 22 per cent of cells getting arrested in sub-G_1 phase and 20 and 31 per cent loss of mitochondrial membrane potential, respectively. These studies thus proved the enrichment of apoptosis inducing ability in ARRF3 and dose dependent increase in

apoptotic potential of INF1 and suggested the occurrence of apoptosis through mitochondrial pathway.

Scanning electron microscopy of HL-60 cells treated with 25 µg/ml of ARRF3 and 10 µg/ml of INF1 in culture and EAC cells collected from the ascitic fluid of animals treated with 80 mg/kg of ARRF3 and 2 mg/kg of INF1 revealed typical morphological changes suggestive of apoptosis such as cell shrinkage, smoothening of cell surface, blebbing of the plasma membrane and formation of apoptotic bodies. Likewise, light microscopy of tumor tissue from Sarcoma-180 (solid) bearing animals treated with 80 mg/kg of ARRF3 and 2 mg/kg of INF1 showed shrinkage of cells, vacuolization, condensation of chromatin, fragmentation of nucleus and apoptotic bodies. Transmission electron microscopy of the same tissue revealed changes typical of apoptosis like marginalization of chromatin, fragmentation of nucleus and loss of mitochondrial cristae.

All extracts and fractions studied for *in vivo* anticancer activity were also evaluated for their toxic effects with reference to myelosuppression in Sarcoma-180 (solid) bearing mice. Animals treated with 250 mg/kg of AILE and AILF2 showed 27.74 and 19.15 per cent myelosuppression whereas 23.28, 14.89 and 15.12 per cent myelosuppression was noticed in groups treated with 120, 100 and 80 mg/kg of ARRE, ARRF2 and ARRF3, respectively. Highest myelosuppression (51.80 and 47.93 per cent) was recorded in animals treated with INE (3 mg/kg) and INF1 (2 mg/kg), respectively.

Conclusions:

The present studies have lead us to derive following conclusions.

1. That the n-butanol fraction from alcoholic extract of *Asparagus racemosus* roots has excellent anticancer activity as evidenced by its promising *in vitro* cytotoxicity, highly significant *in vivo* anticancer activity against most of the tumor models and pro-apoptotic potential established in flow-cytometric and microscopic studies. Its non-significant effect on the bone marrow cellularity further makes it an attractive candidate for anticancer drug development.

2. That the presence excellent anticancer activity in n-hexane fraction from alcoholic extract of *Ipomoea nil* is supported by its cytotoxic potential against cancer cell

lines, exceedingly high tumor growth inhibition at doses as low as 1 and 2 mg/kg and pro-apoptotic potential established in flow-cytometric and microscopic studies. However, it is accompanied by severe myelosuppressive effects.

3. That the overall anticancer activity of alcoholic extract of *Azadirachta indica* leaves and its chloroform fraction varies from poor to moderate. However, little enrichment of anticancer activity in chloroform fraction demands individual compounds present in it to be studied for their anticancer activity so that better insight into the anticancer potential of *Azadirachta india* leaves is obtained.

4. That the aerial parts of *Costus speciosus* and flowers of *Butea monosperma* did not exhibit significant cytotoxic potential *in vitro* against any of the cancer cell lines studied and therefore are likely to be devoid of any anticancer activity.

5. Further studies are indicated to isolate and characterize the active principles from n-butanol fraction of alcoholic extract of *Asparagus racemosus* roots and n-hexane fraction of alcoholic extract of *Ipomoea nil* and to develop them into clinically active anticancer drugs.

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