INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

By

JOHN RAPHAEL T. M.Sc.

AMALA CANCER RESEARCH CENTRE
THRISSUR - 680 555, KERALA, INDIA

APRIL - 2006

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DECLARATION

I, John Raphael T., hereby declare that this thesis has not previously formed the basis of the award of any degree or diploma or other titles of any other University.

Thrissur

19- 4 -2006

Signature of the Candidate

JOHN RAPHAEL T.

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CERTIFICATE

This is to certify that the present report is an authentic account of the work carried out by Mr. John Raphael T., under my supervision and guidance and no part thereof has been presented before for any other degree.

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19 -4 -2006

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DEDICATED TO MY GUIDE

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INTRODUCTION

Cancer is one of the most dreaded disease of the 20th century and spreading further with continuance and increasing incidence in 21st century. World Health Organization in its 1990 report stated that cancer occupied the second position in the list of killer diseases in industrially advanced countries. A combination of life style, exposure to environmental carcinogens and the overall balance between inherited resistance and sensitivity genes is likely to determine the susceptibility of an individual to cancer. Invasion and metastasis are the most insidious and life-threatening aspects of cancer (Sporn, 1996).

Tumorigenesis associated with metastasis formation accounts for 90% of cancer deaths, and represents one of the prime causes of human mortality (Weiss, 1985; Mareel et al., 1993; Sporn, 1996). In general, cancer arises from the stepwise accumulation of genetic changes and the progressive alterations in gene expression, disengaging cells from homeostatic constraints that normally keep the tissue balance in the healthy adult. Several defined events have been described as common to cancer cells including self-sufficiency in proliferation signals, insensitivity to growth inhibitory signals, evasion from cell death, limitless replicative potential, aberrant angiogenesis, as well as tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Metastatic disease is the hallmark of malignant cancer. Metastasis is the spread of cancer cells from a primary tumour to vital organs and distant sites in the cancer patient's body. The design of more effective therapies to treat metastatic cancer requires better understanding of the molecular events and cellular processes that are involved in the process of metastatic formation. Successful formation of metastatic foci consists of several highly complex and interdependent steps. These include separation from the

primary site, circulation through blood or lymph, adhesion to the basement membrane, invasion and proliferation at distant sites (Fidler et al., 1974). Since each step is rate limiting, failure to complete any of these events completely disrupts metastasis formation. Any drug, which can interrupt any of these steps in the cascade, will be useful in the inhibition of tumour metastasis.

Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into world medicine. Modern researches aim to eradicate the death and suffering from cancer, to cure cancer once it starts and ultimately to prevent cancer. Therefore high priority is given to research promoting continuous development of sophisticated molecular technologies and clinical application of these technologies to prevention, diagnosis and treatment of cancer (Boder, 1993).

Most of the cytoreductive cancer chemotherapeutic agents are highly toxic towards normal cells and hence the drug dose which can be used for cancer therapy is limited. Side effects such as nausea, vomiting, mucosal ulceration, interstitial pulmonary felorious, hepatic toxicity, lympocytopenia and alopecia (Belli et al., 1967; Glick et al., 1982) and other symptoms act as discoursing factors for patients subjected to such therapy. Higher doses of administration of chemotherapeutical agents produce severe urotoxicity with haemorrhagic cystitis on urinary bladder (Hutter et al., 1973). Radiotherapy is one of the widely accepted theraputical approaches in cancer. However whole body radiation with a dose of more than 100 rads cause acute effects including hematopoietic syndrome and gastrointestinal syndromes, which involves nausea, vomiting, diarrhea, decreased count of various blood elements such as red blood cells,

granulocytes, lymphocytes and platelets (Manuch et al., 1995). Unfortunately there is no specific drug at present, which can effectively reduce these effects. Therefore it is imperative to look for less toxic but effective therapeutics. Development of effective and nontoxic cancer therapeutics are essential.

Natural product research continues to explore a variety of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry. In recent years many natural compounds derived from plants and or crude plant extracts have been proved to have protective effect against toxic effects of many chemicals and to combat a variety of ailments. Plants have a long history of use in the treatment of cancer (Pettit et al., 1994). The need to find a safe and highly effective cure for neoplastic diseases remains a major challenge for modern medicine. A wide variety of natural compounds appear to possess significant cytotoxic as well as chemopreventive activity. Extracts of plants used in traditional medicine also have a similar property. Many more screening studies are necessary using plant extracts and compounds isolated from them. Naturally occurring compounds that are included in the diet are non-toxic and may partially regulate programmed cell death in several tissues and organs. Elaborate studies with such compounds with respect to their abilities to inhibit metastatic tumour progression and understanding their mechanism of action may provide valuable information for their possible application in cancer therapy. Flavones, flavanols, isoflavones, catechins and tannins present in many plants have also been shown to possess anti cancer activities. Furthermore some of the herbal medicines and their constituents have been reported to inhibit metastatic tumour progression (Leyon and Kuttan, 2005).

The possibility that cancers viewed by immune system as non-self was postulated by Burnet in 1957 (Burnet, 1957). The concept of immune surveillance states that a physiological function of immune system is to recognize and destroy the clones of transferred cell before they grow into tumours and to kill tumours after they are formed. Evidence indicates that the healthy immune system is necessary for control of malignant disease and that immune suppression associated with cancer contributes to its progression. Tumours have developed strategies to successfully evade the host immune system, and various molecular and cellular mechanisms responsible for tumour evasion have been identified (Theresa and Whiteside, 2006). The fate of the host- tumour interactions depends on the balance between the intrinsic metastatic potential of the tumour and strength of the host immune response (Cooper et al., 2001). Therapeutic strategies are being designed to correct the immune imbalance, deliver adequate *in vivo* stimulation, transfer effector T cells capable of *in vivo* expansion and provide protection for the immune effector cells re-populating in the host. Survival of these cells and long-term memory development in patients with malignancy are necessary for improving clinical benefits of cancer immunotherapies (Theresa and Whiteside, 2006).

Immunomodulation is another type of the immuno-therapeutic modalities in clinical immunology. It may be defined as the augmentation of the host nonspecific or specific immune response through the use of a wide variety of biologically active substances. Immunomodulatory responses of an organism acts by interfering with its regulatory mechanism. These responses are antigen independent and may directly induce production of mediators and effector molecules by the immuno competent cell. The

primary target of the immunomodulatory compound is believed to be macrophages, which play a key role in the generation of immune response.

Terpenoids are the class of compounds widely distributed in plant kingdom (Steinmetz and Potter, 1991). Many of them have shown to inhibit chemically induced tumours (Tanaka et al., 2000). Although terpenoids are widely used for medicinal purpose in many Asian countries, their biogenesis and pleiotropic actions has not impacted on the practice of western medicines (Nanjoo et al., 1998).

Present study was aimed to investigate the immunomodulatory and antimetastatic activity of some of the naturally occurring terpenoids using *in vitro* and *in vivo* models. Antioxidant, antitumour, radioprotective and chemo protective activity of these terpenoids were also analysed in the present study.

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CHAPTER 1

REVIEW OF LITERATURE

Cancer is a term used to represent a group of more than 100 types of diseases involving any of the organs in the body. It is recognized by the behavior of a population of abnormal cells within a normal tissue exhibiting a diverse set of phenotypic abnormality including loss of differentiation, increased motility, invasiveness and decreased drug sensitivity (Bishop, 1991). These phenotypic abnormalities arise from a stepwise accumulation of genetic changes that liberates neoplastic cells from homeostatic mechanism that govern normal cell proliferation (Bishop, 1991). The presence of multiple genetic alterations in human cancers strongly indicated that alterations accumulate during tumor progression. Comparative analysis of these genetic alterations in early and late stage tumors led to the hypothesis of a multistage carcinogenesis in colorectal cancer progression which now is a widely accepted genetic tumor progression model (Kinzler and Vogelstein, 1996)

Transformation of normal cell into cancerous cell is referred to as oncogensis. It involves several steps. An initiator that generally transforms a normal cell to cancer cell whereas promoter triggers changes in gene expression. Some genetic oncogenic changes contribute to uncontrolled growth or loss of senescence. Some oncogenes cause uncontrolled growth by activating persistent growth stimulatory signal transduction pathways and also by altering critical nodes in the cell cycle. Uncontrolled growth can be caused by deregulation at the level of DNA transcription factors. After the initial neoplastic transformation the tumour cell undergoes progressive proliferation which is accompanied by further genetic changes resulting in generation of heterogeneous tumour cell population with varying degree of metastatic potential (Shereen Keleg et al., 2003)

I CONVENTIONAL TREATMENT MODALITIES

Surgery, chemotherapy and radiotherapy are the most established strategies used to eradicate cancer cell in patient's body. Physical removal of cancer mass is the foundation of surgery. Radiotherapy and chemotherapy use exposure to toxic ionizing radiations and cytotoxic chemicals respectively to destroy cancer cells without finding and remove them.

I.I Chemotherapy

Cancer chemotherapy had its roots in the work of Paul Ehrlich who coined the term chemotherapy. Modern chemotherapy might have started on 1948 with the introduction of nitrogen mustard (Goodman et al., 1946). Most chemotherapeutic agents currently in use appear to exert their effect primarily on the cell multiplication and tumour growth. Because cell multiplication is characteristic of normal cells as well as cancer cells, most of the chemotherapeutic agents also have toxic effect on normal cells (DeVita, 1991). Inhibition of cell multiplication and tumour growth can take at several levels within the cells such as 1) macromolecular synthesis and function 2) cytoplasmic organization and 3) cell membrane synthesis and function.

Currently chemotherapy has a role in four different clinical setting (DeVita, 1991) as an induction treatment for advanced disease, as an adjuvant to local methods of treatment, as the primary treatment for some patients who present with localized disease in whom local forms of therapy by themselves are inadequate and by direct instillation into sanctuary sites or by site-directed perfusion of specific regions of the body directly affected by the cancer.

The term induction chemotherapy has been used to describe drug therapy given as the primary treatment for patients who present with advanced cancer for which no alternative treatment exists (Holland, 1986).

Adjuvant chemotherapy denotes the use of systemic treatment after the primary tumour has been controlled by an alternative modality such as surgery and radiation therapy (Cokgor et al., 1999). The selection of an adjuvant treatment program for a particular patient usually based on response rates in separate groups of patients with advanced cancers of the same histologic type. Determination of a population of patients as suitable for adjuvant treatment is based on available data about their average risk of recurrence after local treatment alone. Currently adjuvant chemotherapy is considered standard treatment for early stage breast and colorectal cancer (Fuchs and Mayer, 1995).

Use of chemotherapy as the initial treatment for patients who present with localized cancer denotes neo-adjuvant chemotherapy. For chemotherapy to be used as the initial treatment of a cancer partially curable by either surgery or radiation therapy, there must be considerable evidence for the effectiveness of drug program in question against advanced disease of same type. At this time neo-adjuvant therapy has been effectively used in the treatment of anal cancer, bladder cancer, breast cancer etc. (Goldie, 1987; Bonadonna et al., 1990; Jacobs, 1991).

Combination chemotherapy using conventional cytotoxic agents accomplishes several important objectives which not possible with single-agent treatment. Standard single drugs at clinically tolerable doses have been unable to cure cancer (Potter, 1951; Nathanson et al., 1969). In the early years of cancer chemotherapy drug combinations were developed based on known biochemical actions of available anticancer drug rather

than on their clinical efficiency .The era of effective combination chemotherapy began when an array of active drugs from different doses became available for use in combination with for treatment of leukemias and lymphomas (Norton and Day, 1991).

Combination chemotherapy using conventional cytotoxic agents has some advantages over the single agent treatment. It provides maximal cell kill within the range of toxicity tolerated by the host for each drug as long as dosing is not compromised. The combination chemotherapy provides a broader range of interaction between drug and tumour cells with different genetic abnormality in heterogeneous tumour populations (Norton and Day, 1991).

Chemotherapeutical drugs cause many side effects like bone marrow suppression, mucositis and hair loss (Smith et al., 1996). Some of them cause cumulative dose-dependent toxicities to slowly- proliferating or non-proliferating normal tissues like kidney, liver, nervous system and heart. Cytoxicity results when exposed cells attempt cell division before repairing the critical damage. The goal of cytoreductive therapy is often stated as reducing the surviving number of malignant cells to less than one. Since chemotherapy often produces severe toxic side effects, the drug dose that can be administered is determined by the patient tolerance rather than by the therapeutic advantage. Moreover, high cost of chemotherapy reduces the accessibility of this treatment to poor patients. Chemotherapy in fact usually fails in its goal of totally eradicating tumor cells and sparing normal cells.

I.2 Surgery

A surgical procedure used to physically remove malignant tissue is the most effective modality for treating localized disease. There are reports of higher probability of

surgical cure in early stages. Adjacent healthy tissue is also usually removed to provide a surgical 'margin' between the diseased and healthy tissues. This area is used by the pathologists to assess whether the tumour is invading the adjacent tissue or not. Local lymph nodes may also be removed to estimate a probability of systemic disease.

Cancer surgery carries the risk of surgery in general, dangers of anesthesia, loss of hemostasis and infection (Dripps et al., 1988). Resurrection of brain stem tumours of the same size is a higher risk procedure in a tissue that cannot grow and morbidity can be much higher. Besides, experience of surgical team with the procedure can also be an important factor.

I.3 Radiotherapy

Radiotherapy is a very important tool in the fight against cancer. It is used in the treatment of as many as 50% of all cancer patients. More than half a million-cancer patients receive radiation therapy each year either alone or in conjugation with surgery chemotherapy or other forms of cancer therapy.

Radiation therapy may be used to treat almost all types of tumour (Kligerman, 1977). It is useful in cases where surgical removal of cancer is not possible or when surgery might debilitate the patients. Together with image guided treatment planning, radiation therapy is a powerful tool in the treatment of cancer, particularly when the cancer is detectable at early stage (Harris., et al 1984). Radiation therapy can be used following surgery to destroy any cancer cells that were not removed by surgery or prior to surgery to shrink a previously incorporable tumour to a manageable size to enable surgical excisions. Radiation can also be used to destroy any remaining cancer cells after

surgery (Hellman 1980; Harris., et al 1984). Chemotherapy and radiation therapy may also be used together to effectively treat the cancer.

Radiation therapy uses high energy X-rays (ionizing radiations) to stop cancer cells from dividing. During this therapy high-energy ionizing radiation deposit energy in the area being treated, damaging the genetic materials of cells and making it impossible for these cells to divide. Although, radiation damages both cancer cells and normal cells, the normal cells are usually able to repair them and function properly (Thompson and Suit, 1969). Like surgery radiation therapy is a local treatment as it affects only the cells in the treated area.

Radio-sensitizers and radio-protectors are chemicals that modify a cell response to radiation. Radio-sensitizers are drugs that make cancer cells more sensitive to the effect of radiation therapy. But radio protectors are those protect normal cells from the damage caused by radiation therapy. These agents promote the repair of normal cells that are exposed to radiation (Sweeney, 1979). Many of the radiosensitizers and protectors are limited in application because of toxic nature (Sweeney, 1979). The most common side effects are hair loss, tiredness, skin reactions, loss of appetite and nausea (Bloomer and Hellman, 1975). Radiotherapy may also cause a decrease in the number of white blood cells and inducing severe immunosuppression (Praveen Kumar et al., 1996; Jagetia et al., 2002).

II. CANCER INVASION AND METASTASIS

Invasion and metastasis are the most insidious and life threatening aspect of cancer and distinguishing feature of malignant cell (Hart, 1982; Fidler, 1973). Once a neoplasm become invasive it acquires the capacity to disseminate through lymphatic and vascular channels (Hart, 1982).

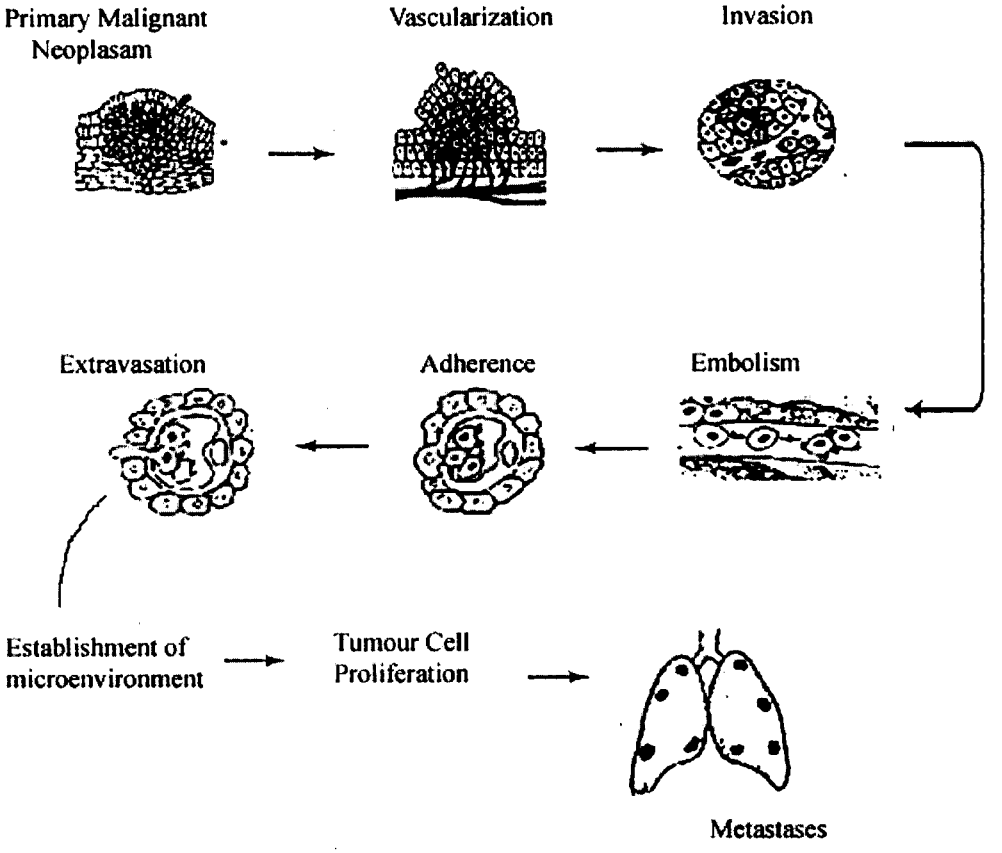
Metastasis is the spread of cancer cells from a primary tumour to vital organs and distant sites in the cancer patient's body. The distribution of metastasis varies widely depending on the histologic types and anatomic location of primary tumour. The most frequent organ location of distant metastasis in many types of cancer appears to be first capillary buds, encountered by the circulating cells (Terranova et al., 1986). On the other hand there are many metastatic sites that cannot be predicted on the basis of anatomic consideration alone but can be considered as an example of organ tropism (McCarthy et al.,1984; Terranova et al., 1986; Muller et al., 2001).

It is now well recognized that most neoplasms consists of several tumour cell populations, which vary, widely in several important biologic characteristics. These characteristics include growth rates, karyotype, production of growth factors and stimulators of angiogenesis, hormone production, susceptibility to cytotoxic agents, immune response and hypoxia (Fidler, 1973; Hart, 1982; Hanahan and Weinberg, 2000). It was logical to assume that the size of the aggressive subpopulation in primary tumour would reflect the propensity of that tumour to metastasis (Borsiget al., 2002). This assumption would be of prognostic significance if a clinical assay of primary tumour were able to detect and determine the presence of the highly aggressive subpopulation.

II .1 Metastatic cascade

The formation of a metastatic lesion is the result of a complicated, multistep process that is collectively referred to as the metastatic cascade(Poste and Fidler 1980). Formation of metastatic foci is a continuous process commencing early in the growth of primary tumours and it increase with time. The process of metastasis is a cascade of linked sequential steps involving multiple host tumour interactions(Fidler, 1973; Hanahan and Weinberg, 2000). By this scheme, the nutritional requirements of a growing tumor likely exceed the available blood supply, leading to establishment of a localized hypoxic state that in turn triggers an angiogenic switch (Hanahan and Folkman, 1996) (as described in Fig 1.1). The result is angiogenesis, a host response that facilitates neovascularization. As a consequence, and with continued tumor growth, some cells will lose expression of cell–cell adhesion molecules, such as E-cadherin, (Bussemakers and Schalken , 1996) and express matrix metalloproteinases (MMPs), such as MMP2,(Fang et al., 2000) which will allow them to detach from the primary tumor site and invade through the basement membrane. Subsequently, the invasive cells may enter the lymphatic or hematogenous circulation where they can be carried to distant sites. In order to avoid detection by the immune system, it is believed that metastatic cells form aggregates with other cells as well as with fibrin deposits and platelets (Albelda, 1993). Ultimately, the metastatic cells extravasate across the blood vessel endothelium and invade the extracellular matrix at the distant site. It is generally accepted that the metastatic cascade can only be complete if the microenvironment at the distant site is suitable for the establishment of a viable macroscopic metastatic lesion. (Fig 1.1)

Fig 1.1 Metastatic cascade



Experimental evidences show that these interactions are highly complicated. Large foundations of experimental work suggest that during each stage of the process, only the fittest tumour cells survive. A very small percentage of circulating tumour cells ultimately initiates successful metastatic colonies. Thus metastasis is a highly selective competition, favoring the survival of a minor subpopulation of metastatic tumour cells that present within the primary tumour (Glinsky et al., 2000).

II .1a Adhesion

The first step of basement membrane invasion requirement is tumour cell attachement. Intravital microscopy shows that initial arrest of cancer cells occurs primarily by size restriction in the capillaries (Chambers et al., 1992).Adhesion among endothelial cells is mediated by several major cell-cell adhesion molecules (CAMs) like members of the immunoglobulin and calcium-dependent cadherin families and integrins (Hanahan et al., 2000). Notably, all of these "adherence" interactions convey regulatory signals to the cell (Aplin et al., 1998). Changes in integrin and immunoglobulin superfamily expression are also evident in invasive and metastatic cancer cells (Skubitz, 2002; Maurer et al., 1998). The importance of integrins in tumor cell dissemination is their role in cellular adaptation to changing tissue microenvironments as found in metastatic organs. This is achieved through various permutations in the spectrum of the more than 22 integrin subtypes. Experimental changes in expression of integrin subunits in cultured cells induces or inhibits invasive and metastatic growth, which confers their role as central determinants of these processes.

II 1b Invasion

Tumor invasion encompasses the process of tumor cell penetration or infiltration into adjacent tissue. This event is also central and related to the development of metastasis. The process of invasion is not only a passive one due to pressure from excessive cellular proliferation but it is an active dynamic process that requires protein synthesis and degradation (Kleiner and Stetler-Stevenson, 1999). Tumour cell must traverse the extra cellular matrix in the process of invasion and must be able to either secrete or activate enzymes that can degrade the major compound of the matrix such as collagen, fibronection and proteoglycan.

Matrix proteolysis has been recognized as a key part of the mechanism of tumour cell invasion. A variety of proteases has been implicated in this process including matrix metalloproteinase, serine proteinase and cystein proteinase etc. The most important family of proteases are matrixin or matrix metallo proteinase.(Kleiner and Stetler-Stevenson, 1999)

II .1c Cell migration

Transendothelial migration is a dynamic process that involves the constant breaking and remaking of intercellular contacts and is accompanied by drastic changes in cell shape and cytoskeletal reorganization in both the tumor cell and its neighboring endothelial cells (Voura et al., 1998; Brandt et al., 1999). Many motility factors for cancer cells and non malignant cells were described first as growth factors. Motility factors converts a cell from static to a motility status, a transition that is characterized by the appearance of membrane ruffling, lamella, and pseudopodia. Several motility factors have been described for cancer cells including autocrine motility factor. Autotoxin and

scatter factors may also act as positive and negative growth factors . Insulin like growth factors stimulates chemokinesis and chemotaxsis. Cell adhesion molecule also play a role in the formation of heterotypic contacts between cancer cells and endothelial cells (Voura et al., 2001). Antibody and peptide inhibition studies suggest the major involvement of multiple CAMs in cell migration. N-cadherin considered to be a potential candidate because transendothelial migration can be retarded by antibodies against N-cadherin (Sandig et al., 1997).

II.1c. Angiogenesis

As the tumour grows and central tumour cells become hypoxic, the tumour initiates its own blood supply. This process is called angiogenic switch. Oxygen and nutrients supplied by the vasculature are crucial for cell function and survival. Angiogenesis is permissive for local and systemic expansion of the tumor mass and can be induced by multiple molecules that are released by both cancer cells and stromal cells (Bergers et al., 1999). Angiogenesis itself encompasses a cascade of sequential processes emanating from microvascular endothelial cells, which are stimulated to proliferate and degrade the endothelial basement membrane of parental vessels, migrate, and penetrate into host stroma and initiate a capillary sprout (Holmgrenet al., 1995). The recruitment of endothelial cells during angiogenesis and the formation of vascular tumors depends on the breakdown of basement membranes, which occurs under the control of numerous activating factors that were shown to be overexpressed in pancreatic cancer, including vascular endothelial growth factor (VEGF), bFGF, angiogenin, members of the TGF and FGF gene families, and interleukin-8 (IL-8) (Bergers et al.,1999)

II 2. Matrix metalloproteinases and their role in cancer metastasis

Since collagen represents the major structural protein of all tissues and the chief obstacle of migration of tumour cells, it has long been postulated that collagenolytic enzymes play a pivotal role in facilitating the dissemination of cancer (Kleiner and Stetler-Stevenson 1999). Matrix metalloproteinase are a family of closely related metal-dependent endopeptidase which once activated, degrade a variety of extra cellular matrix components. Secreted MMPs with specificity for which interstitial collegen (types I-III) basement membrane (type IV) or type V collagen has been purified from several different types of tumour cells (Kleiner and Stetler-Stevenson, 1999).

The expression and activity of MMPs are increased in almost every type of human cancers and this correlates with advanced tumour stage increased invasion and metaslasis and shortened survival. Considerable experimental data from many laboratires indicated that interaction between host cells and tumour cell have a dynamic symbiotic effect in controlling the breakdown of connective tissue stroma during cancer invasion by different types of cancer. Recent studies have implicated gelatinase A, gelatinase B and matrilysin in playing major roles in cancer invasion and metastasis (Kleiner and Stetler-Stevenson, 1999).

II.2a Regulation of Metallo proteinase

The MMP are secreted as inactive zymogens (pro MMPs). They are kept inactive by an interaction between a cysteine-sulphydryl group in propeptide domain and the zinc ion bound to the calatytic domain. Activation requires the poteolytic removal of the pro-petide domain.

Extracellular MMP regulation is carried out mainly by an edogenous inhibitor Tissue Inhibitor of Metalloprotenase (TIMPS). Cytokines and growth factors also appear to play an important role in the modulation of MMP secretions in different tissue especially during inflammation and wound healing. Over expression of TIMP inhibitors and MMP 2, 3,13 and 14 promotes invasion of cell lines through either collagen type I optic nerve explants or matrigel (Matsuyama et al., 2002). Levels of TIMP 1 or TIMP 2 production by tumour cell has been inversely correlated with invasive potential of various experimental tumours (Matsuyama et al., 2002).

II .3 The plasminogen activator /plasmin system

In addition to the MMP family, the plasminogen activator/ plasmin system has been implicated in tumor invasion and metastasis. Plasmin participates in tissue degradation and is activated from the inactive precursor plasminogen by two types of plasminogen activators – uPA (urokinase plasminogen activator) and tPA (tissue plasminogen activator) (Wang, 2001). The proteolytic activity of uPA plays a dominant role in cell migration, angiogenesis, and tumor metastases and is tightly regulated by proteolytic cleavage. It is released from various cell types as an inactive proenzyme (pro-uPA) which upon cleavage by proteinases becomes enzymatically active (Schmitt et al., 1992). uPA binds to a specific cell surface receptor the Urokinase Plasmino gen Activator Receptor (uPAR). Upon binding, uPA converts the zymogen plasminogen to plasmin, an enzyme which degrades fibrin and numerous other components of the extracellular matrix, such as type IV collagen, fibronectin and laminin (Dano et al., 1985). This likely enables tumor cells to migrate through tissue barriers (Dano et al., 1985; Friess et al., 1997). Several studies provided evidence that the expression of active

uPA by malignant cells correlates with their invasive potential (Bramhall et al., 1997). Elevated levels of uPA/uPAR have been reported in numerous tumors, including pancreatic cancer (Cantero et al., 1997)

.II .4 Metastasis as a Therapeutic Target

The recognition that invasion and even metastasis are early events that leads to the logical application of these disciplines to clinical translation. Thus regulation of adhesion, proteolysis, migration and targeted signaling may be direction for translational application.

Many research studies have documented an important role for cell adhesion in tumour progression, invasion, and metastasis (Hanahan et al., 2000). Recent studies in the area therefore has been given rise to attempt to interfere with adhesiveness in attempt to control tumour invasion and metastatic spread. Many studies have investigated various anti galactoside binding lectins (Platt and Rraz, 1992) and agglutinin binding sugar chains (Platt and Rraz, 1992) and their role in modulation of metastatic spread. Additional recent studies of adhesion molecules and cancer metastasis have emphasized the role of CD44. Certine studies have indicated that anti CD44 monoclonal antibody inhibited the formation of metastatic tumours and prolonged survival in animals bearing human melanoma xenograft metastasis (Guo et al., 1994)

Many growth factors can stimulate both tumour and endothelial cell behaviors ranging from proliferation to attachment, motility and proteolysis. For that reason they are logical targets for therapeutic intervention. Vascular endothelial growth factors have been targeted through the genus drug development approaches. Small molecular receptor antagonists and monoclonal antibodies are the two class of molecule developed against

growth factor receptor. These molecules bind the growth factor binding site of its receptors. An antibody directed against VEGF receptor is in clinical trails (Harris, 2000).

Regulation of TIMP/MMP balance is critical to the localization inhibition of matrix breakdown for both physiologic invasion of angiogenesis and the malignant invasion of metastasis. The tissue inhibitors of metalloproteinases (TIMPs) represent a family of ubiquitous proteins which are natural inhibitors of the matrix metalloproteinases (MMPs). Each MMPs each have different substrate specificities within the ECM and are important in its degradation. The analysis of MMPs in human mammary pathology showed that several MMPs were involved in degradation of the ECM: collagenase (MMP1), which degrades fibrillar interstitial collagens, gelatinase (MMP2), which mainly degrades type IV collagen, and stromelysin (MMP3) which has a wider range of action (Bramhall et al., 1996; Bramhall et al., 1997). There are four members of the TIMP family. TIMP-1 and TIMP-2 are capable of inhibiting tumor growth, invasion, and metastasis which has been related to MMP inhibitory activity. Furthermore, both TIMP-1 and TIMP-2 are involved with the inhibition of angiogenesis. Unlike other members of the TIMP family, TIMP-3 is found only in the ECM and may function as a marker for terminal differentiation. Finally, TIMP-4, is thought to function in a tissue-specific fashion in extracellular matrix hemostasis (Gomez et al., 1997).

Tissue inhibitor of metalloproteinase-2 (TIMP-2) is a 24kD protein that is also known as metalloproteinase inhibitor 2. The gene encoding TIMP-2 has been described by Stetler-Stevenson et al. (Stetler-Stevenson et al., 1990). Metalloproteinase-2 (MMP2) which plays a critical role in tumor invasion is complexed and inhibited by TIMP-2.

Thus, TIMP-2 could be useful to inhibit cancer metastasis (Musso et al., 1997). When B16F-10 murine melanoma cells (a highly invasive and metastatic cell line) were transfected with a plasmid coding for human TIMP-2 and injected subcutaneously in mice, TIMP-2 over-expression limited tumor growth and neoangiogenesis *in vivo* (Valente et al., 1998).

Administration of MMP regulators that block the synthesis of MMPs prevent MMPs from interacting with molecules that direct their activities to the cell surface is one of the newly developed cancer therapy.(Matsuyama et al., 2002; Gress et al., 1995) Bastimastat and marimastat hydrxamate molecule targeted to interact with MMPs at the activation site by blocking chelation of the metal ion thus mimicking the physiologic action of TIMPs

The loss of balance in the cellular communication process may allow for deregulation leading to tumourigenecity, invasion and metastasis. Therapeutic efforts in cancer prevention and treatment are being focused at the level of signaling pathways or selective modulatory proteins .Investigations into the signaling path ways underlying metastasis have suggested that protein kinase activity, calcium homeostasis and ras activation are important signals and therefore many be key regulatory sites for therapeutic intervention. Several natural products have been found to inhibit protein tyrosine kinase activity and many possess anti-proliferate or anti-invasive property (John Mann, 2002).Another recently developed therapeutic target in metastasis is ras oncoprotein signaling cascade (van't Veer et al., 2002) and several agents have been taken for clinical trial. Investigators have further demonstrated its utility as a therapeutic target

though studies that tie ras to the action of cytosketon and its function (van't Veer et al., 2002).

III . IMMUNE SYSTEM AND CANCER

Cancers arise from the uncontrolled proliferation and spread of clones of transformed cells. From an immunologic perspective cancer can be viewed as altered self cells that have escaped normal growth regulated mechanism. The concept of immune surveillance states that a physiologic function of immune system is to recognize and destroy clones of transformed cells before they grow into tumour and to kill tumours after they are formed. In experimental animals tumour antigens can be shown to induce both humoral and cell mediated immune responses resulting in the destruction of tumour cells (Rosenberg et al., 2004).

III. 1 Immune surveillance

The immune surveillance hypothesis suggest that a major function of immune system is to control the development of cancer (Shevach, 2004). The host provides number of effector mechanism against tumour cells. The important effectors include tumour specific antibodies, mononuclear phagocytes, natural killer cells; cytotoxic T lymphocytes and lymphokine activated killer cells.

III. 1a Natural killer cells (NK cells)

NK cells are distinct subpopulation of lymphocytes that without prior sensitization or requirement of MHC restriction can kill some cancer cell as well as nonmalignant non self cells. In some cases Fc receptors on NK cells can bind to antibody coated tumour cells leading to antibody depending cellular cytoxicity (ADCC)

III 1b Macrophages

Macrophages and neutrophils from normal donour are generally slightly cytotoxic to tumour cells in *in vitro*. However macrophages and neutrophils can be activated by bacterial products *in vitro* to cover selective cytolysis or cytostasis of malignant cells (Srivastava et al., 1998; Suto and Srivastava, 1995). TNF produced by activated macrophages can account for all of the classical tumoricidal effects against some tumours *in vitro*. Activated macrophages also synthesize nitrogen oxides from L arganine and reactive nitrogen intermediates that also appear to be important mediators of killing of tumour cells. Macrophages also express Fc receptors enabling them to mediate ADCC

III 1 c Complement

The compliment system is composed of a group of serum proteins most of which are β globulins with protease activity. Binding of compliment components to the appropriate immunoglobulin subclass initiates a cascade of compliment activation and macromolecular aggregation that results in the release of anaphylotoxin which cause neutrophil chemotoxis, neutrophil activation, increased vascular permeability, release of histamine from host cell and smooth muscle contraction.(Szebeni et al., 1998)

III. 1d Antibodies and B cells

The role of B cells in regulating tumour immunity is poorly understood. In certain tumour model in which CD4+ helper T cells are required for tumour rejction, B cells appear to be necessary for T-cell priming and tumour resistance (DeFranco,1999).

Human antisera and monoclonal antibodies with autologus tumors have been isolated . Normal or malignant cells of hematopoietic origin are generally lysed quite effectively by antibody and heterologus components *in vitro*, however certain normal or

malignant cells derived from solid tissue may be much less affected even when expressing higher levels of antigen. The reason for this striking difference depending on the source of compliment, the antigen distribution or repair of compliment mediated lesions may be involved. Some tumour cells are killed by a process involving coating with antibody (opsonization) and subsequent phagocytosis by macrophages .This process may be enhanced by the presence of complement. Alternatively antibody coated tumour cells may be killed in the absence of phagocytosis by ADCC when cultured with macrophages, NK cells or neutrophils (Sliwkowski et al., 1999).

III .1e Lymphocyte.

It has been demonstrated convincingly that T cell mediated immunity is of critical importance for the rejection of virally and chemically induced tumours by immunized mice or for the rejection of allogenic and UV light induced tumours by normal mice (Jardetzky et al., 1994). The relative importance of various T cell subsets in tumour rejection has been the subject of repeated and probably necessary controversies. T cells may use their NKG2 receptor to counteract cutaneous carcinogenesis and to kill skin carcinoma cells *in vitro* (Mingari et al., 1997)

III. 2 Tumour escape mechanism

Human tumors, like viruses, have evolved an elaborate assembly of tricks designed to escape from the immune system, that are are "borrowed" from viruses (Lybarger et al., 2005). In general, tumors employ two strategies to avoid recognition: they either "hide" from immune cells thus avoiding recognition or they proceed to disable or eliminate immune cells. It has been recognized for a long time that tumors are adept at shedding surface antigens or down-regulating expression of key molecules necessary for

interactions with immune cells (Whiteside et al., 2006). In this way, tumors can evade the host's immune response by being either a poor stimulators of Tcells or a poor targets for tumor-specific T cells (CTL). Expression of molecules such as TAA, HLA class I molecules or antigen processing machinery components (APM) is often down-regulated or altered in tumor cells (Ferris et al., 2006). As a result of abnormalities in the APM components, which might include their down-regulation, absence or mutation (Meissner et al., 2005), peptides are not generated from tumour associated antigens (TAA) or are generated in a form not allowing for the formation of HLA class I-peptide complexes recognized by T cells (Meissner et al., 2005). Tumors are not effective antigen-presenting cells, and they frequently mis-process and mis-represent processed TAA, so that immunogenic peptides cannot be made or are defective and thus do not fit into the HLA class I groove.

A wide variety of soluble immunosuppressive factors such as TGF, IL-10, ROS, enzymes, and inhibitory ligands such as FasL or TRAIL, that are released by tumor cells or other cells in the tumor microenvironment, suppressor cell populations, i.e., regulatory T cells (CD4+CD25) or myeloid-derived suppressor cells have been shown to play a key role in down-regulation of anti-tumor host immunity (Shevach, 2004; Gabrilovich, 2004). Generally, immunosuppressive effects of tumors are best seen locally, at the tumor site. Functional aberrations of TIL freshly isolated from human tumors are well documented in the literature (Whiteside et al., 1993)

Certain tumour specific antigens have been disappearing from the surface of tumour cells. Such antigen loss variants are common in rapidly growing tumours and can be readily induced in tumour cell lines by culture with tumour-specific antibodies or

cytotoxic T lymphocytes (CTLs). Malignant transformation of cell is often associated with a reduction (or even complete loss) of class I MHC molecule and a number of tumours have been shown to express decreased level of class I MHC molecule. Since CD8+ CTL recognize only antigen associated with class I MHC molecule, any attraction in the expression of class 1 MHC molecule on tumour cells may exert a profound effect on CTL mediated immune response.

Among less known but clearly important immunosuppressive effects tumors mediate is the ability to induce T-cell apoptosis (Whiteside, 2002). Different mechanisms may account for the high frequency of T-cell apoptosis observed in patients with cancer. Binding of Fas ligand (FasL) to the Fas receptor has been known for some time to induce apoptosis of T cells responding to autologous antigens and maintain tolerance to normal tissue antigens. Furthermore, chronically stimulated T cells are likely to undergo activation-induced cell death (AICD) mediated by the Fas/FasL pathway, or they may die because appropriate cytokines are not secreted (Van Parijs et al., 1996)

The mechanisms of escape evolved by human tumors are varied and ingenious. They appear to target components of the innate as well as adaptive immune system; they operate at the local as well systemic levels, and interfere with molecular pathways responsible for the key cellular functions of immune cells. Furthermore, progressing tumors co-opt tissue cells to participate in creating a microenvironment especially unfavorable for immune interventions in situ. As a result of these mechanisms, tumors have become adept at avoiding immune surveillance, and it might be predicted that their escape from the host's immune system is likely to be difficult to overcome by immune therapies.

IV. CANCER IMMUNOTHERAPY.

As a result of the limited efficacy of conventional radiotherapy and chemotherapy regimens for treating advanced cancers and the significant morbidities associated with surgical treatment of localized disease, other approaches for treating different cancers are actively under investigation. Among these, immunotherapeutics represent a spectrum of alternative modalities that have proven to be effective in many malignancies such as renal cell carcinoma and malignant melanoma.(Parmiani et al., 2002; Glaspy, 2002)

In general, immunotherapies are designed to augment or manipulate the host immuneresponse to eradicate neoplastic cells. One major hurdle for this approach involves the high similarity between the genome and proteome of a normal cell and it's corresponding malignant counterpart. Not surprisingly, very few cancer-specific genes or proteins have been identified. This is in sharp contrast to immunotherapies targeted toward microbial pathogens in which many proteins are unique to the infectious agent. The practical ramifications of the high genotypic and phenotypic identity exhibited between normal and neoplastic cells involves the ability of an immune based therapy to break immunological tolerance and to avoid toxicity directed toward normal host cells. Nonetheless, the immune system does exert significant anticancer effects as demonstrated by the presence of cytotoxic lymphocytes infiltrating tumors,(Rosenberg, 2001b) the increased incidence of malignancies such as cervical carcinoma in immunocompromised individuals, (Rosenberg, 2001b) and the graft-versus-leukemia effect observed in allogeneic bone marrow transplantation.(Weiden et al., 1981)

However, the very existence of neoplastic diseases and the almost certain progression without intervention underscores the failure of the natural surveillance systems to effectively police every tumorigenic event. The result of allowing even one critical transforming incident to progress unimpeded can be catastrophic for the human host. Thus, the primary objective of oncological immunotherapeutic approaches is to enhance the potency and effectiveness of the immune response toward the recognition and eradication of neoplastic disease.

Cancer immunotherapies can be broadly categorized into those utilizing active or passive mechanisms. Active immunotherapy entails vaccinating patients with antigens and adjuvants that activate tumor-specific T cells, the major cellular immune effector component. T cells identify target cells through a membrane-bound protein known as the T-cell receptor (TCR). The TCR recognizes short peptide antigens in association with major histocompatibility complex (MHC) molecules displayed by antigen-presenting cells (APCs). Thus, generating tumor-specific immunity is consequently dependent on an appropriate target antigen and the effective presentation of that antigen to the patient's immune system. The critical roles for APCs in this process have recently been appreciated as APCs such as dendritic cells are responsible for the uptake, processing, and presentation of antigens to cytolytic–T (CD8) and helper-T (CD4) cells in the context of MHC class I and class II molecules (Guermonprez. et al. 2002). To generate an immune response, initial efforts to produce cancer vaccines utilized irradiated neoplastic cells derived from the patient (autologous) or from other individuals (allogeneic) to inoculate cancer patients. (Sedlacek, 1994)

However, this approach could be expected to have greater side effects due to cross-reactivity with normal host antigens, and the immune response may not be optimized due to the potential low expression level of any particular target. Subsequently, for some malignancies, focused efforts to identify and characterize cancer-specific antigens have distinguished well-defined targets for T cell recognition (Scanlanet al. 2002; Mullins. et al. 2001).

IV .1 Immuno conjugates in Immunotherapy.

Immuno conjugates are cell targeting molecules such as mAbs, cytokines or soluble receptors that have been genetically or biochemically coupled to cytotoxic moieties (Niculescu-Duvaz and Springer, 1997). Thus cell-targeting portion of an immuno conjugates is used as a delivery system for toxins, radio isotopes, drug enzymes that can activate pro-drugs, liposomes or effector cell recruiting strictures.

IV .2 Cellular strategies

The cellular arm of the immune system plays a key role in maintaining antitumour immunity. In cellular therapy immune cell with antitumour activity are transferred to a tumour-bearing host. Cellular immunotherapeutic strategies can be aimed directly or indirectly at the tumour cells. Successful cellular therapy depends on the types of cells transferred and their effector functions, the ability of the cell to reach tumour site and their ability to overcome tolerance or immune suppression in the host.

IV .2a. Tumour Infiltrating Lymphocytes (TIL)

TILs are lymphocytes that infiltrate growing tumours. They are lymphocytes that have been obtained from tumour tissue and are considered to be a component of an

inflammatory host response to the tumor.. The resulting single cell suspension is cultured for several weeks after the TILs can be harvested.

IV 2b. Lymphokines activated killer cells.

Lymphocytes from a tumour bearing patient are cultured in IL2 to expand and activate cytotoxic LAK cells primarily NK cells. They are then infused in to the patients with or without more IL2. Although some tumour regression occurs with this approach, significant toxicity is evident when high doses of IL2 are used (Lanfrenier and Rosenburg, 1985).

IV 2c. Macrophage activated killer cells.

Monocytes isolated from peripheral blood of tumour bearing patients are cultured *in vitro* with cytokine, which activate these cells for enhanced cytotoxicity before re-injection into patients.

IV 3. Administration of immunomodulators.

Agents that enhance the immune response of the host against cancer, infections disease or immunologic disorders are referred to as immunomodulators (Tzianabos, 2000). Immunomodulators belongs to a highly heterogeneous group of molecule with different mechanism of action. Immunomodulators can be administrated with antigen to increase their local retention and these by facilitate their slow release onto the body. In cancer treatment these immune response modifiers are widely used (William, 1997).

With recent advances in the understanding of how cells communicate with each other to signal effector functions, it has become possible to conceive of strategies to manipulate these signaling pathways in order to influence host responses. Compounds

that are capable of interacting with the immune system to upregulate or down regulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers. Several classes of these compounds, such as proteins, peptides, lipopolysaccharides, glycoproteins, and lipid derivatives, have all been characterized as molecules that have potent effects on the host immune system. Peptides such as cytokines and chemokines are well-known examples of such molecules. Whether certain compounds enhance or suppress immune responses can depend on a number of factors, including dose, route of administration, and timing of administration of the compound in question. The type of activity these compounds exhibit can also depend on their mechanism of action or the site of activity.

IV. 4 Cytokines

Cytokines are soluble mediators secreted by virtually all nucleated cells in the body and particularly by the cells of immune system (William 1997). They are relatively small proteins that influence the behaviour of other cells expressing appropriate receptors. Cytokines have both autocrine and paracrine activities and virtually all act on multiple cellular targets, Cytokines are involved in both the growth and death of malignant cells. Antiapoptote signals generated by cytokines can promote cell survival and signal transduction pathways involved in the pathogenesis of neoplasia. Conversely cytokines are crucial for the activation and development of immune response against tumour cells. Therefore these two effects must be manipulated in a manner that will be therapeutically useful. (William, 1997)

IV. 4a Interleukins (IL)

Interleukins is a generic name for a hormone like substance produced by the body. It stimulates the growth of blood cells important to body's immune system. They are particularly important as they regulates inflammatory and immune response.

IL was among the earliest cytokines identified as it has so many potent activities. IL-1 is a powerful inducer of inflammatory process. The most profile IL-1 producing cells are macrophages following stimulation with variety of microbial product or other agents including cytokines (Rosenburg, 1993). Blocking IL-1 activity via receptor antagonist soluble receptors or newly screened drugs shows promise in controlling inflammatory disease such as rheumatoid arthritics and septic shock probably most effectively if combined with blockade of other inflammatory cytokines such as TNF, and IL-6 (Rosenburg, 1993).

IL-2 was the first cytokines to be molecularly characterized. It is a molecule whose functions are highly pleotropic. It is involved in the activation of antigen-specific T and B cells and it also triggers innate immunity by stimulating several functions of NK cells and macrophages. IL-2 can circumvent defective or suboptional antigen mediated activation and thus overcomes tolerance. This findings suggests that IL-2 may be useful in tumour immunotherapy by enhancing the activity of NK cell or by activating tolerant or poorly responsive antitumor T cells (Kawakami and Rosenburg, 1997).

IV 4b Interferons.

Interferons are family proteins that are produced by cells in response to viral infections or stimulation with double stranded RNA antigens or mitogens (Kurzrock et al., 1991). Interferons have antitumour activity against a variety of tumour types

including hairy cell leukemia chronic myelogenous leukemia, cutaneous T cell lymphoma and Kaposi's sarcoma (Devita et al., 1991).Three types of interferons are available. Interferon alpha has the greatest efficacy in the treatment of hematologic malignment diseases such as hairy cell leukemia and lymphoma (Pegram et al., 1998). IFN-α inhibits cell growth by inducing G1 arrest. The success of IFN-α therapy is greater when it is combined with other anticancer agents (Quesada et al., 1998).

IFN-γ is best known for its ability to augment the cytotoxic activity of CTLs and NK cells and to increase the expression of MHC molecules on various cells. It also activates monocytes and macrophages .IFN-γ has been used in patients with metastatic renal cell cancer and although the response rate was only 15% some response are durable (Motzer et al., 1928).

IV 4c. Tumour necrosis factor (TNF)

TNF-α was originally identified as a cytokine responsible for endotoxin induced necrosis (Bazzoni, and Beutler, 1996). Several independent groups reported that therapy with recombinant TNF-α was effective against several types of murine models of hepatic (Nishiyama et al, 1989; Scheringa et al., 1989) and pulmonary metastasis (Schultz, and Altom, 1990). TNF-α and TNF-β have been shown to exhibit direct antitumour activity, killing some tumour cells and reducing the rate of proliferation of others while sparing normal cells. In the presence of TNF-α. or TNF-.β, a tumour undergoes visible hemorrhagic necrosis and tumour regression. TNF-α has also been shown to inhibit tumour-induced vascularization (angiogenesis) by damaging the vascular endothelial cells in the vicinity of a tumour, thereby decreasing the flow of blood and oxygen that is necessary for progressive tumour growth. TNF has potent antitumour activity against

large tumour burdens in some murine models (Haranaka et al., 1984; Creasey et al; 1986). However, humans can only tolerate 2% of the systemic TNF dose (by weight) required in mice, due to dose limiting hypotension (Feinberg et al, 1988; Mortiz, et al, 1989). High doses of TNF, administered locally via direct tumour injection (Bartsch, 1989) or isolated limb perfusion (Lienard, 1992) can result in dramatic tumour regression in some cancer patients.

IV 5. Plant derived immunomodulators

Administration of artificially prepared immunomodulatory agents including various cytokines produce certain toxic side effects and it affect their theraputical efficacy. Immunomodulatory agents that are free from side effects and which can be administrated for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. Use of plant products as immunostimulants getting more and more importance in the field of cancer research because of their nontoxic nature. Some of the plants with known immunomodulatory activities are *Viscum album* (Kuttan and Kuttan, 1992), *Panax ginseng* (Singh et al.,1984), *Tinospora cordifolia* (Mathew and Kuttan, 1997), etc; components such as polysaccharides, lectins (Tzinabose, 2000) present in plants have been shown to stimulate the immune system. Curcumin, an active ingredient present in *Curcuma longa*, proved to be a immunopotentiator (Antony et al., 1999). Administration of an extract from the powdered root of the plant *Withania somnifera* was found to stimulate Immunological activity in Balb/c mice (Davis and Kuttan 2000).It also reported to be increase cytokines production in normal as well as tumour bearing animals (Davis and Kuttan, 1999) and proved as potent activator for CTL production both *in vivo* as well as *in vitro* .(Davis and Kuttan

2002a). Intraperitoneal administration of *Withania extract* was found to enhance the proliferation of lymphocytes, bone marrow cells and thymocytes in responses to mitogens. Both PHA and Con A mitogens along with Withania treated splenocytes, bone marrow cells and thymocytes could stimulate proliferation twice greater than the normal (Davis and Kuttan 2002b). After treatment with five doses (20 mg/dose) of naturally occurring sulphur compounds such as diallyl sulphide (DAS), diallyl disulphide (DADS) and allyl methyl sulphide (AMS) the total white blood cell (WBC) count was found to enhanced significantly in Balb/C mice (Kuttan, 2000; Manesh and Kuttan, 2003). Administration of alcoholic extract of *Piper longum* (10 mg/dose/animal) as well as piperine (1.14 mg/dose/animal) were reported to be a immunostmulant and increased the total WBC count , the number of plaque forming cells in spleen, bone marrow cellularity and alpha-esterase positive cell number in Balb/c mice (Sunila and Kuttan, 2004).

V PLANT PRODUCTS IN CANCER THERAPY

Cancer chemoprevention is defined as pharmacological intervention with synthetic or naturally occurring compounds that may prevent, inhibit or reverse carcinogenesis, or prevent the development of invasive cancer (Wattenburg, 1993). Reports from the World Health Organization (World Health Organization. 1990) and the UK Department of Health (Department of Health. 1994, Department of Health. 1998) also provide evidence of beneficial effects of fruits and vegetables. Higher consumption of vegetables and fruit significantly reduces the risk of many chronic diseases including cancers of the mouth pharynx, oesophagus, lung, stomach, and also of colon and rectum (World Cancer Research Fund 1997). The cancer inhibitory potential of human nutrients

derived from plants, as well as of non-nutrient constituents (phytochemicals) has been confirmed in various animal models (Crowell, 1999). Dietary consumption of foods and herbal medicines is a convenient method of administrating potentially beneficial phyto-chemicals in a cost-effective manner.

Medical benefits from plant and plant products forms have been recognized for centuries. Herbs have been used in traditinal medicine for thousands of years to cure diseases and heal wounds, even though their biogenesis and pleiotropic actions has not impacted on the practice of western medicines (Nanjoo et al., 1998). Several medicinal plants are being screened for their antitumour properties in India, China, Korea, Brazil and some other countries. *Selaginella tamariscina* a traditional medicinal plant for therapy of advanced cancer patients in the 'Orient' which has been shown to modify gene expression and cytokine production (Kuo et al 1998). There are several evidences that water extract of this plant efficiently increased p53 gene expression and induced G1 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 (Lee et al.,1999). Hot water extract of the bark of *Nikko maple* (*Acer nikoensa*), a traditional crude drug, has been known for its cytotoxicity (mediated through apoptosis) in susceptible and resistant mouse leukaemia P-388 cell lines, and this extract also increased the expression of sialylated glycoconjugates in apoptotic cells(Nitta et al., 1999). *Uncaria tomantosa* (Willd.) DC, also known as 'Cat's claw', has been used in South American traditional medicine. The native Indians particularly of the Amazon region use tea made of the bark or roots for the treatment of a variety of health disorders including cancer, arthritis and infectious diseases. Organic solvent extracts of this plant

were shown to have cytostatic, contraceptive and anti-inflammatory activity (Keplinger, 1982). Sheng et al. reported that water extract of this plant induced cytotoxicity in human leukaemic cell lines HL- 60, K-562 and human EBV-transformed B-lymphoma cell line Raji (Sheng et al., 1998). Extracts of *Solanum muricatum* (*Pepino*) have been shown to induce apoptotic cell death in prostate (PC-3, DUI-45), stomach (MKN-45), liver (QSY-7721, SKHEP- 1), breast (MDA-MB-435), ovarian (OVCAR), colon (HT-29) and lung (NCI-H-209) cancer cells and some normal (NHP, HUVEC, WI-38) cells in culture.. *Alpinia oxyphylla Miquel* (*Zingiberaceae*) used in traditional Oriental herbal medicine has been shown to induce apoptosis-mediated cytotoxicity in HL-60 cells in culture (Lee et al., 1998). *Salvia miltiorrhiza* is a traditional Chinese herbal medicine commonly used to treat liver diseases in China for centuries. This plant extract exerted clear cytotoxic effects and strongly inhibited the proliferation of human hepatoma cell line, HepG (2) cells (Liu, et al 2000). Water-soluble macromolecular components of *Artemisia capillaris Thunberg* exhibited inhibition of cell proliferation and apoptosis when studied on human hepatoma cell line (SMMC-7721) (Hu, et al., 2000). *Phyllanthus orbicularis,* a plant of genus *Phyllantus*, is used in Indian traditional medicine for its antiviral activity against Hepatitis B virus and A and B flu virus. The aqueous extract of this plant induced apoptosis in Chinese hamster ovary (CHO) cells (Sanchez-Lamar et al., 1999). 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). Bussing et al. observed an induction of apoptosis and generation of reactive oxygen intermediates in granulocytes by an aqueous extract of the seeds (Bussing et al, 1999). This 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 (IFN-γ).

Natural products in modern medicine have been the mainstay of cancer chemotherapy for the past 30 years. Vinblastine and vincristine (Johnson 1968; Gidding et al., 1999) were first introduced plant products as cancer therapeutics and have contributed to long-term remissions and cures with childhood leukaemia, testiculartеratoma, Hodgkin's disease and many other cancers. Several structural analogues are also in clinical use, and most notable of these are vinorelbine and vindesine (Fahy, 2001). Taxol, a natural product obtained from the bark of the Pacific yew *Taxus brevifolia* which shows efficacy against refractory breast and ovarian cancers. Camptothecin, from the Chinese ornamental tree *Camptotheca accuminata*, was blighted by severe bladder toxicity, but chemical manipulation of its structure subsequently produced analogues, including topotecan (Hycamptin) and irinotecan (Camptosar), that have been approved for clinical use (Jonsson et al., 2000). These days camptothecin is valued as a biological tool to understand the functions of the enzyme topoisomerase I, for which it is a specific inhibitor (Chen and Liu, 1994).The human diet contains a complex mixture of plant polyphenols and it is believed that human individuals may consume as much as one gram of plant phenols per day in their diet (Tanaka1994; Chung et al.,1993; Meyer et al., 1993). Studies have shown that cytotoxic effect of these phenols against different tumours (Inone et al., 1994). Curcumin, a phenolic compound that has been identified as the major pigment in turmeric, induces apoptosis in transformed rodent and human cells in culture, (Wall et al.,1993; Samaha et al., 1997; Kuo et al., 1996; Jiang et al., 1996). In past etiological studies, intake of certain kinds of polyhydroxyphenols such

as flavonoids or lignans in the diet has been correlated with low incidence of colon cancer and breast cancer (Setchell et al., 1981; Adlercreutz et al., 1982) The past decade has seen a dramatic resurgence inresearch on carbohydrates involved in diseases and their potential use as therapeutics (Persidis, 1997). Several plant polysaccharides have been described with *in vitro* and *in vivo* immunostimulating activity (Tomoda et al., 1994). Their major effect seems to be the activation of macrophage cytotoxicity against tumour cells. Likewise, other branched plant heteropolymers have been reported to enhance cytotoxicity of NK cells by inducing the production and/or release of cytokines (Mueller et al.,1990a; Mueller et al.,1990b; Hauer et al., 1993). Some polysaccharides have shown potent activity against various tumours when tested in implanted animals (Yamada et al., 1990). The mechanism proposed has been the blockage of metastasis by covering galactose-specific binding sites (Hagmar et al., 1991). These activities may have possible therapeutic implications in cancer treatment, from the approach of modulating the immunological functions or by blocking metastasis. Roots of *Ashwagandha* (*Withania somnifera)* a common ingredient of many Ayurvedic preparations have shown very promising cancer therapeutic effects in clinical study (Davis and Kuttan, 2000). *Withania samnifera* has shown to be immunomodulator and metastatic inhibitor (Leyon and Kuttan, 2004a). *Boerhaavia.diffusa* a medicinal plant has proved its antimetastatic activity (Leyon et al., 2004). Similarly Piperine a natural product from *Piper longum Linn* has shown to be a potent inhibitor of metastasis (Pradeep and Kuttan,2002) nuclear factor-kappaB (NF-kappaB), c-Fos,CREB, ATF-2 and proinflammatory cytokine gene expression in B16F-10 melanoma cells (Pradeep and Kuttan,2004). It also proved to inhibit the matrix metalloproteinase production in highly metastatic B16F-10 melanoma

cells. Intraperitoneal administration of the synthetically prepared natural compounds tetrahydrocurcumin (THC), salicyl curcumin (SC) and curcuminIII (C-III) proved to reduce the number of tumour directed capillaries induced by B16F-10 melanoma cells in C57BL/6 mice (Leyon and Kuttan 2003). Administration of Indian medicinal plant *Tinospora cordifolia* proved to inhibit angiogenesis and also inhibit the production of pro inflammatory cytokines and the direct endothelial cell proliferating agent vascular endothelial cell growth factor (VEGF) during angiogenesis (Leyon and Kuttan, 2004b. Administration of the polysaccharide fraction from *Tinospora cordifolia* was found to be very effective in reducing the metastatic potential of B16F-10 melanoma cells and also inhibit the production of the matrix metalloproteinase (Leyon and Kuttan, 2004c). The natural products from *cruciferase* family allyl and phenyl isothiocyanates proved their anti metastatic activity in highly metastatic B16F-10 melanoma cells and it also proved to inhibit the production of pro inflammatory cytokines and matrix metalloproteinase (Manesh and Kuttan, 2003). Beta-carotene a naturally occurring polyterpene proved to inhibit the lung metastasis induced by B16F-10 melanoma cells in C57BL/6 mice (Pradeep and Kuttan, 2003) Curcumin, an active ingredient present in *Curcuma longa* shown to inhibit lung metastasis induced by B16F-10 melanoma cells (Menon et al., 1999)

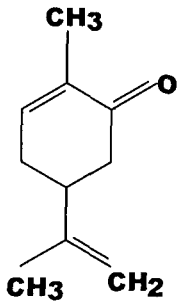
The production of monoclonal antibodies led to a new approach to targeting cancer cells in the 1980s opened an approach that combined this new means of tumour targeting with natural products: antibody-directed enzyme-prodrug therapy (ADEPT). This technique uses an antibody specific for a tumour cell that is conjugated to an enzyme ((Niculescu-Duvaz and Springer, 1997) . The conjugate is administered to the

patient and, in principle, it accumulates at the tumour site as the antibodies bind to antigens on the tumour cell surface. After a short period, during which excess conjugate clears from the system, a non-cytotoxic prodrug is given and this is activated by the enzyme in the antibody–enzyme conjugate to yield a cytotoxic anticancer drug. In principle, the cytotoxic agent is only revealed close to the tumour cells, so nonspecific cytotoxicity is reduced. The other advantage is that one molecule of enzyme catalyses the production of numerous molecules of drug. Many naturally occurring cytotoxic agents have been converted into prodrugs, and for much of the past decade they have been used with the ADEPT technology more as experimental tools than as clinically viable drugs.

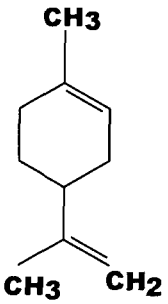
VI TERPENOIDS

Terpenoids perhaps are the most structurally varied class of plant natural products. The name terpenoid, or terpene, derives from the fact that the first members of the class were isolated from turpentine. All terpenoids are derived by repetitive fusion of branched five-carbon units based on isopentane skeleton. These monomers generally are referred to as isoprene units because thermal decomposition of many terpenoid substances yields the alkene gas isoprene as a product and also because suitable chemical conditions can induce isoprene to polymerize in multiples of five carbons, generating

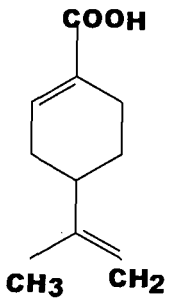
Fig 1.2a Terpenoids



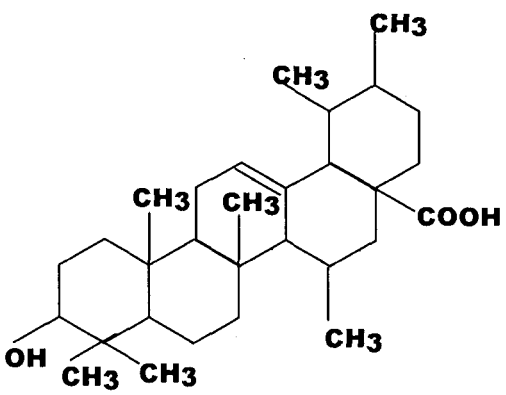
Carvone



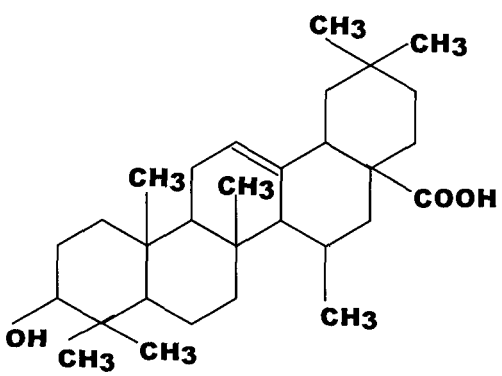
Limonene



Perillic acid

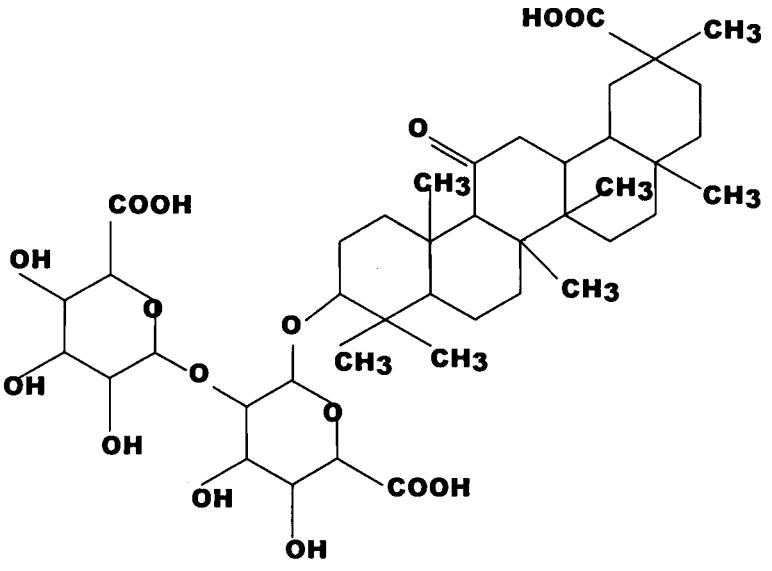


Ursolic acid

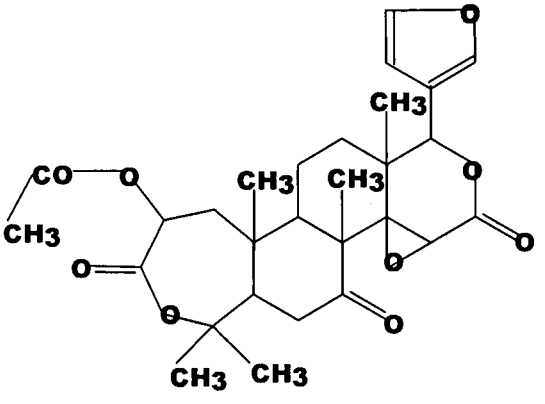


Oleanolic acid

Fig 1.2b Terpenoids



Glycyrrhizic acid



Nomilin

numerous terpenoid skeletons. The enzyme isoprene synthase is present in the leaf plastids of numerous plant species, but the metabolic rationale for the light-dependent production of isoprene is unknown C10 terpenoids, although they consist of two isoprene units, are called monoterpenes, The triterpenes, which contain 30 carbon atoms, are generated by the head-to-head joining of two C15 chains, each of which constitutes three isoprene units joined head-to-tail. This large class of molecules includes the brassinosteroids, the phytosterol membrane components, certain phytoalexins, various toxins and feeding deterrents, and components of surface waxes, such as oleanolic acid of grapes. These compounds are important intermediates in the biosynthesis of steroids. Most of the terpenes are colourless fragrant liquids having boiling points between 150°C and 200°C. They are lighter than water and are ready steam volatile. They dissolve in organic solvent but usually not in water. Chemically they are considered hydrocarbons and are highly reactive in nature. Although terpenoids are widely used for medicinal purposes in many Asian countries, biogenesis and pleiotropic actions has not impacted on the practice of western medicines (Nanjoo et al., 1998).

Monoterpenes are nonnutritive dietary components found in the essential oils of citrus fruits, cherry, mint and herbs. They function physiologically as chemoattractants or chemorepellents (McGarvey and Croteau, 1995), and they are largely responsible for the distinctive fragrance of many plants. These 10 carbon isoprenoids are derived from the mevalonate pathway in plants but are not produced by mammals, fungi or other species. In citrus fruits (Chayet et al., 1977), peppermint and other plants, d-limonene is formed by the cyclization of geranylpyrophosphate in a reaction catalyzed by limonene synthase (Alonso et al. 1992; Kjonaas and Croteau, 1983). Limonene then serves as a precursor to

a host of other oxygenated monocyclic monoterpenes such as carveol, carvone, menthol, perillyl alcohol and perillaldehyde (Karp et al. 1990; McGarvey and Croteau, 1995). In addition, d-limonene is a prevalent flavoring agent for fruit juices, soft drinks, baked goods, ice cream and pudding. Orange oil, naturally consisting of 90–95% d-limonene, is a commercially available food flavoring agent. Furthermore, because of its pleasant citrus fragrance, d-limonene is commonly added to cosmetics, soaps and other cleaning products. Thus, human exposure to monoterpenes through the diet or environment is widespread.

VII TERPENOIDS IN CANCER THERAPY

A number of dietary monoterpenes have antitumor activity, exhibiting not only the ability to prevent the formation or progression of cancer, but to regress existing malignant tumors. Limonene has well established chemopreventive activity against many cancer types. Limonene has been shown to inhibit the development of spontaneous neoplasms in mice receiving 1200 mg/kg orally; dietary limonene also reduces the incidence of spontaneous lymphomas in p532/2 mice (Hursting et al. 1995). Furthermore, when administered either in pure form or as orange peel oil (95% d-limonene), limonene inhibits the development of chemically induced rodent mammary (Elegbede et al. 1984, Elson et al. 1988, Maltzman et al. 1989; Wattenberg 1983), skin (Elegbede et al. 1986b), liver (Dietrich and Swenberg 1991), lung and forestomach (Wattenberg et al. 1989 and 1991) cancers (reviewed in Crowell and Gould 1994; Elson and Yu 1994, Elson 1995). In rat mammary carcinogenesis models, the chemopreventive effects of limonene are evident during the initiation phase of 7-12-dimethylbenz [a]anthracene (DMBA)2-

induced cancer (Elson et al. 1988) and during the promotion phase of both DMBA- and nitrosomethylurea (NMU)-induced cancers (Elson et al. 1988, Maltzman et al. 1989). Dietary limonene also inhibits the development of ras oncogene–induced mammary carcinomas in rats (Gould et al. 1994).There are many reports (Kawamori et al., 1996) that the development of azoxymethane-induced aberrant crypt foci in the colon of rats was significantly reduced when they were given 0.5% limonene in the drinking water. Caraway seed oil, and its principal monoterpene, carvone, prevent chemically induced lung and forestomach carcinoma development when administered before the carcinogen (Wattenberg et al. 1989). In addition, carveol (Crowell et al. 1992a) and menthol (Russin et al. 1989) have chemopreventive activity against DMBA-induced rat mammary cancer when fed as 1% of the diet only during the initiation phase. Geraniol, an acyclic dietary monoterpene, has *in vivo* antitumor activity against murine leukemia, hepatoma and melanoma cells (Shoff et al. 1991; Yu et al. 1995) when administered before and after tumor cell transplantation. In addition, perillyl alcohol has promotion phase chemopreventive activity against chemically induced liver cancer in rats (Mills et al. 1995) and is very effective at preventing tumor recurrences or secondary tumors in animals treated in a chemotherapy regimen (Haag and Gould, 1994). Dietary monoterpenes have promising chemotherapeutic activity against established rodent pancreatic and mammary tumors. Both limonene (Elegbede et al. 1986a, Haag et al. 1992) and perillyl alcohol (Haag and Gould 1994) have chemotherapeutic activity against rat mammary tumors, causing the complete regression of .80% of established DMBA- or NMU-induced mammary carcinomas. Chander et al. (1994) reported that combination chemotherapy of NMU-induced rat mammary tumors with limonene and the aromatase

inhibitor 4-hydroxyandrostenedione was more effective than either drug alone. Perillyl alcohol has chemotherapeutic activity against pancreatic cancer at doses that cause little toxicity to the host (Stark et al. 1995). Perillyl alcohol reduced the growth of transplanted hamster pancreatic tumors to less than half that of controls. Moreover, a significant portion of perillyl alcohol– treated pancreatic tumors completely regressed, whereas none of the control tumors regressed (Stark et al. 1995). Phase I clinical trial testing of the cancer chemotherapeutic activity of limonene (McNamee 1993) and perillyl alcohol (Phillips et al. 1995) is in progress.The limonoids, limonin and nomilin are oxidized triterpenes, which are responsible for bitter taste of citrus fruits. They have shown to inhibit azoxymethane induced colon carcinogenesis in rats (Tanaka et al., 2000). Nomilin was found to inhibit benzo[a] pyrene (BP) induced neoplasis in fore stomach of ICR/Ha mice. Ursolic acid is a pentacyclic triterpenes compound isolated from many types of medicinal plants are is presannt in human diet. It has been reported to possers a wide range of pharmacological properties and is one of the most promising chemopreventive agent (Rocha et al., 2004; Shih et al., 2004**).** Ursolic acid (3ß-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid derived from berries, leaves, flowers, and fruits of medicinal plants, such as *Rosemarinus officinalis, Eriobotrya japonica, Calluna vulgaris, Ocimum sanctum, and Eugenia jumbolana* (Liu, 1995) . Ursolic acid has been shown to suppress tumorigenesis (Huang 1994) , and inhibit tumor promotion (Tokuda et al., 1986; Ohigashi et al., 1986; Nishino et al., 1988) . Several of these effects of ursolic acid are mediated through suppression of the expression of lipoxygenase, COX-2, MMP-9, and iNOS (Simon et al., 1992; Najid et al.,1992; Ringbom et al.,1998 Subbaramaiah et al., 2000; Cha et al., 1998; Cha et al., 1996; Suh et al., 1998) all of which are genes regulated

by NF- [{kappa}] B. In addition, ursolic acid and its derivatives have been shown to induce apoptosis in a wide variety of cancer cells including breast carcinoma, melanoma, hepatoma, prostate carcinoma, and acute myelogenous leukemia (Es-Saady et al., 1996; Es-Saady et al., 1996 (b); Cho et al., 2000; Hollosy et al., 2000; Hollosy et al., 2001; Konopleva et al., 2002; Choiet al., 2000) through inhibition of DNA replication (Kimet al 2000) , activation of caspases (Cho et al., 2000; Hollosy et al., 2001; Konopleva et al., 2002) inhibition of protein tyrosine kinases (Hollosy et al., 2000)and induction of Ca2+ release (Baek et al., 1997; Lauthier et al., 2000). Ursolic acid inhibits the cell proliferation of human lung cancer cell line A549 showed that the blocked cell cycle progression in the G1 phase (Hsu et al., 2004).It also decreased the protein expression of cyclin D1, D2, and E and their activating partner cdk2, 4, and 6 with concomitant induction of p21/WAF1. Ursolic acid is able to inhibit key steps of angiogenesis *in vitro*, including endothelial cell proliferation, migration, and differentiation. (Cardenas et al., 2004). Oleanolic acid and ursolic acid have known anti-inflammatory and anticarcinogenic activity (Deepak and Handa, 2000; Liu, 1995; Nanjoo et al., 1998).

A wide variety of natural compounds appear to possess significant anticancer as well as chemopreventive activity. Extracts of plants used in traditional medicine also have a similar property. Clearly, knowledge of the active principles associated with common plant products is of utmost importance. Many more screening studies are necessary using plant extracts and compounds isolated from them. Elaborate studies with such compounds with respect to their abilities to induce apoptosis and understanding their mechanism of action may provide valuable information for their possible application in

cancer therapy and prevention. Having the option to administer an agent through the diet or as a prescription drug is a major advantage.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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APRIL - 2006

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

I. Terpenoids

Carvone, Limonene, Perillic acid, Ursolic acid, Oleanolic acid, Glycyrrhizic acid and Nomilin - Sigma Chemicals, St.Louis, USA

II. Chemicals:

Dulbeco's Modified Eagle Medium (DMEM)
Eagle's Minimum Essential Medium (MEM)
Rosewell Park Memorial Institute Medium (RPMI-1640)
Hanks balanced Salt Solution (HBSS)
Fluid Thioglycollate Medium
Trypsin
Sodium caesinate
- Himedia Mumbai India.

Foetal calf serum - Biological Industries, Kibbutz, Bethhaemeck, Israel

Concanavalin A
Collagen Type I
Hydroxyproline
Sialic acid (n-acetyl nuraminic acid)
- Sigma Chemicals, USA

Phytohaemagglutinin (PHA)

Lipopolisaccharide (LPS) - Difco Laboratories, USA

Pokeweed Mitogen (PWN)

Hepes Buffer

Acrylamide

Bis- Acrylamide - Sisco Research Laboratories, Mumbai

PPO

POPOP

Radioactive Materials:

i) ^{3}H-Thymidine - Board of Radiation and Isotope Technology, BARC, Mumbai

ii) Sodium Chromate ($Na_2Cr^{51}O_4$)

(Specific Activity > 50mci/mg)

Crystal Violet, Trypan blue - Romali, Mumbai

Coomassie brilliant blue

Paraffin Wax - E Merck, India

Eosin

Leishman's Stain

Alpha naphthyl Acetate	Loba Chemie, Mumbai, India
Pararosaniline	
Policarbonate Filters	Nucleopore, Cambridge, USA
Goat Serum	From local slaughter house

Diagnostic, Research and Reagent Kits

a) Drabkin's reagent	Haemochek, Thane, India
b) Folin Ciocaltau	E.Merck, Germany
c) Gamma glutamyl transferase	AVT diagnostics, Madras
d) IL-2, GM-CSF, TNF- α, IL-6, IL-Iβ --ELISA Kits	Endogen, USA (Elisa Kits)

III. Reagents

a) Phosphate Buffered Saline (PBS)

NaCl - 8.00 g

KCl	- 0.20 g
$Na_2HPO_4.2H_2O$	- 1.44g
KH_2PO_4	- 0.02g
Distilled Water	- 1000ml

pH was adjusted to 7.2 with I N HCl or NaOH

b) PBS EDTA solution

EDTA	- 20mg
PBS	- 100ml

Sterilized by autoclave

c) Trypsin solution

Trypsin	- 200mg
Glucose	- 20mg
PBS-EDTA	- 100ml

Sterilized by filtration

d) Alsever's solution

Dextrose	- 2.05g
Sodium Citrate	- 0.80g
NaCl	- 0.42g
Distilled water	- 100ml

pH adjusted to 6.1 with 10% acetic acid.

e) Griess Reagent

A. 0.1% N- (1-Naphthylethylene diamino dihydrochloride) (NNED)

B. 1% Sulfanilic acid in 5% H_3PO_4

Solution A and B was mixed in 1:1 ratio

f) Stains

i) Trypan blue

Trypan blue	- 100mg
Saline (0.9%)	- 100ml

ii) Eosine

Eosine	- 500mg
Saline (0.9%)	- 100ml (Final volume)

Eosine was dissolved in 5ml of distilled water and made up to 100ml with ethanol.

iii) Harris haematoxylin

Haematoxylin	- 5g
Ethyl alcohol	- 50ml
Potassium alum	- 50mg
Potassium iodide	- 50mg
Distilled water	- 950ml

Hamatoxylin was dissolved in alcohol using gentle heat. The alum was dissolved in distilled water by heating with frequent stirring and keep overnight at 4^0C. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium was added and filtered.

iv) Crystal violet

Crystal violet	- 50mg
Methanol	- 20ml
Distilled water	- 80ml

g) Scintillation Fluid

PPO	- 2.5g
POPOP	- 0.25g
Naphthalein	- 100g
Dioxan	- 1000ml

IV) Animals

Balb/C, Swiss albino (6-8 weeks) and C57BL/6 mice (4-6 weeks) old were purchased from National Institute of Nutrition, Hyderabad. Animals were maintained in ventilated cages and fed with normal mouse chow (Sai Durga Feeds, Pune) and water *ad libitum*

All the animal experiments were done according to the rules and regulations of Animal Ethics Committee Govt. of India

V) Cell lines

L929 (Mouse lung fibroblast) cells, K562 (Human leukemic cells) EL 4 and B16F-10 (Metastatic melanoma) cells were obtained from National Centre for Cell sciences, Pune, India.

Daltons Lymphoma Ascites (DLA) cells (arose as a spontaneous Carcinoma of thymus) were obtained from Cancer Institute, Adayar. Ehrlisch ascites tumour cells (EAC) were obtained from Cancer Research Institute, Mumbai. Both cell lines were maintained as ascites tumour in Swiss albino mice.

VII) Instruments

1.Blind well chamber (Modified Boyden chamber)	-Nucleopore, Cambridge,USA
2. Cooling Centrifuge	-Remi, Chennai
3. CO_2 Incubator	-Napco, Canada
4. Disc electrophoresis tank	-Balaji Scientific Services, Madras
5. ELISA reader	-Stat Fax
6. Gamma ray spectrometer	-Wallac-Wizard
7. Inverted microscope	-Leica, Germany
8. Laminar flow hood	-Klenzaids Contamination control PVT.Ltd. Gujarat
9.Liquid Scintillation Counter	- Rack Beta, LKB, Walac
10. Research Microscope	- Meiji, Japan
11. Rotary Microtome	- Lab Agencies, Kerala
12. Sonicator	- Labline Instruments,Illinois, USA
13. Spectrophotometer	- SL 150, Elico Pvt.Ltd,India
14. Tissue homogenizer	-Yorco Scientific Industrues, Delhi

B. Methods

I. Tissue culture

I a. Sterilization of glass wares

All glasswares and filtration apparatus used for tissue culture purposes were soaked in a solution of Extran (1%) overnight, cleaned using brush and washed thoroughly under running water. All the glasswares were rinsed in distilled water and dried in a hot air oven. These were then autoclaved at a pressure of 15 pounds/square inch for 15 minutes, dried and used for experiments.

I.b. Preparation of Culture Media

DMEM (9.98g/l) MEM (10.3g/l) and RPMI (10.3g/l) were prepared in autoclaved double distilled water, pH adjusted to 7.2, and filtered under negative pressure using a 0.22μm cellulose filter. Sterility of the medium was tested using fluid thyoglycollate medium. For this 10ml sterile thioglycollate (929.8g/l) medium was inoculated with I ml of medium prepared and incubated at 37^0 for 6 days, and checked for visible contamination. Antibiotics-Penicillin (100u/ml) and streptomycin (100mg/ml) were added to the medium prior to use.

I.C. Maintenance of Cell lines in Tissue Culture

L-929 cells

The spent medium was removed from the confluent bottles and the cells were washed thrice with 2ml of PBS-EDTA. 1 ml of trypsin solution containing 0.02% EDTA was added and incubated for 3-4 minutes at 37^0C and the bottles were tapped to dislodge the cells. MEM (5ml) containing 10% goat serum and antibiotics (complete medium) was added. Cells were dispersed to single cell suspension by repeated pipetting and an aliquot of cell suspension was

added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37^0C. Cells were subcultured every week. (Freshney, 1987)

K-562 cells

The cell suspension was mixed well, dispersed the clumps by repeated pipetting. The cells were counted and $1x10^6$ cells were seeded to fresh bottles containing 10 ml of RPMI-1640 medium with 10% FCS and antibiotics and incubated at 37^0C and subcultured every third day (Freshney, 1987).

B16F-10 melanoma cells

The spent medium was removed from confluent bottles and the cells were washed three times with PBS. 1ml trypsin solution free of EDTA was added and incubated for 3-4 minutes at 37^0C. Bottles were then tapped to dislodge the cells. DMEM containing 10% FCS and antibiotics (complete medium) was added and the cells were dispersed to single cell suspension by repeated pipetting. An aliquot of the cell suspension was added to fresh bottles containing 10ml of complete medium and incubated at 37^0C. The cells were subcultured every week (Freshney, 1987).

II. Maintenance of experimental animals

C57BL/6 mice, Balb/C mice and Swiss albino mice were used for the experiments. They were housed in ventilated cages and fed with pelleted mouse chow (Sai feeds, Pune) and water *ad libitum*.

III. Maintenance of tumours in animals

Dalton's Lymphoma Ascites (DLA) cells and Ehrlisch Ascites Carcinoma (EAC) cells were propagated in the peritoneal cavity of Swiss albino mice and maintained by transplanting

the cells every two weeks. Tumour cells were aspirated from the peritoneal cavity, washed with PBS and $1x10^6$ cells were injected intraperitoneally to induce ascites tumour.

B16F-10 melanoma cells were propagated in C57BL/6 mice as transplantable solid tumours. $1x10^6$ cells were injected subcutaneously to the hind limb of the mouse. After 10-15 days, the animal was sacrificed, tumour mass mashed and processed in PBS. $1x10^6$ viable cells were injected to another set of animals.

IV. Preparation of B16F-10 cells for *in vivo* studies

For *in vivo* studies, tumour mass from animal was resected and processed to get a single cell suspension. Tumour mass was mashed and the pieces were forced through a fine steel mesh using PBS. The cells were separated from RBC's and then suspended in PBS to required cell number and used for *in vivo* experiments after determining cell viability

V. Preparation of cells for *in vitro* studies

a. B16F-10 Cells

Tumour cells were harvested from sub confluent cultures (70-80% confluency). The spent medium was removed, monolayers were washed three times with PBS and the cells were harvested by mechanical dislocation using a cell scraper. Cell concentration was adjusted to required number. Cell suspension with 90% viability were used for *in vitro* experiments.

b. L-929 Cells

The spent medium was removed, monolayers were washed three times with PBS and the cells were layered by 1ml of trypsin containing EDTA and incubated at 37^0C for 3-4 minutes. Cells were dislodged by tapping the bottles and a single cell suspension was prepared by adding 5ml MEM (complete medium) and repeated pipetting. Cell viability checked by trypan blue dye exclusion method (Kuttan et al., 1985) and used for experiments.

VI. Determination of cell viability

Cell viability was determined by trypan blue dye exclusion method (Kuttan et al., 1985). 0.1 ml of cell suspension was mixed with 0.1ml of 1% trypan blue, kept for 2-3 minutes and loaded on a haemocytometer. Viable cells exclude trypan blue dye, while non-viable cells take up the dye and thus appeared blue in colour. The number of stained and unstained cells was counted separately.

$$\% \text{ Dead cells} = \frac{\text{Number of dead cells}}{\text{Number of viable cells + Number of dead cells}} \times 100$$

VII. *In vitro* Cytotoxicity studies

Tumour cell ($1x10^6$) were mixed with various concentrations of test compounds and the final volume was adjusted to 1ml with PBS. Control tubes contained tumour cells only. The assay mixture was then incubated at 37^0C for 3h and the percentage of dead cells was determined by trypan blue dye exclusion method (Kuttan et al., 1985).

VIII. Long term *in vitro* Cytotoxicity studies by tissue culture

B16F-10 melanoma cells growing in log phase was used for this experiment. Cells were collected by trypsinization and 5000cells/well were seeded into 96well flat bottomed tissue culture plate containing 250µl complete medium and incubated at 37^0C. After 24hours of incubation, various concentrations of the test compounds were added to the bottles and then incubation was continued for 48hours.4 hour before termination 20µl of MTT solution (5mg/ml) was added to each well. After the incubation period the plates were centrifuged, supernatant was removed and 100µl DMSO was added to each well. The plates were then incubated at room temperature for 15 minutes and the optical density was measured at 570nm with reference of 690nm. The percentage dead cells was determined using the formula,

$$\% \text{ dead cells} = \frac{\text{O.D of drug treated}}{\text{O.D of Control}} \times 100$$

IX. Hematological parameters

1. Determination of Haemoglobin (Cheesbrough et al., 1976)

Principle

Ferricyanide forms methamoglobin with haemoglobin, which is converted to cyanmethaemoglobin by cyanide, which has absorption at 540nm.

Procedure

0.02 ml of blood was mixed with 5ml of Drabkin's reagent and allowed to stand for 5minutes at room temperature. Optical density (OD) was measured against reagent blank. Haemoglobin content was calculated using the formula,

$$\text{gm \% of Hb} = \frac{\text{OD of the test}}{\text{OD of Std.}} \times \frac{251 \times \text{conc. of Std.}}{1000}$$

2. Determination of total count of leukocytes

Principle

The cells were diluted in Turk's fluid, which contains a week acid (acetic acid) to lyse RBC and a stain (crystal violet), for staining the leukocytes. The number of cells in the large four corner square was coumted (Cheesbrough et al., 1976).

Procedure

Blood (0.02ml) was mixed with 0.38ml of Turk's fluid and kept at room temperature for 2-3 minutes. The cells were mixed gently and loaded on to the haemocytometer, allowed to settle at the bottom of the chamber for 2 minutes and counted under a microscope using 10x objective. The total WBC counts was determined using the formula,

$$\text{Total leucocyte counts/mm}^3 = \frac{\text{No. of cells counted x dilution factor x depth factor}}{\text{Area counted}}$$

Where,

Dilution factor = 1/20

Depth = 1/10mm

Area counted = 4sq.mm

$$\text{Therefore, Total leukocyte counts/mm}^3 = N \times \frac{20 \times 10}{4} = N \times 50$$

3. Differential count of leucocytes (Cheesbrough et al., 1976)

Procedure

A thin film of blood was made by spreading a drop of blood evenly across a clean glass slide using a glass spreader and air dried. Few drops of Leishman's stain was poured over the smear and kept for 3minutes. The stain was diluted with distilled water and kept for seven minutes, washed with tap water and allowed to air dry. Various types of cells were scored

using the morphology under oil immersion with 100x objective and a total of 100 cells were counted.

X. Immunological parameters

X.1. General Techniques

a) Collection and preparation of SRBC (Mehra et al)

Sheep blood was freshly collected from the slaughters house in equal volumes of sterile Alsever's solution and stored at 4^0C for not more than one week. Cells were washed three times with PBS (pH 7.2). Supernatant discarded and the pellet was suspended in Hanks balanced salt solution (HBSS)

b) Trypsinization of SRBC

10 parts of 4% SRBC and one part of 1%trypsin solution were incubated at 37^0C for 30 minutes. After incubation cells were washed twice in PBS (pH 7.2) and resuspended at a concentration of 2%.

c) Preparation of anti SRBC antibody

A young healthy rabbit was injected intradermally with 2% SRBC in saline which was mixed with Freunds complete adjuvant in a ratio of 1%. A booster dose was given four weeks after the initial dose. Next day after the booster dose, animal was bled and serum separated; heat inactivated and checked the antibody titre by the haemagglutination method (Singh et al., 1984). According to the antibody titre value, serum was diluted and used for the experiments.

d) Preparation of spleen cells

All the procedures were done under sterile condition. The animals were sacrificed, the skin was cleaned with rectified spirit. An incision was made on the left side just below the rib and spleen was removed with out any adherent tissue. Spleen was cut into small pieces and teased over a stainless steel mesh in cold PBS or HBSS. Clumps were allowed to settle in a centrifuge tube, kept in ice bath for 2minutes. Supernatant was collected, washed three times with HBSS and resuspended in RPMI-1640 medium at required concentrations.

e) Preparation of bone marrow cells

All the procedures were done under sterile conditions. Mice were sacrificed by cervical dislocation and fixed on a board with fore and hind limbs fully stretched. The skin and flesh overlying the limbs were removed and the femur was exposed. The shaft of the femur was separated from both ends and the bone marrow was flushed out of the cavity by passing a jet of medium with 2% FCS through the ends of the shaft using a 26G needle and syringe. Flushed bone marrow was made into a single cell suspension by repeated pipetting. It was then centrifuged and suspended at required cell concentrations in RPMI-1640 medium.

f) Preparation of thymus cells

All the procedures were done under sterile conditions. Animals were sacrificed by cervical dislocation. The skin was cleaned and body was incised at the upper part above the heart. Bilobed thymus was detached, suspended in HBSS, and processed. The same way for spleen and the thymocytes were suspended in RPMI-1640 medium containing 10%foetal calf serum.

g) Preparation of peritoneal macrophages

Peritoneal macrophages were elicited by injecting 0.2ml of 5% sodium caesinate solution. After five days animals were sacrificed by cervical dislocation. All the procedures were done under sterile conditions. Mice were fixed on a board, skin was removed and the peritoneum was exposed. The peritoneal cavity was distended by injecting 5ml of PBS or HBSS. The peritoneal cavity was gently prodded and the peritoneal fluid containing macrophages was aspirated. The cells were washed and suspended in RPMI-1640 to the desired cell concentrations.

X.1.1. Determination of circulating antibody titre

Principle

The non-agglutinated SRBC will settle to the bottom of the well as a clear 'button' while agglutinated cells settle as a diffused 'mat'. The maximum dilution of anti-sera at which clear agglutination observed gives the titre of the antibody (Singh et al., 1984).

Procedure

Anti-sera (0.1ml) were serially diluted in round bottom 96 well tissue culture plates containing 0.1ml PBS/well (pH 7.2). 0.1ml of trypsinized SRBC was added to each well, mixed gently and incubated at room temperature for 3hours. The dilution at which clear agglutination seen was noted.

X.1.2. Determination of antibody forming cells

Principle

Antibody produced by the lymphoid cells from animals immunized with SRBC cause lysis of red cells in its vicinity (plaques) in a semi-solid support in the presence of complement (Jerne and Nordin, 1963)

Procedure

0.5 ml of Agarose (0.5%) was distributed in to tubes kept at 45^0C and 0.05ml SRBC (7%) and 0.05ml spleen cells ($8x10^6$ cells/ml) were added and mixed well. The contents were poured over a glass slide, spread in an area of $10cm^2$ and the gel was allowed to solidify. The slides were kept in an incubation rack filled with fresh rabbit serum (1:10 diluted with PBS, pH 7.2) as a source for complement and incubated for 1hour at 37^0C. The number of plaques were counted using a colony counter and represented as plaque forming cells/10^6 spleen cells.

X.1.3. Assay for lymphocyte, thymocyte and bone marrow cell proliferation

Principle

Mitogens can stimulate resting lymphocytes to undergo a series of biochemical and physical changes and are converted to blast-like cells. This process leads to cell division and can be quantitated by ^{3}H thymidine uptake method (Mustafa, 1993)

Procedure

All the techniques were sterile during the experiment. Spleen cells/thymus cells/bone marrow cells ($5x10^4$) were incubated with and with out mitogens in a final volume of 200µl of RPMI-1640medium in 96 well flat bottom titre plates supplemented with 10% FCS and antibiotics in a humidified atmosphere containing 5% CO_2 at 37^0C for 48 hours. The concentrations of the mitogens added were; PHA-2.5µg/ml; Con-A -10µg/ml PWM 10µg/ml; and LPS 10µg/ml. 1µCi of ^{3}H thymidine was added to each well and incubated further for 18 hour under the same conditions. The cultures were centrifuged at 1500rpm for 10minutes. Supernatant discarded and the pellets were dissolved in 200µl of 6N NaOH and incubated at 37^0 for 2hour. Then the contents were transferred to 5ml scintillation fluid, kept overnight in dark. Counts per minutes (CPM) was measured in a liquid scintillation counter.

^{51}Cr-release assay was used to determine the cytotoxicity mediated by immune effector cells such as natural killer cells and cells expressing Fc receptors (ADCC) and was performed in round bottom titre plates (Gupta, 1993).

Principle

^{51}Cr binds to cytoplasmic proteins after diffusing through the cell membrane and is released only when the cell membrane is sufficiently damaged.

Labelling of target cells

The target cells, K-562 (10^6) and SRBC (10^7) were washed twice in RPMI-1640 and resuspended in few drops of FCS. 100μCi of $Na_2{}^{51}CrO_4$ was added and incubated at 37^0C for lh on a shaker. The cells were washed in medium twice and allowed to incubate in large volumes (5ml) of medium for 1h at 4^0C. Cells were washed twice in medium and resuspended in complete medium at a concentration of $1x10^5$ cells/ml.

a. Determination of Natural Killer cell- mediated cytotoxicity

Labelled target cells (K-562, 0.1ml) and equal volumes of various dilutions of spleen cells (to yield effector target ratios of 100:1, 50:1, and 25:1) were added to 96 well round botton titre plates. Final volume was adjusted to 0.2ml with RPMI-1640 supplemented with 10% FCS and incubated at 37^0C for 4h. Titre plates were centrifuged for 15 minutes, supernatant (100μl) collected and radioactivity measured in a gamma ray spectrometer (Gupta, 1993).

The following control tubes were kept along with each experiment.

Spontaneous release (SR)- wells contained only target cells and medium.

Total release (TR) - wells contained target cells, medium and 0.1 ml of 1N HCl.

Calculations

$$\% \text{ Lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

b. Determination of antibody-dependent cellular cytotoxicity (ADCC) (Gupta, 1993)

0.1ml of labeled SRBC (target cells) and 0.1ml of spleen cells (effector cells) were added to get an effector-target ratios of 100:1, 50:1 and 25:1.

0.05ml of anti-sera against SRBC was added and incubated at 37^0C for 4h. The final volume was made up to 0.2ml with complete medium and the 4h ^{51}Cr release assay was performed as explained above.

c. Determination of antibody-dependent complement-mediated cytotoxicity

Principle

When tumour cells are incubated with specific antibodies in presence of complement, the classical pathway will be activated leading to the lysis of target cells (Mehrotra, 1993)

Procedure

Anti-serum was diluted in RPMI-1640 to get 1:1, 1:2 and 1:4 dilutions of the antibody and 0.1 ml of the serum was mixed with 10^6 EAC cells. 0.05ml of 1:10 diluted fresh rabbit serum as a source of complement was added and the final volume was made up to 2ml and incubated at 37^0C for 3h. The cells were centrifuged and 1 ml of the supernatant was discarded and cytotoxicity was assessed by trypan blue exclusion method (Kuttan et al, 1985)

Determination of α-naphthyl acetate esterase activity (Bancroft and Cook , 1984)

The enzyme hydrolyses the substrate to form an invisible primary reaction product. The complex is coupled with the diazonium salt to produce coloured final reaction product.

Procedure

Animals were sacrificed by cervical dislocation. Skin and flesh removed from the femur of the animal. Bone marrow cells were flushed in PBS with 10% goat serum using a syringe. Cells were counted and thin smears prepared on glass slides. The smear was air dried and fixed using 10% formaldehyde. Slides were incubated in a reaction buffer containing pararosaniline, sodium nitrate and α-naphthyl acetate at room temperature. Smear slides were counterstained with haematoxylin for 2minutes. α –esterase positive cells take up a yellowish brown colour and cells were counted under microscope using oil immersion.

XI. Antimetastatic studies

Determination of the metastatic potential of B16F-10 melanoma cells in animal model

Studies on the metastatic ability of tumour cells (*in vivo*) were done in C57BL/6 mice. Pulmonary colony forming ability of B16F-10 cells were carried out as described by Fidler (). C57BL/6 mice were injected with B16F-10 cells ($1x10^6$) through the lateral tail vein. Animals were sacrificed on 21st day. Metastasis of the lungs were determined by a) counting the metastatic foci on the surface of the lungs.b) By measuring biochemical parameters such as lung collagen hydroxyproline, lung uronic acid, lung hexosamine, serum sialic acid, and serum γ-glutamyl transpeptidase c). Histopathological analysis of lungs and f) By determining the rate of survival.

XI.a. Biochemical parameters

Estimation of protein

Principle

This assay relies on the formation of protein copper complex and reduction of Phosphomolybdate-Phosphotungstate reagent (Folin Ciocaltau reagent) by tyrosine and tryptophan residues of protein (Lowry et al, 1951)

Reagents

Solution A

1. Sodium potassium tartarate - 1ml(2%)
2. $CuSO_4$ - 1ml(1%)
3. Na_2CO_3 - 98ml(2% in 0.1N NaOH)

Solution B

Folin's phenol reagent - 1N, diluted 1:1 with distilled water

Procedure

20µl sample and different concentrations of standard BSA (150µg, 100µg, 50µg and 25µg) were made up to 1.2ml with distilled water. To this, 6ml of solution A was added and then incubated at room temperature for 10minutes. 300µl solution B was then added to the

reaction mixture vortex mixed and incubated at room temperature for 30minutes. Optical density read at 660nm.

Estimation of Hydroxyproline (Bergman and Loxley, 1970)

Principle

Hydroxyproline present in samples were oxidized by chloramineT. The coloured product is more stable in the presence of high concentrations of isopropanol.

Reagents

1.Oxidant solution

Sodium acetate	- 5.7g
Trisodium citrate	- 3.75g
Citric acid	- 0.55g
Isopropanol	- 38.5m
Distilled water	- 61.5m

2.Ehrlisch's reagent

p-dimethyl amino benzaldehyde	- 4.4g
Perchloric acid	- 10.2g (60%)
Isopropanol	- 25ml (Final volume)
Chloramine T	- 1.75g/25ml oxidant solution
	solution prepared on the day of use

Procedure

Lung tissue (1g) was homogenized using 4ml isotonic saline and hydrolysed in 6 N HCl. The tubes were sealed and incubated at 110^0C for 24h. 1ml hydrolysate was neutralized with KOH then made up to 5ml with H_2O. To 0.5ml neutralized sample 2.5ml isopropanol and 1ml oxidant solution was added by mixing and kept at room temperature for 4minutes. 2ml Ehrlisch's reagent was added to the tubes and incubated at 60^0C in water bath for 21minutes. Then the tubes were kept at room temperature for 1h. The absorbance was taken at 560nm.

Extraction and estimation of uronic acid

Extraction of uronic acid from the tissue was carried out according to the method of Schiller et al (Schiller et al., 1961). Digestion of the tissue was carried out with crude papain (10mg/g dry weight of tissue) in 5ml of 0.5M acetate buffer of pH 5.5 containing 0.005M cysteine and 0.005 M disodium salt of EDTA at 65^0C for 24h. An aliquot of the sample containing approximately 5-15 mg uronic acid was taken for estimation. Uronic acid was estimated by the method of Bitter and Muir (Bitter and Muir, 1962).

Procedure

Aliquote (5ml) of sulphuric acid reagent (prepared with 0.025M sodium tetraborate in conc. H_2SO_4) was taken in tubes and cooled at 4^0C for some time. 1ml of sample or standard glucaronolactone solution containing 5-20mg was layered on the acid. Tubes were closed with ground glass stoppers and the rack was shaken first gently and then vigorously. Tubes were kept in a boiling water bath for 10minutes and cooled at room temperature. 0.2ml of carbazole reagent (0.125% carbazole in absolute alcohol) was added and the tubes were shaken heated in

a boiling water bath for 15minutes and cooled. The pink colour thus developed was read at 530nm.

Uronic acid content of the tissues were expressed as µg/100mg net weight.

Preparation of sulphuric acid reagent

0.025M sodium tetraborate in concentrated H_2SO_4

Acetate Buffer (0.1M)

A. 0.2M solution of acetic acid

B. 0.2M solution of sodium acetate

Estimation of Hexosamine (Elson and Morgan, 1933)

Lyophilized tissue samples (5mg) were hydrolyzed with 2N HCl (5ml) at 100^0C for 6h. HCl was then removed by evaporation; the residue was dissolved in water and made up to a known volume.

Procedure

Aliquots containing 10-15µg hexosamine were treated with 1ml of freshly prepared 2% acetyl acetone in 0.5N Na_2CO_3 in capped tubes and kept in boiling water bath for 15minutes. After cooling, 5ml of 95% ethanol and 1ml of Ehrlisch's reagent (1.33% p-dimethylaminobenzaldehyde in 1:1 ethanol: Conc.HCl mixture) were added and mixed thoroughly. The purple red colour developed was read after 30minutes at 530nm. Water blank and standard glucosamine solutions of various concentrations were also treated similarly to get a standard curve.

Hexosamine contents of tissues were expressed as µg/100mg dry weight.

Preparation of 2% acetyl acetone in 0.5M Na_2CO_3.

2ml of acetyl acetone in 100ml of 0.5M Na_2CO_3.

Preparation of Ehrlisch's reagent

1.33g of p-dimethyl aminobenzaldehyde (PDAB) was dissolved in 100ml of 1:1 ethanol: conc. HCl.

Estimation of protein bound serum sialic acid (Skoza and Mohos, 1976)

Principle

Acid hydrolysis of serum for liberation of sialic acid forms a coloured compound with thiobarbituric acid.

Reagents

1. H_2SO_4	- 0.2N
2. Periodic acid	- 25μM in 62.5 mM H_2SO_4
3. Sodium arsenite	- 0.2% in 0.5M HCl
4. Thiobarbituric acid	- 6% (pH 9.0)
5. Dimethyl sulphoxide (DMSO)	- 400 μl

Procedure

200μl of sample was mixed with equal volume of 0.2N H_2SO_4 and hydrolyzed for 1h at 80^0C. To this hydrolysate 50μl periodic acid (25μM) was added and incubated for 30minutes at 37^0C. To this reaction mixture 50μl of sodium arsenite was added, followed by 100μl of thiobarbituric acid and was heated in a boiling water bath for7.5minutes. After heating, 400μl of DMSO was added to intensify the colour and read at 549nm and 532nm

Estimation of γ-glutamyl transpeptidase (Tate and Meister , 1974)

Principle

γ-glutamyl transpeptidase catalyses the transfer of γ-glutamyl moiety of a γ-glutamyl donor to a variety of acceptors.

2_γ- (glutamyl-p-nitroanilide)⟶ γ-glutamyl- γ-glutamyl-p-nitroanilide + acceptor⟶ γ-glutamyl acceptor + p-nitroaniline

Reagents

L- γ-glutamyl-p-nitroanilide	- 2.5nM
Glycyl glycine	- 20mM
Tris-HCl (pH 8.0)	- 0.05M
NaCl	- 75mM

Procedure

The standard assay mixture contained (1ml) 0.05M Tris HCl, 75mM NaCl, 2.5mM-L- γ-glutamyl-p-nitroanilide and 20mM glycyl glycine along with 25µl sample. The rate of release of p-nitroaniline was measured at an optical density of 410nm using a spectrophotometer.

XI.b. Histopathological Analysis

The tissue as soon as they are removed were fixed in 10% neutral formalin for at least 4h. The tisues were dehydrated in alcohol series, cleaned in xylene and embedded in paraffin. About 5-6µm thick sections were taken on a glass slide and stained with haematoxylin and eosin and visualized under the microscope for histological changes (Culling, 1976)

XI.c. Determination of the rate of survival

Animals were injected with B16F-10 melanoma cells ($1x10^6$) intravenously. The mortality of the animals was noted and the percentage increase in life span (% ILS) was calculated from the formula,

$$\%ILS = \frac{T - C}{C} \times 100$$

Where, 'T' is the number of days drug treated animals survived and 'C' is the number of days control animals survived.

XII. *In vitro* antimetastatic studies

XII.a. Collagen matrix invasion assay

Invasion of collagen matrix by tumour cells was carried out using modified, Boyden chambers (Blind well chambers) as described by Albini et al (Albini et al., 1987). The lower compartment of the chamber was filled with DMEM without FCS and polycarbonate filters coated with 25µg Type I collagen were placed above this in blind well chamber. B16F-10 cells ($1x10^5$/0.1ml) prepared and suspended in FCS with out FCS. The cell suspension was added to the upper chamber and incubated at 37^0C for 1h in a 5% CO_2 atmosphere. After the incubation period medium from the upper chamber was removed and the cells on the upper side of the filter was removed by a cotton swab. The filter was then fixed in methanol for 1minute and stained for 3minutes with crystal violet. Cells that penetrated the polycarbonate filters were counted in 10 fields under a microscope. Results were calculated as % inhibition of invasion using the formula,

$$\% \text{ inhibition of invasion} = 100 - \left[\frac{\text{mean no. of migratory cells in test}}{\text{mean no. of migratory cells in control}} \right] \times 100$$

XII.b. Tumour cell motility assay

B16F-10 cells ($1x10^5$/0.15ml) were seeded on the upper compartment of blind well chamber, containing polycarbonate filter without collagen coating. Chambers were incubated at 37^0C for 24h. Migrated cells were collected from the lower chamber and counted using a haemocytometer. Results were calculated as,

$$\% \text{ Motility} = \frac{\text{Mean no.of migrating cells in test}}{\text{Mean no.of migrating cells in control}} \times 100$$

Tumour cell adhesion assay (Inokuchi and Jimbo , 1991)

Principle

Transformed cells have higher adhesive attachment rates to a variety of homotypic or heterotypic cell susbstrates. Metastatic cells are always found to have higher rates of homotypic attachment (Nicolson, 1978)

Procedure

B16F-10 melanoma cells ($5x10^3$ cells/well) were added to 96 well flat bottom titre plates, pre coated with collagen type I (25µg/well) and incubated for 5h at 37^0C in 5%CO_2 atmosphere. After incubation, medium was removed and the wells were washed with PBS.

Adhering cells were fixed with 5% formaldehyde and stained using crystal violet for 20minutes each. The cells were counted under an inverted microscope.

XII.c. Gelatin Zymography (Billing , 1991)

Principle

Proteases of tumour cell lysate were initially resolved on SDS- poly acrylamide gels, which were incorporated with gelatin. Following incubation of the gel in the activation buffer, protease separated on the gel will breakdown the gelatin and appears as transparent zones or clearings against a dark back ground (upon staining)

Reagents

1. 0.25M sucrose- 0.01M Tris-HCl buffer, pH 7.4

Sucrose	- 85.87g
Tris-HCl	- 1.21g
Distilled water	- 1000ml (Final volume)

2. 0.1M Tris-HCl, pH-8.0, 10mM $CaCl_2$

$CaCl_2.2H_2O$	- 1.47g
Tris	- 12.1g
Distilled water	1000ml (Final volume)

Distilled water- 1000ml (Final volume)

pH adjusted with Conc.HCl

3. Trypsin solution

Trypsin -75µg/ml in 0.1M Tris-HCl, with 10mM $CaCl_2$, pH 8

4. Activation buffer (0.1M Tris-HCl, 10mM $CaCl_2$, pH 7.8)

Tris HCl -12.1g

$CaCl_2.2H_2O$ - 1.47g

Distilled water- 1000ml (Final volume)

5. Preparation of gels

5.a) Resolving Gel

11% Polyacrylamide gels with 0.1%SDS and 0.6% gelat

29.2% acrylamide + 0.5% bisacrylamide - 11ml

0.1M Tris-HCl, pH 8.8 - 1.2ml

20% SDS - 0.15ml

20% Ammonium per sulphate - 0.10ml

Gelatin (180mg/2ml distilled water, heated to dissolve) - 2ml

Distilled water - 6.505 ml

TEMED - 0.045ml

Mix and pour at room temperature.

5.b) 5% Stacking gel

29.2% acrylamide +0.5% bis acrylamide - 1.67ml

0.1M Tris-HCl, pH 8.8 - 1.75ml

20%SDS - 0.10ml

20% Ammonium per sulphate - 0.10ml

Distilled water	- 6.36ml
TEMED	- 0.02ml

Mix and pour above the resolving gel at room temperature

6. Sample buffer (2x)

Glycerol	- 1ml
1M Tris-HCl, pH 6.8	- 0.25ml
20% SDS	- 1ml
Bromophenol blue (Tracking dye)	- 1.65mg

Made up to 5ml with distilled water

7. Running buffer

Tris base	- 3g
SDS	- 2g
Glycine	- 14.2g

Made up to 1L with Distilled water

8. 2% Triton X-100

Triton X-100	- 2ml
0.1M Tris HCl, pH 7.8	- 100ml (Final volume)

9. 10mM EDTA solution

EDTA- Na_2	- 372.24mg
0.1M Tris-HCl, pH 7.8	- 1000ml (Final volume)

Procedure

Gelatin Zymography was followed according to the procedure of Billings (Billings, 1991) with some modification. The medium from sub-confluent (70%) bottles of B16F-10 tumour cells were removed, cells were then washed with serum free medium and incubated in serum free medium (DMEM) at 37^0C for 24h.

After the incubation, medium was collected, centrifuged, and supernatent was used for zymographic analysis. After determining the protein concentration, supernatant (equvalent to 50μg protein) containing the proteases were activated with trypsin (75μg/ml, 5μl trypsin solution for 100μg protein) for 30 minutes at room temperature. Trypsin treated and untreated samples (equivalent to 50μg protein were mixed with an equal volume of sample buffer (2x), with out reducing agent, loaded on 0.1%SDS -11% polyacrylamide gels containing 0.6% gelatin and electrophoresed at 10^0C for 3h with a constant current of 2mA per tube until the tracking dye (Bromophenol blue) reach the periphery of the gels.

The gels were then washed with 2% Triton X-100 on a shaker at 20-25^0C for three changes of 30 minutes each, to remove the SDS which could interfere with proteolytics activity. This was followed by 2h washing with activation buffer and the gels were finally incubated in the same buffer at 37^0C for 18h, in the presence and absence of test compounds. Gels were then fixed and stained with Gelcode Blue stain reagent and clear bands were visualized against a dark background.

XIII Determination of *in vitro* antioxidant activity

XIII a Superoxide scavenging activity

Superoxide scavenging activity was determined by the light induced superoxide generation by riboflavin and subsequent reduction of nitroblue tetrazolium as described by

McCord and Fridovich (McCord and Fridovich, 1969). The reaction mixture contained EDTA (6μM) containing 3 μg NaCN, riboflavin (2μM), NBT (50μM), various concentrations of the test material and phosphate buffer in a final volume of 3 ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 min and the optical density was measured at 530 nm before after illumination. The percentage inhibition was evaluated by comparing the absorbency value of the control tubes and experimental tubes.

XIII b Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the test material was measured by studying the competition between deoxyribose and test compounds for hydroxyl radicals generated from the Fe^{3+}/ ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose which eventually results in the formation of thiobarbituric acid reacting substances (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1μM), EDTA (0.1 mM), H_2O_2 (1mM), ascorbate (0.1 mM) KH_2PO_4 -KOH (20 mM, pH 7.4) and various concentrations of the sample in a volume of 1 ml was incubated for 1 h at 37^0C. Deoxyribose degradation was measured as thiobarbituric acid reactive substances by the method of Ohkawa et al. (Ohkawa et al., 1979). The inhibition produced by different concentrations of the sample as well as the concentration required for 50% inhibition was calculated.

XIII c Lipid peroxidation assay

Lipid peroxidation was induced in mice liver homogenate by the method described by Bishayee and Balasubramonian (Bishayee and Balasubramonian , 1971) in the presence of different concentrations of the test material and estimated by thiobarbituric acid reactive

substances by the method of Ohkawa et al (Ohkawa et al., 1979). Different concentrations of the test material was incubated with 0.1 ml of rat liver homogenate (25%) containing 30 mM KCl, Tris- HCl buffer (0.04 M, pH 7.0), ascorbic acid (0.06mM) and ferrous ion (0.16 mM) in a total olume 0.5 ml for 1h. At the end of the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS (8.1%), 1.5 ml thiobarbituric acid (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and kept in water bath at 95 ^{0}C for 1h. After cooling, 1ml distilled water and 5 ml butanol-pyridine mixture (15:1 v/v) was added. After vigorous shaking, the tubes were centrifuged and the upper layer containing the chromophore was read at 532 nm. The percentage inhibition was calculated and the concentration required for 50 % inhibition was calculated.

XIII d Nitric oxide inhibition activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al., 1982). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the compound (from 1 to 100 µg/ml) was incubated at 25° C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was determined at 546 nm.

XIV Determination of CTX and radiation induced toxicity

a Estimation of tissue glutathione (GSH)

The method described by Moron et al (1979) was used. Glutathione in the tissue homogenate was determined by using 5'5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) to give a yellow coloured complex with absorption maximum at 412 nm.

Procedure

25 µl of 25% trichloroacetic acid (TCA) was added to 0.5ml of homogenate to precipitate the protein. The tubes were cooled in ice for 5 min and the mixture was further diluted with 0.6 ml of 5% TCA and centrifuged at 1000 rpm for 10 min. From the supernatant, 0.3 ml was taken for the assay. The volume of the aliquot was made up to 1ml with 0.2 M sodium phosphate buffer (pH 8.0), and 2ml of the freshly prepared DTNB solution (0.6 mM in 0.2 M phosphate buffer, pH 8.0) were added to the tubes. The intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 min. Standard curve was prepared using reduced glutathione. Values were expressed in nmol/mg protein.

b Estimation of serum glutamate -pyruvate transaminase (GPT) activity

The method of Bergmayer and Bernt was used (Bergmayer and Bernt, 1980) to assay GPT. The enzyme GPT catalyses the reaction between 2-oxo-glutamate and L-alanine forming L- glutamate and pyruvate. The pyruvate produced thus react with 2-dinitrophenyl hydrazene giving a product, with absorption maximum at 520 nm.

Reagents: Phosphate buffer pH 7.4, substrate 1.78 g of DL alanine and 30 mg of ketoglutarate dissolved in 20 ml buffer containing 1.25 ml of 0.4 N NaOH. The solution was made up to 100 ml with buffer, pH 7.4 and kept at 4^0C. Dinitrophenyl hydrazene (DNPH, 20 mg % in 1N HCl), NaOH (0.4N), pyruvate standard 1%.

Procedure: 0.5 ml of the substrate was incubated for 3 min at 37 ^{0}C. Serum (100µl) was added after incubation. mixed well and incubated for 3 min at 37 ^{0}C. 0.5 ml of DNPH was added to this mixture and kept at room temperature for 20 min. The reaction was stopped by adding 5 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5min. Absorbance was measured at 520 nm. The enzyme activity was expressed as a measure of pyruvate formed, which was calculated form the standard curve of pyruvate.

c Estimation of Alkaline phosphatase (ALP) activity

The method of King and Kind was used for this assay (King and Kind, 1954). 4-amino antipyrene reacts with compounds containing phenolic groups. In presence of an alkaline oxidising agent, to give purple colour which can be measure at 520 nm.

Reagents: Alkaline phosphate buffer, pH 10 (6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate in 1 liter of distilled water). Disodium phenyl phosphate (100 mM), NaOH (0.5 N), 4- aminoantipyrene (0.6%), potassium ferricynide (2.4%), phenol standard 1 mg/ml.

Procedure: 1ml of substrate was incubated with 1 ml bicarbonate buffer and inoculated for 3 min at 37 ^{0}C. After incubation 100 µl serum was added, vortexed well and incubated for 15 min at 37 ^{0}C. After incubation 0.8 ml of 0.5 N NaOH, 1.2 ml $NaHCO_3$, 1ml of amino antipyrene and 1 ml potassium ferricyanide were added, mixed well and absorbance

was measured at 520 nm. The enzyme activity was expressed as the amount of phenol produced,which was calculated from the standard graph of phenol.

XVI. Statistical Data

Values were expressed as mean ± S.D. The statistical analysis was done by one-way ANOVA followed by Dunnett's test using Graphpad InStat software

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

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CHAPTER 3

IMMUNOMODULATORY ACTIVITY OF NATURALLY OCCURRING TERPENOIDS

1. INTRODUCTION

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintain a surveillance mechanism to recognize certain components that are considered nonself. Immunomodulators are substances, which modify the activity of the immune system. They have biphasic effects, some tend to stimulate immune system which are low, while other inhibits host defence parameters which are normal or already activated (Sities et al., 1991).

One of the major reasons for the rapid progression of human cancers is the ability of tumour cells to escape from the immune surveillance mechanisms of the body. Cancer cells may secrete immunosuppressive factors to modify the host immune responses. These factors can suppress immune response, thereby impairing the inflammatory responses, chemotaxis of phagocytes, and the complementary cascade. Some of these factors seem to be non-specific and lead to a generalized decline of immunity.

Immuno suppression is one of the major side effects of conventional cancer therapies. Drugs that could alleviate these side effects will be highly useful in cancer treatment. Use of plant products as immunomodulators are getting more and more importance in the field of cancer research. Some of the plants with known immunomodulatory activities are V*isum album*, (Kuttan and Kuttan, 1992), *Panax ginseng* (Singh et al., 1984), *Tinospora cordifolia* (Mathew and Kuttan, 1999), *Piper longum* (Sunila and Kuttan, 2005) etc. Compound such as polysaccharides, lectins (Hajito et al; 1989), proteins and peptides (Kuttan and Kuttan, 1992) present in plants have been shown to stimulate the immune system.

Terpenoids are the class of compounds widely distributed in plants. They occur widely in fruits and vegetables consumed by humans (Steinmetz and Potter, 1991). Although they are widely used for medicinal purposes in many Asian countries, their biogenesis and pleotropic action has not impacted on the practice of modern medicine (Nanjoo et al., 1998). In the present chapter the effect of terpenoids on immune system has been investigated.

2. MATERIALS AND METHODS

2.1 Animals: -

Male Balb/C mice 4-6 weeks old (20-25g) were used for this study.

2.2 Chemicals: -

Pararosaniline hydrochloride and α-naphthyl acetate were used for this study. All other chemicals and reagents were of analytical grade.

2.3 Terpenoid compounds

Carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin

Drug administration: - The compounds were suspended in light paraffin oil. Carvone and limonene were administered at a concentration of 100 μmoles/kg body wt/dose/animal, perillic acid, ursolic acid, oleanolic acid and glycyrrhizic were administered at a concentration of 50 μmoles/kg body wt/dose/animal and nomilin 10 μmoles/kg body wt/dose/animal. Five doses of the compounds were administered intraperitoneally for all the parameters.

For determining the effect of terpenoids on the hematological parameters, bonemarrow cellularity, α- esterase positive cell number, circulating antibody titre plaque forming cells and delayed type hyper sensitivity reaction, for each study nine groups of Balb/c mice (6nos/group) were used . Group I II, III, IV,V,VI and VII animals were treated with carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin respectively as explained above. Group VIII was treated with vehicle (paraffin oil) and group IX was kept as normal control.

2.4 Determination of the effect of terpenoids on the haematological parameters

The animals were treated with the terpenoid compounds as explained above. Blood was collected from tail vein and parameters such as total WBC count (Haemocytometer), differential count (Leishman's stain), body weight and Hb content (Cyanmet hemoglobin method) were recorded prior to the drug treatment and every 3rd day for one month.

2.5 Determination of the effect of terpenoids on the bone marrow cellularity and α-esterase positive cells

The animals were treated with the terpenoid compounds as explained above. The animals were sacrificed after 24 h of the last dose of drug treatment; bone marrow cells from femur were collected and made into a single cell suspension. The bone marrow cellularity was determined using haemocytometer.

The number of α-esterase positive cells were determined by the azo dye coupling method as explained in chapter 2 (Bancroff and Cook,1984). A smear of bone marrow cells from the above preparation was made on clean glass slides, stained with α-naphthyl acetate and pararosaniline hydrochloride and counter stained with heamatoxiline. The number of α-esterase positive cells were expressed out of 4000 cells.

2.6 Determination of the effect of terpenoids on circulating antibody titre:

All the animals were immunized with sheep red blood cells (20% SRBC in 0.1ml) along with the first dose of drug administration. After the 5^{th} dose of drug treatment blood was collected from the tail vein, serum separated and heat inactivated at 56°C. Antibody titre was determined using SRBC as antigen as explained in chapter 2 (Singh et al., 1984).

2.7 Determination of the effect of terpenoids on PFC in spleen.

After the 5 doses of drug treatment all the animals were immunized (i.p) with SRBC ($2.5x10^8$ cells/20µl/animal). All the animals were sacrificed on different days, spleen processed, made to single cell suspension and used to determine the antibody producing cells by Jern's Plaque assay as explained in chapter 2 (Jerne and Nordin, 1963).

2.8 Determination of the effect of terpenoids on delayed type hypersensitivity (DTH) reaction

All the animals were immunized (i.p) with SRBC ($1x10^8$ /20µl/animal) in the presence and absence of terpenoid compounds. The compounds were administered on the same day of immunization and continued for 5 days. After the 5^{th} dose of terpenoid administration all the animals were injected with a challenging dose of SRBC ($1x10^8$ /20µl) on the left hind paw. DTH was determined by measuring the thickness of paw after 24 h (Langrange et al., 1974).

2.9 Determination of the effect of terpenoids on the proliferation of spleen cells bone marrow cells and thymocytes *in vivo*.

Nine groups of Balb/c mice (6 mice/group) were sensitised with SRBC ($1x10^8$ /20 µl) subcutaneously on the ventral side. Group I, II, III, IV,V,VI and VII animals were treated with 5 doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid

glycyrrhizic acid, and nomilin respectively. Group VIII was treated with vehicle (paraffin oil) and group IX was kept as antigen alone treated control. 24h after the drug treatment, the animals were sacrificed and spleens, thymus and bone marrow were processed to single cell suspensions (Chapter2). Triplicate cultures were set to determine the proliferation in the presence and absence of various of mitogens such as Concanavalin A (ConA 10 μg/ml) and Phytohemagglutinin (PHA 2.5 μg/ml), by thymidine uptake method (chapter–2)

2.10 Determination of the effect of terpenoids on the proliferation of spleen cells Bone marrow cells and thymocytes *in vitro.*

Spleen, thymus and bonemarrow from normal Balb/c mice were taken and processed to single cell suspensions as described in Chapter 2. Cells (50000/well) were incubated with various mitogens (ConA-10μg/ml, PHA-2.5 μg/ml, LPS-10 μg/ml and PWM-10g/ml) in the presence and absence of different concentrations of terpenoids (0.5μg/ml, 1μg/ml and 5μg/ml) in 96 well flat-bottomed tire plate. Cultures were set up in triplicate and the proliferation was assessed by thymidine uptake method as described in chapter-2.

2.11 Determination of the effect of terpenoids on cell mediated immune response

Balb/c mice (4-6 weeks old) were divided into 15 groups (15 animals/group). Group 1 was treated with EAC cells and kept as tumour bearing control animals. Group II to XV animals were treated with 5 doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid, and nomilin respectively for 5 consecutive days. Group II to VII animals were kept as drug treated controls. Along with the last dose of drug treatment, EAC (10^6cells/animal) were injected intraperitoneally to group IX to XV animals. At various time intervals animals from each group were sacrificed and a single

cell suspension of spleen cells were made and used as effecter cells to determine the NK-cell activity and ADCC (chapter 2) by ^{51}Cr-release assay.

Blood was collected from the above animals by heart puncture, sera separated, heat inactivated at 56^0c for 30 minutes and used as anti-EAC antibody for ACC by trypan blue exclusion method as described in chapter-2

3 RESULTS

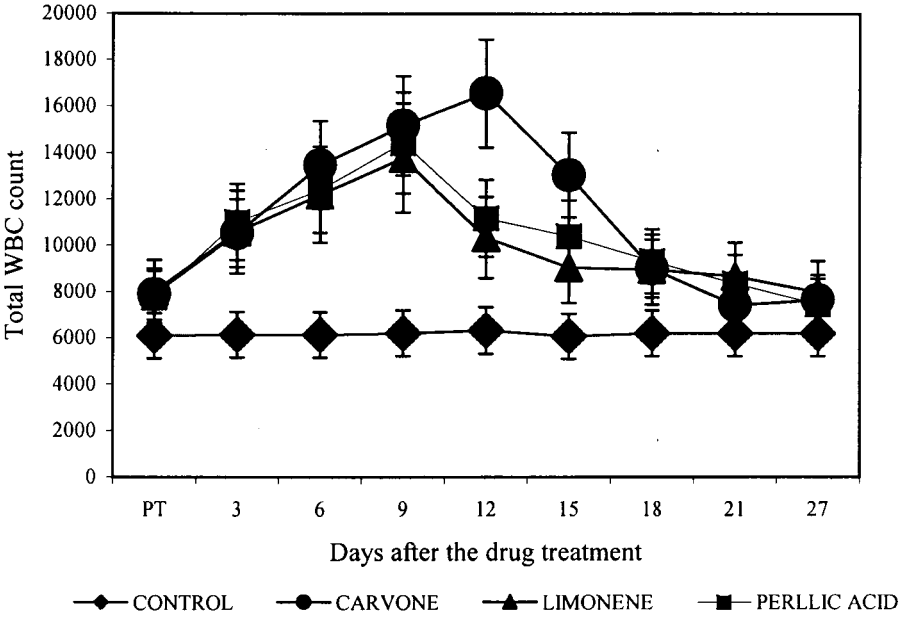
3.1 Effect of terpenoids on the haematological parameters

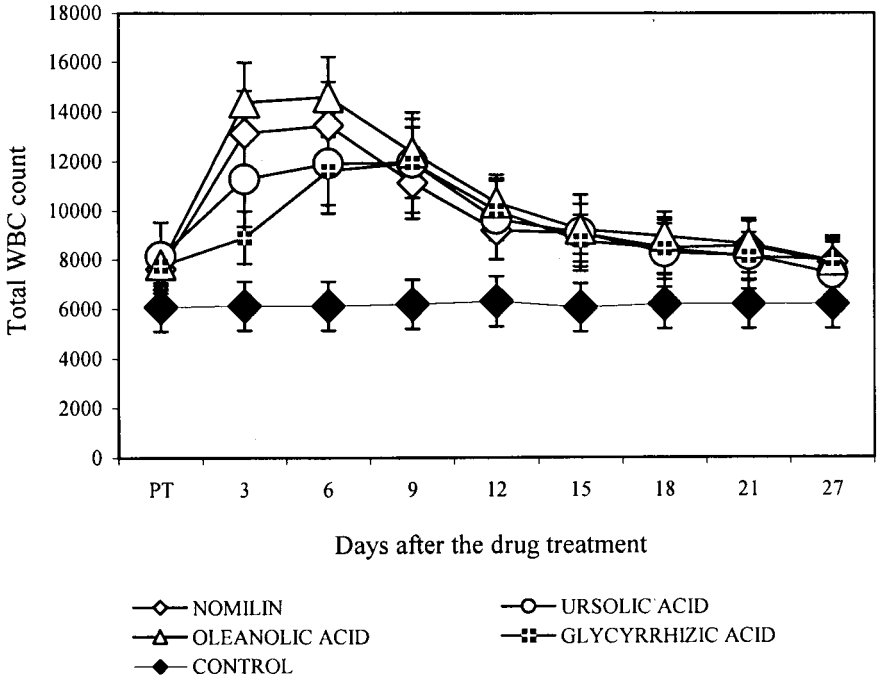
Administration of terpenoids increased the total WBC count in Balb/c mice (Fig 3.1). Among monoterpenoids the maximum count was observed in the case of carvone on the 12th day after drug administration (16560 cells/cmm). In the case of limonene (13783 cells/cmm) and perillic acid (14437.5 cells/cmm) the peak was observed on the 9th day. Administration of triterpenoids also increased the total WBC count in Balb/c mice (Figure3.1b). The maximum WBC count was obtained in the animals treated with oleanolic acid (14616 cells/cmm) nomilin (13475cells/cmm) and ursolic acid (11933 cells/cmm) on 6th day. In the glycyrrhizic acid treated animals the maximum count (11962 cells/cmm) was observed on the 9th day.

Haemoglobin content was moderately increased by the administration of terpenoid compounds.

There was no appreciable change in the differential count and body wt. (data not shown) after the terpenoid administration.

Fig 3.1 Effect of terpenoids on total WBC count





3.2 Effect of triterpenoids on the bone marrow cellularity and α-esterase positive cells.

The effect of triterpenoids on the bone marrow cellularity and α-esterase positive cells is given in table 3.1. Terpenoid treated groups of animals showed a remarkable increase in bone marrow cell number compared to control animals (15.5 x 10^6 cells/femur). The maximum increase in the number of bone marrow cell was observed in animals treated with oleanolic acid (28.8 x 10^6 cells/femur) and nomilin (27.6 x 10^6 cells/femur) . The bone marrow cellularity were also increased in glycyrrhizic acid (22 x10^6 cells/femur) and ursolic acid (20.1 x 10^6 cells/femur) treated groups. In carvone, limonene and perillic acid treated groups the number of bone marrow cells were 22.6 x 10^6 cells /femur, 23.2X10^6 cells/femur and 25.6X10^6 cells/femur respectively.

The number of α-esterase +ve cells were also found to be significantly increased in the bone marrow of animals treated with ursolic acid (1360 cells/4000 bone marrow cells) oleanolic acid (1496 cells/4000 bone marrow cells) and glycyrrhizic acid (1232 cells/4000 bone marrow cells) compared to the normal animals (1063 cells/4000 bone marrow cells).The number of α--esterase positive cells was also found to be increased significantly in monotrerpenoid treated animals (carvone 1614 cells; perillic acid 1255.3 cells and limonene 1410 cells/4000 bone marrow cells).

3.3 Effect of terpenoids on circulating antibody titre.

There was a significant increase in the amount of circulating antibody in animals treated with the terpenoid compounds (Fig.3.2). The maximum antibody titre of 1024 was observed in the limonene, nomilin, oleanolic acid ursolic acid and treated animals on 9th

Table 3.1 Effect of terpenoids on bone marrow cellularity and α-esterase activity

	Bone marrow cellularity(cells/femur)x10^6	α--Esterase activity (no: of α--esterase +ve cells/4000cells)
Normal	15.5±2.3	1063±69.4
Vehicle	15.4±0.43	1050±58.5
Carvone	22.6±1.7*	1614±108.6*
Perillic acid	25.6±2.08*	1255.3±21.6
Limonene	23.2±2.3*	1410±192.5*
Glycyrrhizic acid	22±0.7*	1232±43.3*
Ursolic acid	20.1±1.15*	1360±79.5*
Oleanolic acid	28.8±1.08*	14 96±245*
Nomolin	27.65±1.95*	1254.3±97.38

Treated animals received 5 doses of terpenoids. Bone marrow cells were collected from femur, and the cell number was determined using a haemocytometer and expressed as total live cells/femur. A smear using the above bone marrow preparation was made on a clean glass slide and stained with Harris haematoxylin to determined the non specific α-esterase activity according to the method of Bancroft and Cook.

(*P<0.01 Compared with normal)

day after the antigen administration. The antibody titre remained as 1024 till 15^{th} day in nomilin treated animals. In glycyrrhizic acid and limonene treated animals the maximum antibody titre 1024 was observed only on 12^{th} day. But in carvone treated group the maximum antibody production (256) obtained on the 9^{th} day.

3.4 Effect of terpenoids on plaque forming cells (PFC) in spleen

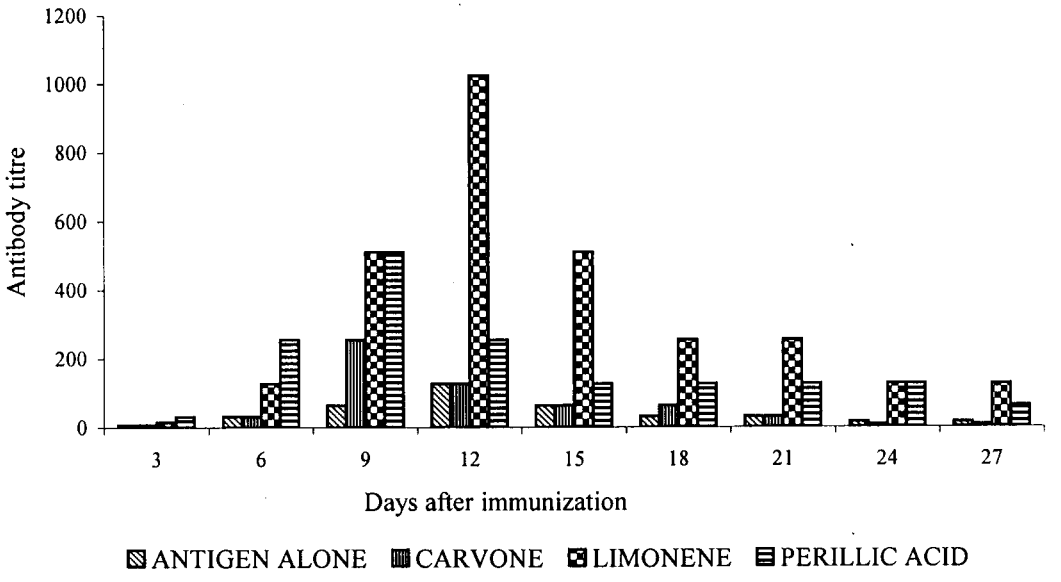
The effect of terpenoid compounds on the number of plaque forming cells is shown in figure 3.3. The maximum number of plaque forming cells was observed in animals treated with ursolic acid (539 cells/10^6 spleen cells) followed by oleanolic acid (444 cells/10^6 spleen cells), nomilin (377 cells/10^6 spleen cells) and glycyrrhizic acid (351cells/10^6 spleen cells) on 5^{th} day after immunization.

Among monoterpenoids treated groups the maximum number of plaque forming cells was observed in the limonene treated group (611.2/10^6 spleen cells) on the 5^{th} day after the immunization. Administration of carvone (262.5/10^6 spleen cells) and perillic acid (596/10^6 spleen cells) enhanced the number of plaque forming cells and on the 4^{th} and 5^{th} day respectively.

3.5 Effect of terpenoids on delayed type hypersensitivity (DTH) reaction

The effect of terpenoids on delayed type hypersensitivity is given in table 3.2. Terpenoids were found to inhibit the delayed type hypersensitivity reaction remarkably. The maximum inhibition of DTH reaction was observed in carvone (100%) and glycyrrhizic acid (95%) treated group and followed by limonene (92%), oleanolic acid (88%) and perillic acid (87%). Whereas in nomilin and ursolic acid treated group the inhibition of DTH reaction was found to be 76%.

Fig 3.2 Effect of terpenoids on circulating antibidy titre



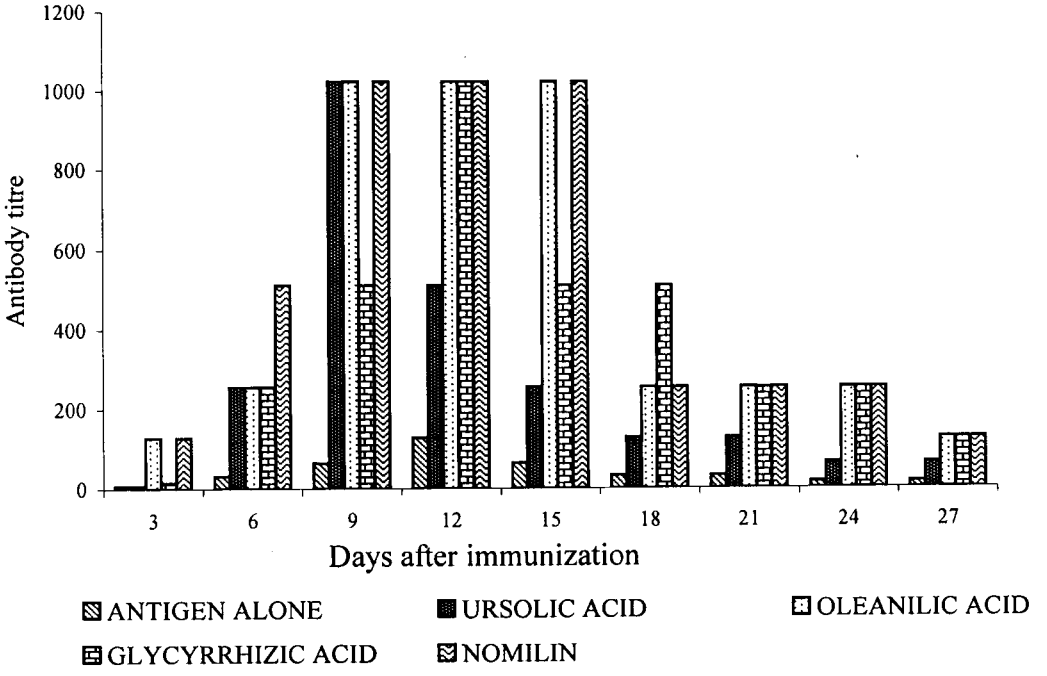
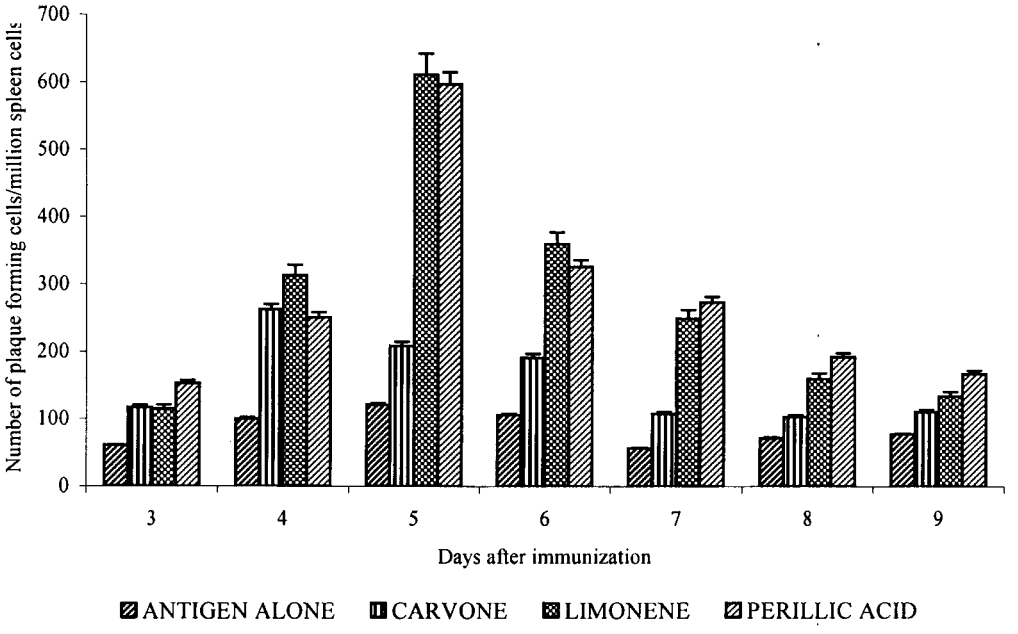


Fig 3.3 Effect of terpenoids on plaque forming cells in spleen



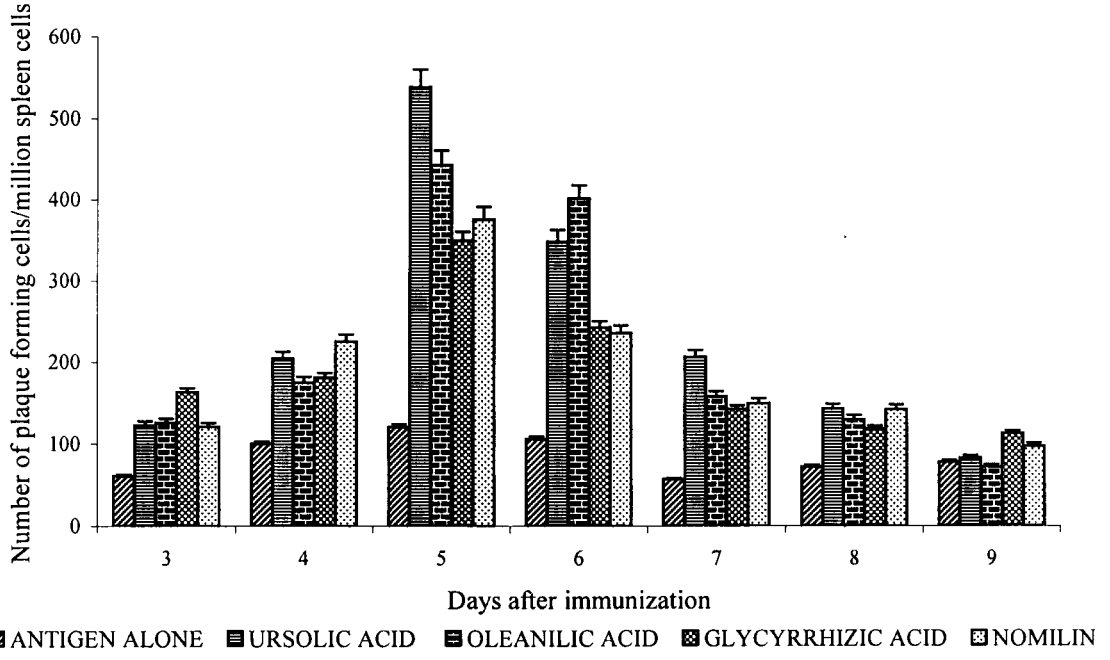


Table 3.2. Effect of terpenoids on delayed type hypersensitivity reaction

Treatment	Initial paw thickness (cm)	Paw thickness after 24 hrs (cm)	% of inhibition compared to antigen alone
Antigen alone	0.18±0.009	0.35±0.02	
Paraffin oil (vehicle)	0.14±0.01	0.33±0.01	0%
Carvone	0.17 ± 0.02	0.17 ± 0.02*	100%
Perillic acid	0.22 ± 0.02	0.25 ± 0.03*	87%
Limonene	0.23 ± 0.01	0.25 ± 0.02*	92%
Glycyrrhizic acid	0.186±0.01	0.194±0.01*	95%
Ursolic acid	0.18±0.01	0.22±0.02*	76%
Oleanolic acid	0.20±0.07	0.22±0.01*	88%
Nomilin	0.19±0.01	0.23±0.01*	76%

All animals were senzitised with SRBC ($1x10^8$ cells/20µl) subcutaneously on ventral side. Treated animals received five doses of various terpenoids. One group kept as untreated control and another group as paraffin treated control animals. After the 5^{th} dose, all the animals injected with challenging dose of SRBC ($1x10^8$ cells/20µl) on left hand paw. DTH determined by measuring the paw thickness after 24h.

(*$P<0.05$ Compared with antigen alone treated control)

3.6 The effect of terpenoids on the proliferation of spleen cells Bone marrow cells and thymocytes *in vivo.*

The effect of various terpenoid compounds on the *in vivo* proliferation of spleen cells, thymocytes and bone marrow cells is shown in figure 3.4. Administration of all of these terpenoids enhanced the mitogen-induced proliferation

The spleen cells from ursolic acid treated animals showed maximum cell proliferation (CPM-5816) followed by nomilin (CPM-5346) treated groups by the mitogen Con A(10μg/ml). Administration of terpenoids also enhanced the mitogenic potential of PHA. The maximum enhancement in splenocyte proliferation by the mitogen PHA was observed also in the case of ursolic acid (CPM 5190) and nomilin (CPM 4819) treated groups

Thymocytes from the terpenoid treated groups in presence mitogen Con A showed significant enhancement proliferation. The maximum enhancement in thymocyte proliferation in the presence of mitogen Con A was observed in ursolic acid (CPM 4835) and carvone (CPM 4850) treated groups. Terpenoid administrations also enhanced the thymocyte proliferation by the mitogen PHA. Where as normal thymocytes in the presence of Con A and PHA did not show a significant increase in the proliferation (CPM 3779 and 3576 respectively)

Administration of various terpenoids also enhanced the mitogen induced bone marrow proliferation. The maximum enhancement in bone marrow proliferation was observed in perillic acid treated groups in the presence of mitogen PHA (CPM 5601). Other terpenoids moderately enhanced the bone marrow proliferation by the various

Fig 3.4a Effect of naturally occurring terpenoids on the proliferation of thymocytes *in vivo*

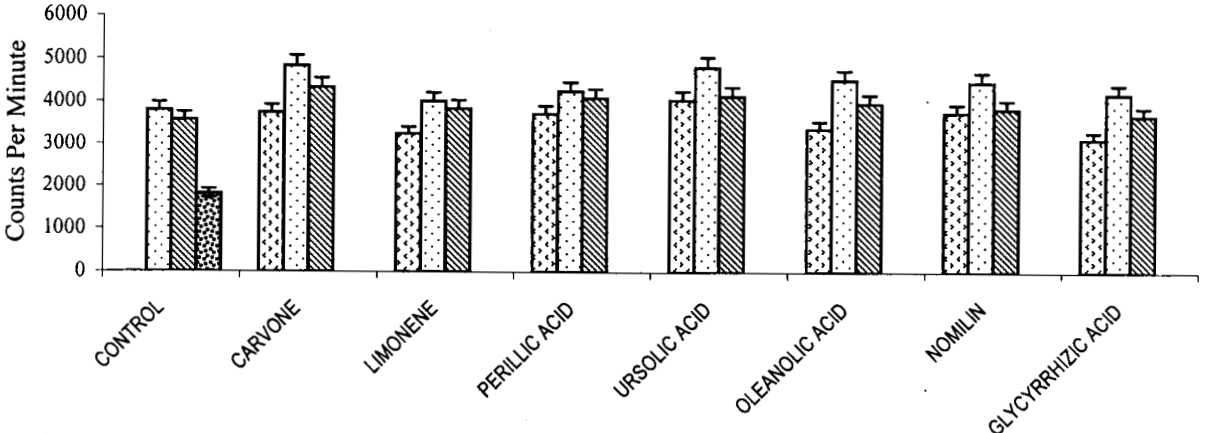


Fig 3.4b Effect of naturally occurring terpenoids on the proliferation of Spleen cells *in vivo*

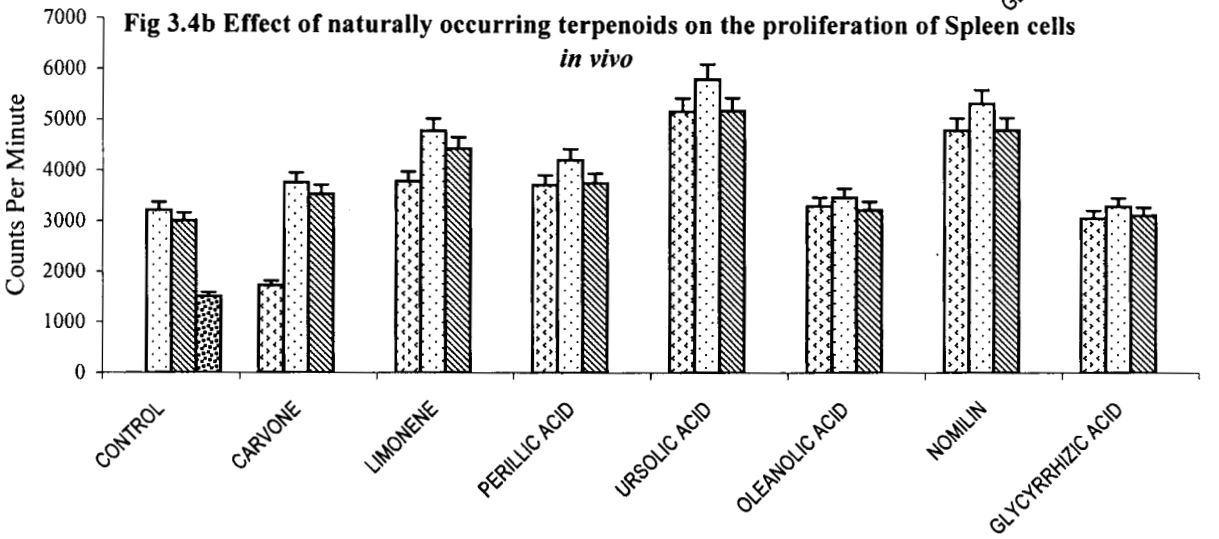
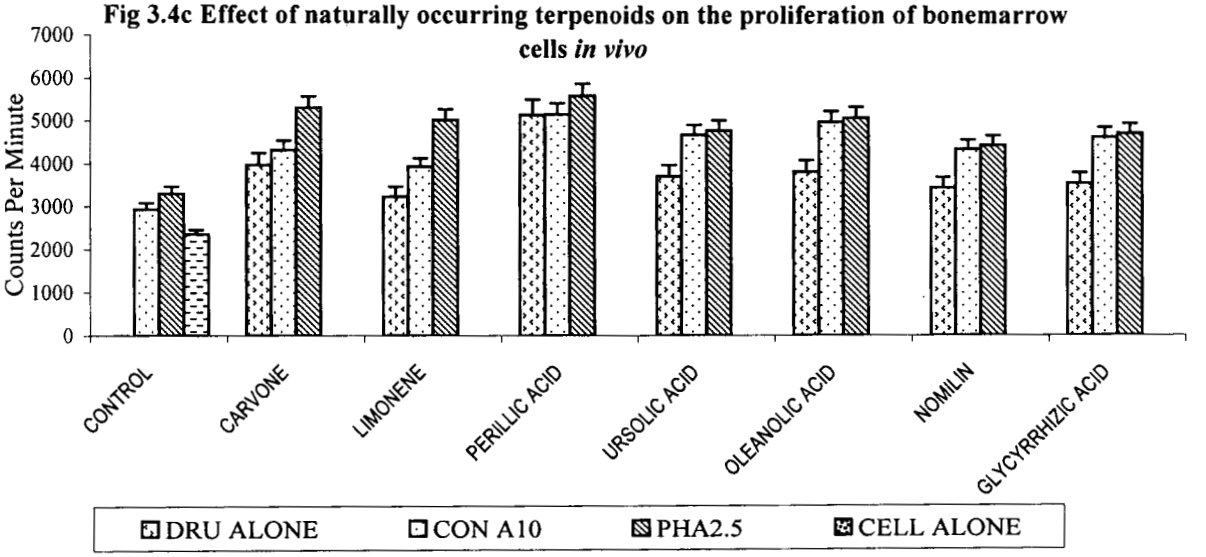


Fig 3.4c Effect of naturally occurring terpenoids on the proliferation of bonemarrow cells *in vivo*



mitogens. The bone marrow cells from normal animals showed only CPM 3299 and 2362 respectively by the treatment with mitogen PHA and Con A.

3.7 The effect of terpenoids on the proliferation of spleen cells bone marrow cells and thymocytes *in vitro.*

The effect of various terpenoid compounds on the proliferation of spleen cells, thymocytes and bone marrow cells is shown in figure 3.5. Among the different terpenoid compound studied perillic acid treated cells showed maximum spleen cell proliferation (CPM-6612.1) when the cells were stimulated with 10μg/ml LPS .Other terpenoids also enhanced the spleen cell proliferation by the presence of mitogen PHA, LPS, Con A and PWM. Maximum enhancement on bone marrow proliferation was obtained when ConA stimulated cells were treated with ursolic acid (CPM-5439) at the same concentration followed by nomilin+ Con A (CPM 5060) and carvone+ Con A (CPM 5017) compared to control (CPM-1334). Other mitogens also enhanced the bone marrow proliferation in the presence of various terpenoid compounds.

Maximum increase in the thymocyte proliferation was seen when the cells were treated with ursolic acid+ ConA (CPM-6939) followed by nomilin + Con A (CPM-6449) at a concentrations of 5 μg/ml compared to control (CPM-837).

3.8 Effect of naturally occurring terpenoids on natural killer cell activity of normal and tumor bearing mice

The effect of terpenoids on the NK cell activity of normal and tumour bearing animals is shown in figure 3.6. There was a significant enhancement in NK cell activity in these terpenoids treated normal as well as tumour bearing animals. In control animals the maximum lysis was obtained on 13^{th} day (21%). In ursolic acid (48%), nomilin (42%),

Fig 3.5a Effect of naturally occurring terpenoids on the proliferation of thymocytes *in vitro*

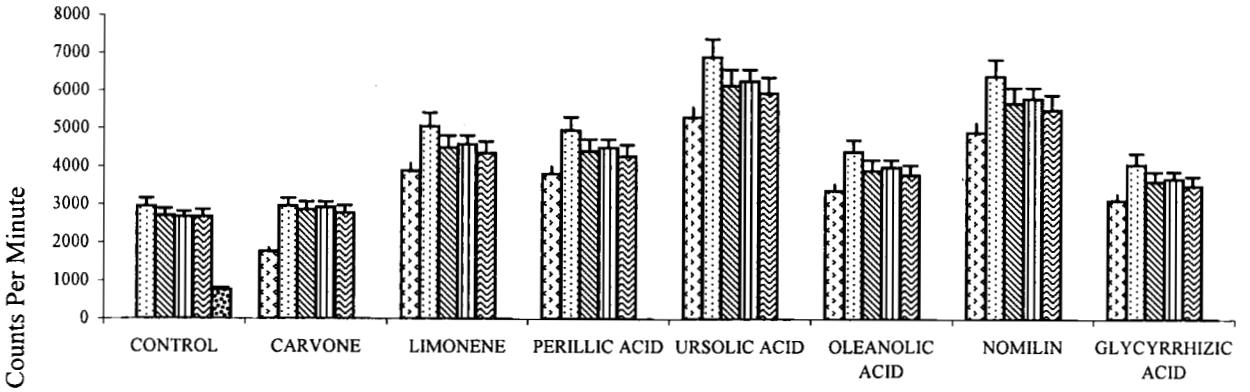


Fig 3.5b Effect of naturally occurring terpenoids on the proliferation of bonemarrow cells *in vitro*

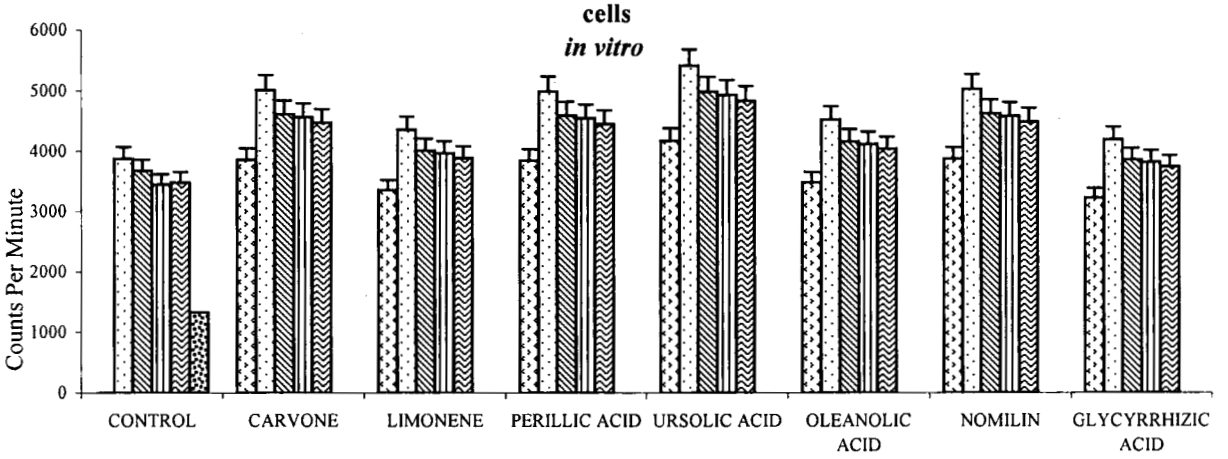
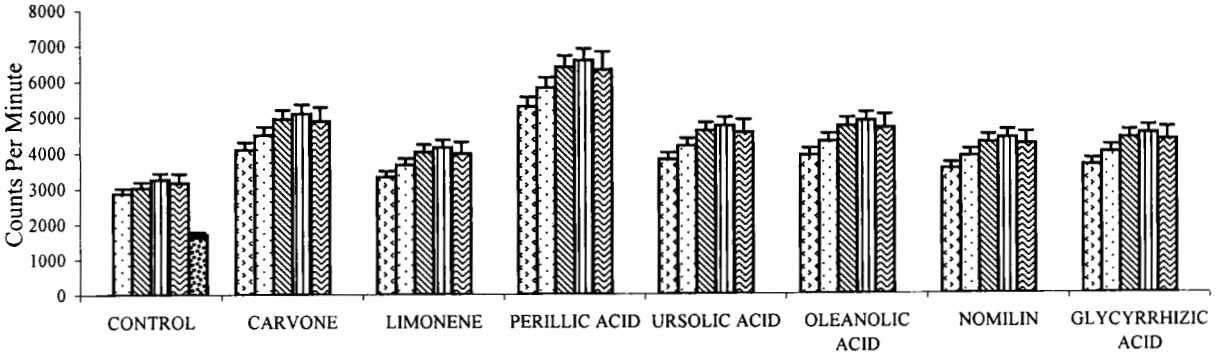


Fig 3.5c Effect of naturally occurring terpenoids on the proliferation of spleen cells *in vitro*



limonene (47%), carvone (48%) and perillic acid (46.2%) treated groups the maximum cell lysis was obtained on 3rd day. Where as in oleanolic acid (49%) and glycyrrhizic acid treated (47%) groups the maximum cell lysis was obtained on 5th day

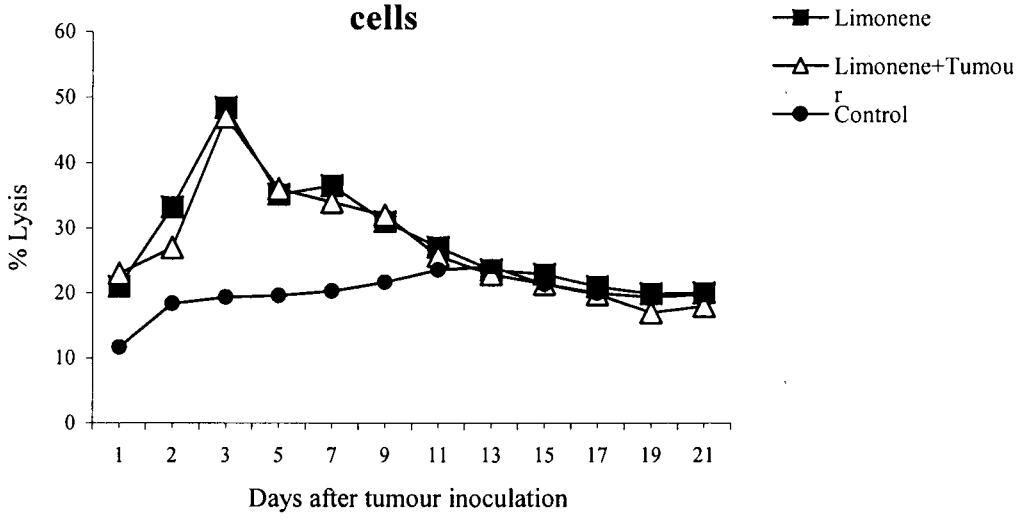
3.9 Effect of terpenes on ADCC in normal and tumour bearing animals

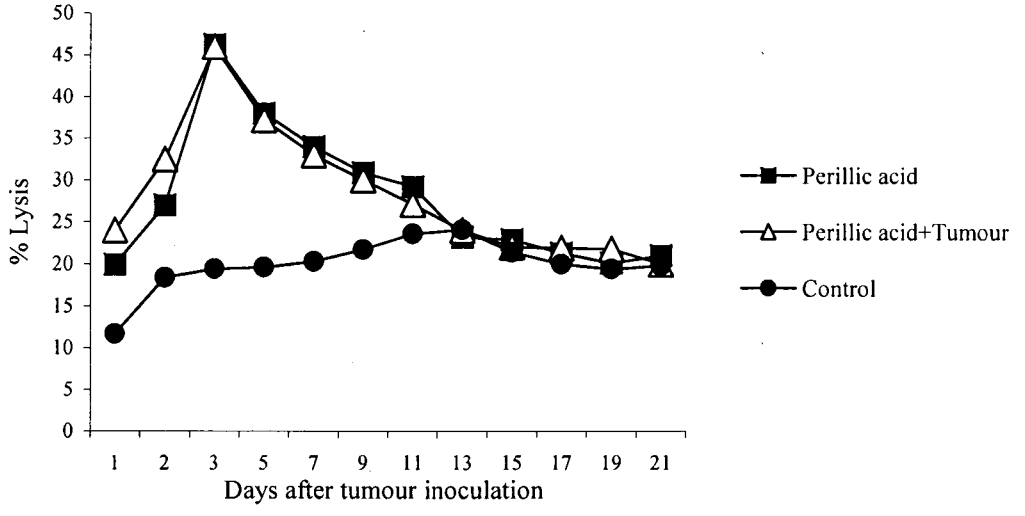
The effect of terpenoids on ADCC activity is given in figure 3.7. Administration of terpenoids clearly enhanced the ADCC activity in normal as well as tumour bearing animals. In tumour alone treated control the maximum lysis was only on 15th day (19 %). But in the case of carvone and perillic acid treated group, the maximum lysis was 38% and 45% respectively and it was obtained on 9th day. Where as in limonene treated group the maximum lysis of 43.9% was obtained on 7th day after the tumour inoculation. Administration of triterpenoid compounds also enhanced the ADCC activity in normal as well as tumour bearing animals. Glycyrrhizic acid and ursolic acid treated groups showed maximum ADCC activity(46%) on 7th day of tumour inoculation followed by oleanolic acid (42%) and nomilin (41%) on the same day.

3.10 Effect of terpenoids on ACC in normal and tumour bearing animals

The effect of terpenoids on complement-mediated cytotoxicity is given in table 3.3. For tumour alone treated group the maximum ACC activity was obtained on 19th day (13.8% cell lysis). But administration of all of these monoterpenoids enhanced the ACC activity and maximum activity was obtained much earlier than that of tumour only group. The maximum ACC activity in terpenoid treated groups were obtained on 17th day of tumour inaculation. The maximum ACC activity was obtained in the case of carvone (33 %) treated groups and followed by glycyrrhizic acid (28%), nomilin (26%), ursolic acid (27%) and perillic acid (25 %) treated groups .

Fig 3.6a Effect of monoterpenes on NK cells





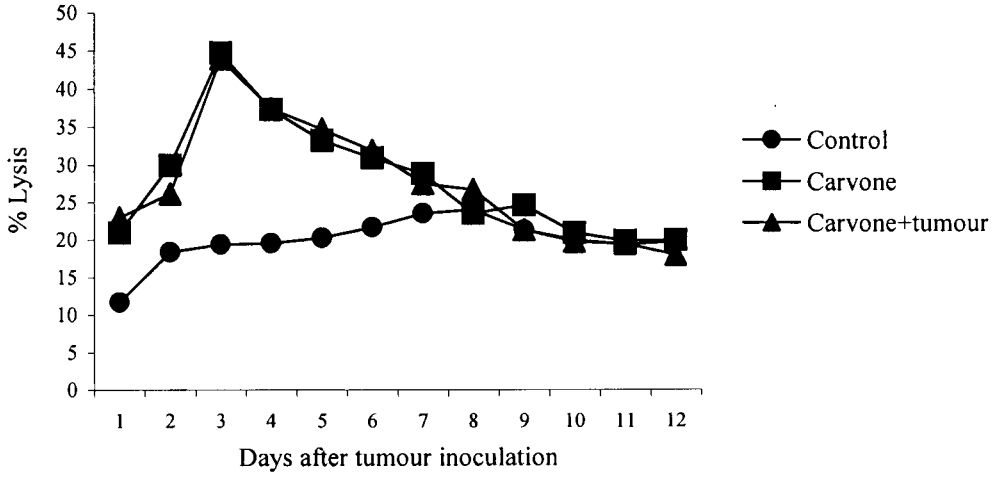
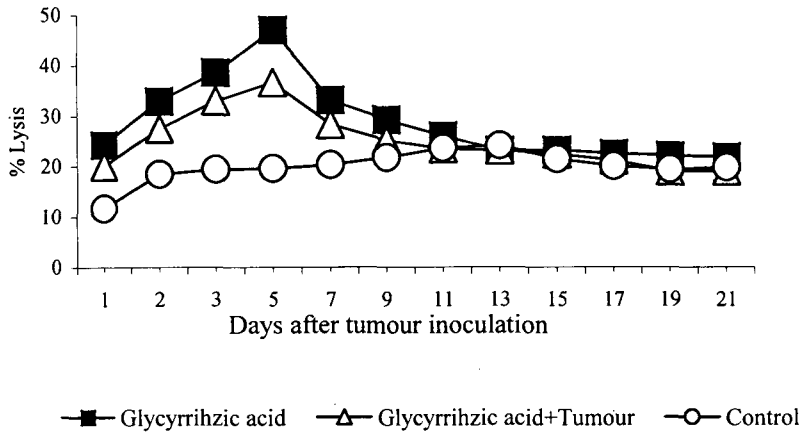
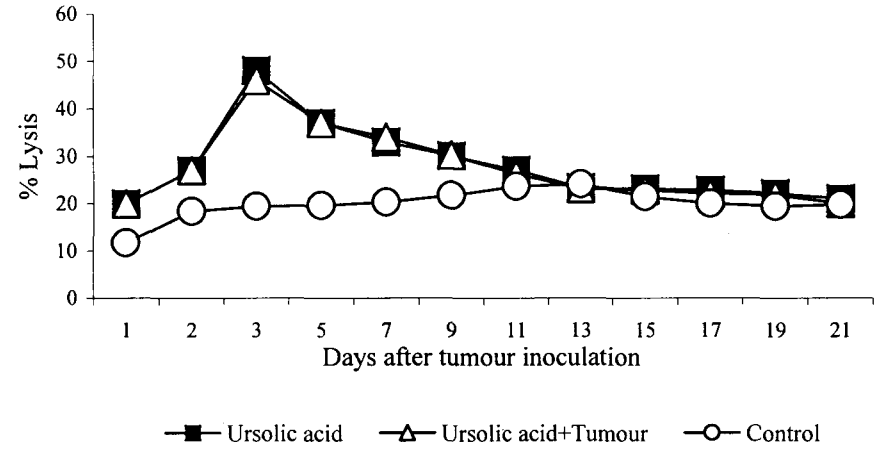
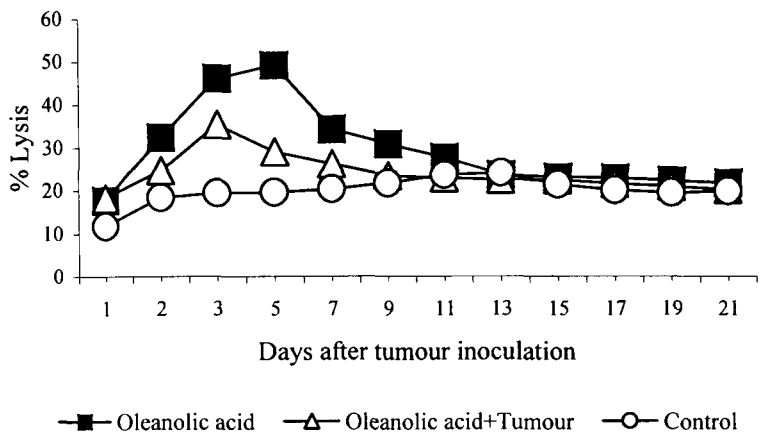


Fig 3.6b Effect of triterpenoids on NK cells







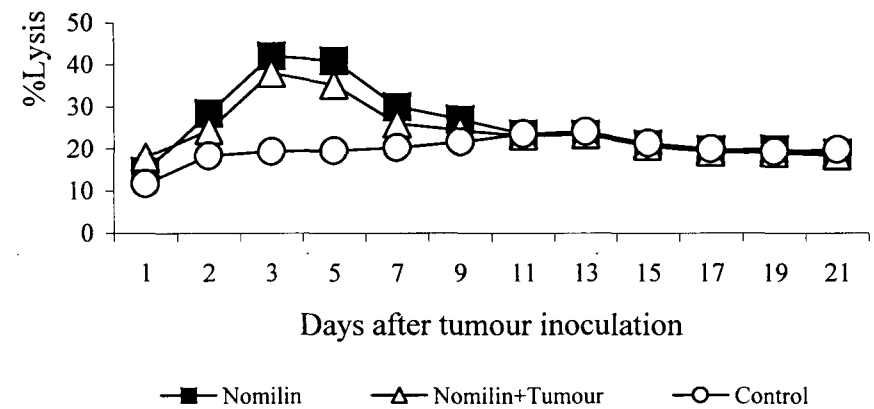
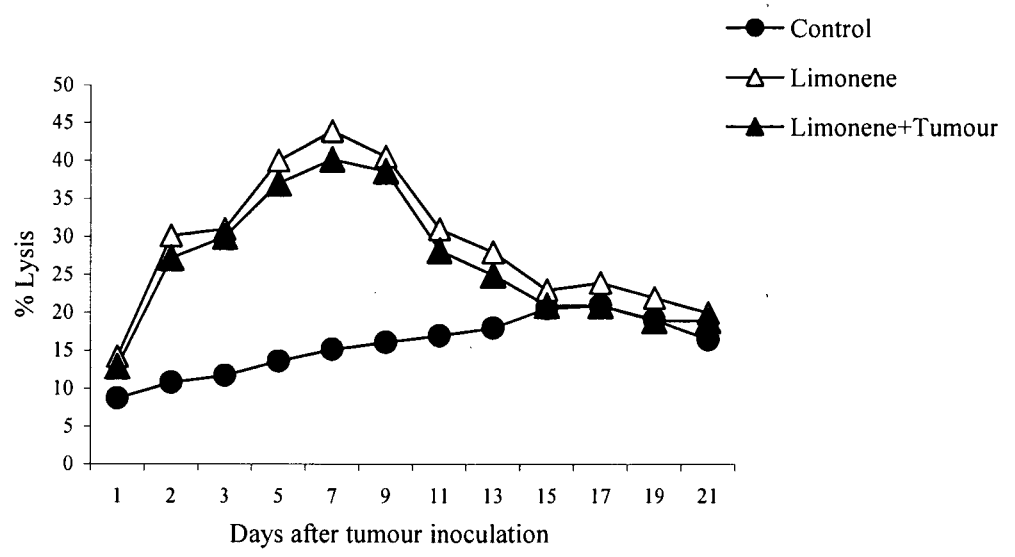
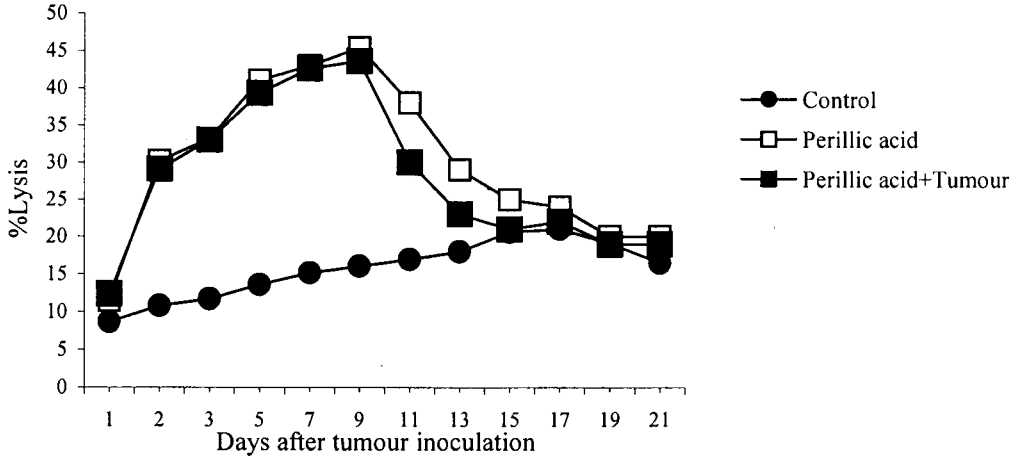


Fig 3.7a Effect of monoterpenes on ADCC





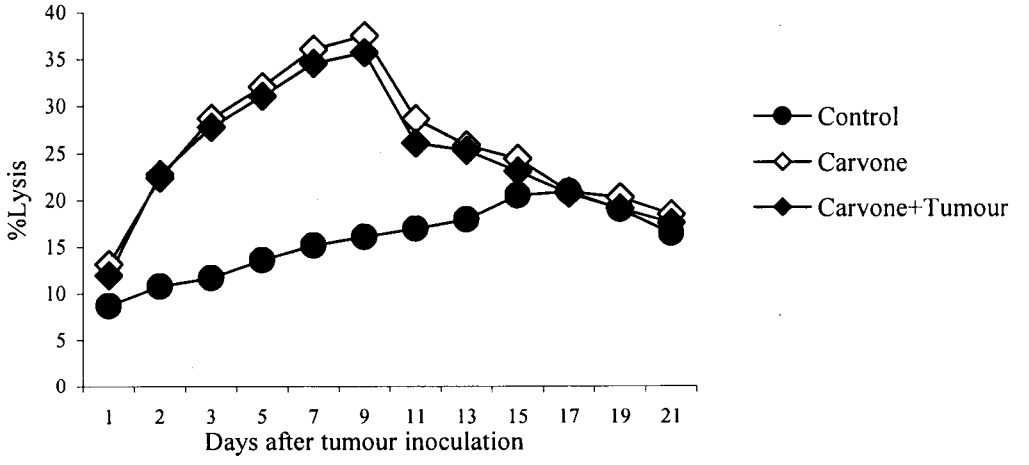
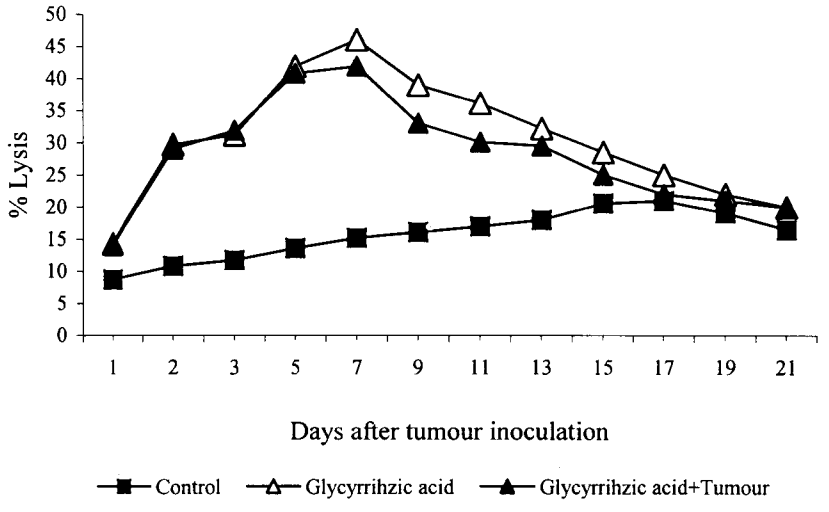
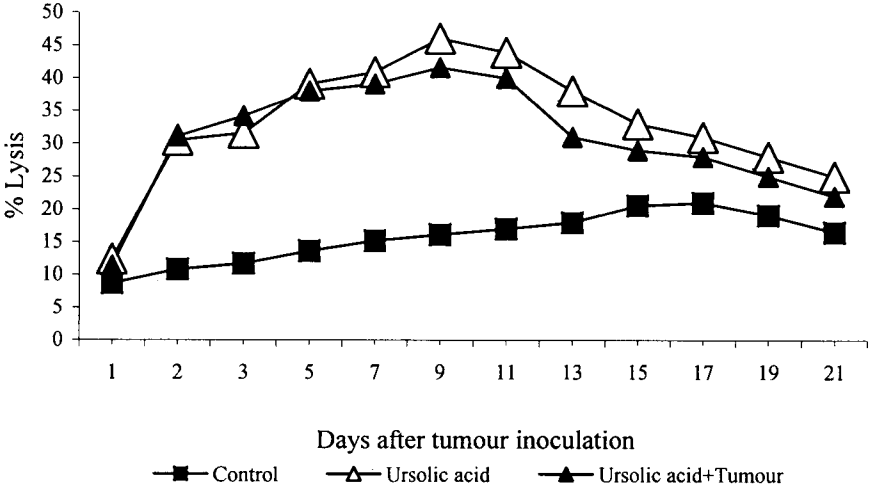
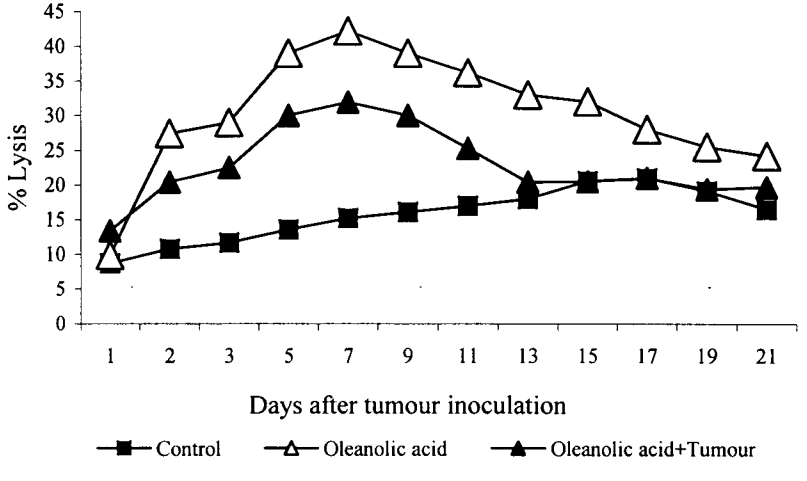


Fig 3.7b Effect of triterpenoids on ADCC







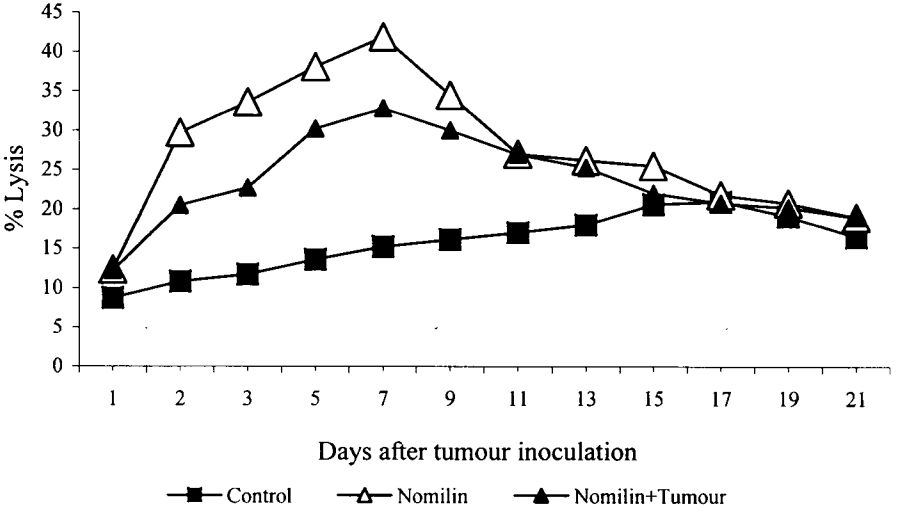
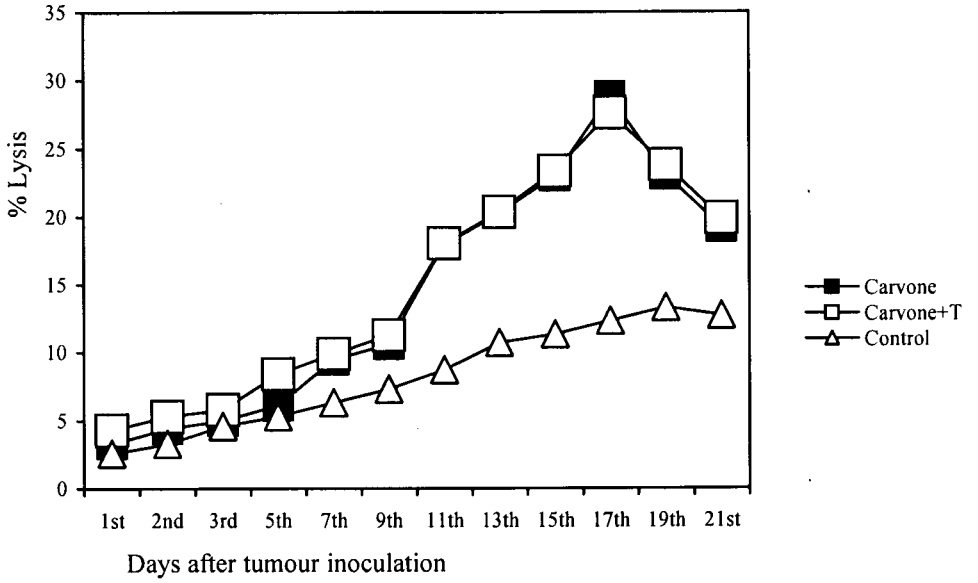
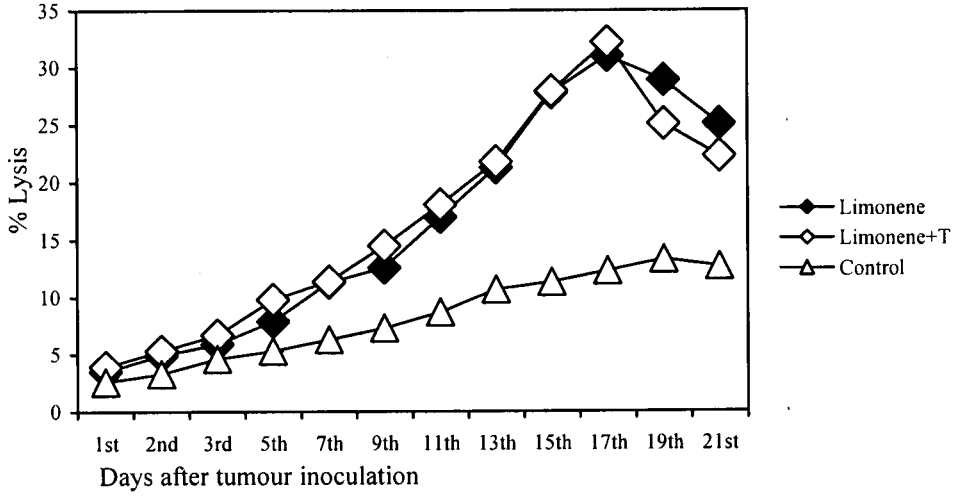


Fig 3 8a. Effect of monoterpenoids on ACC





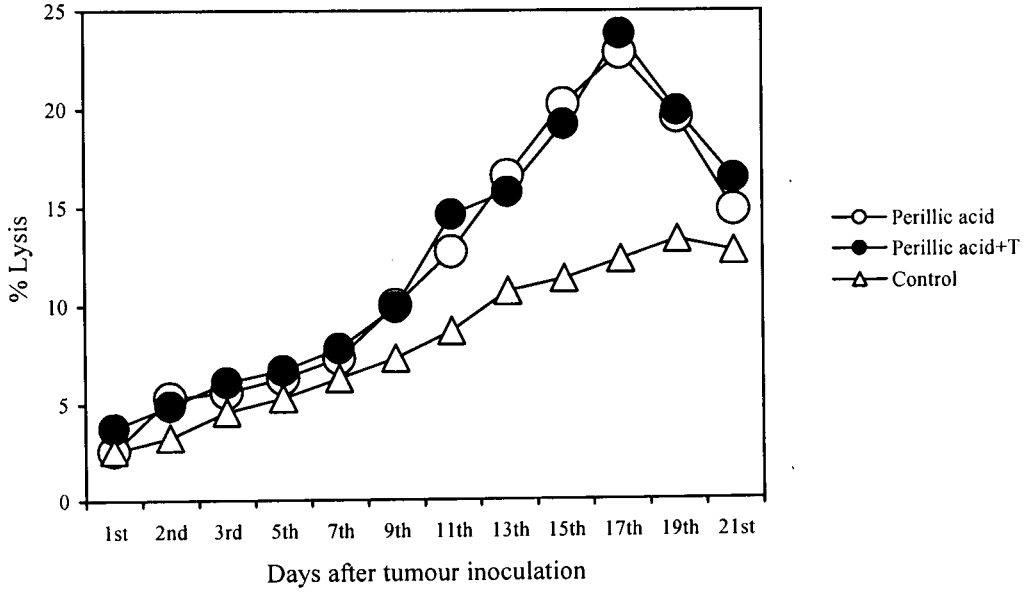
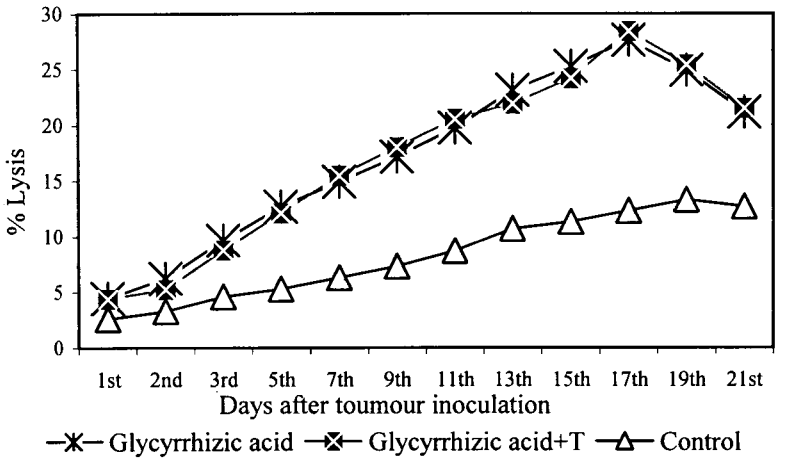
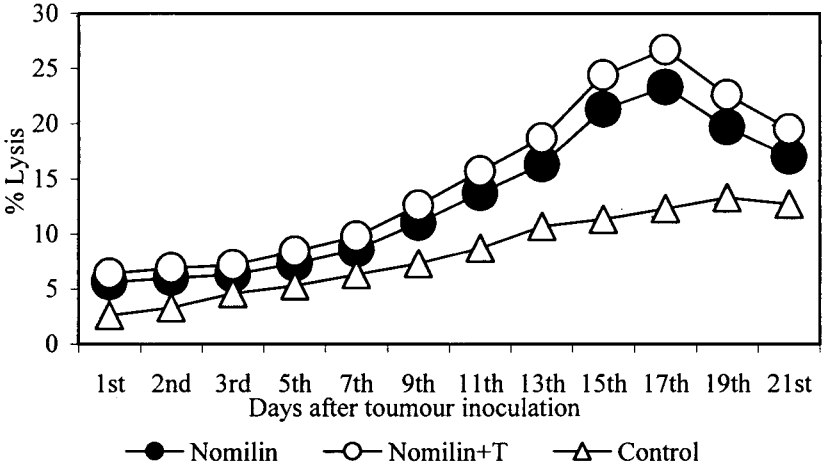
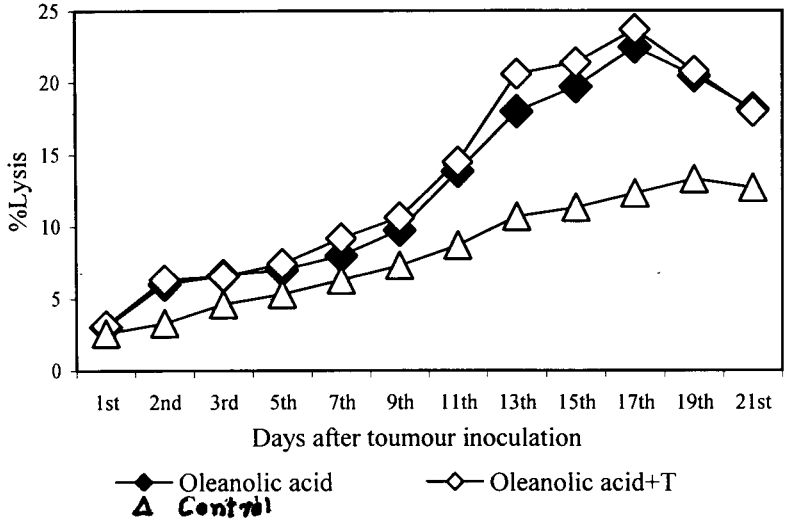
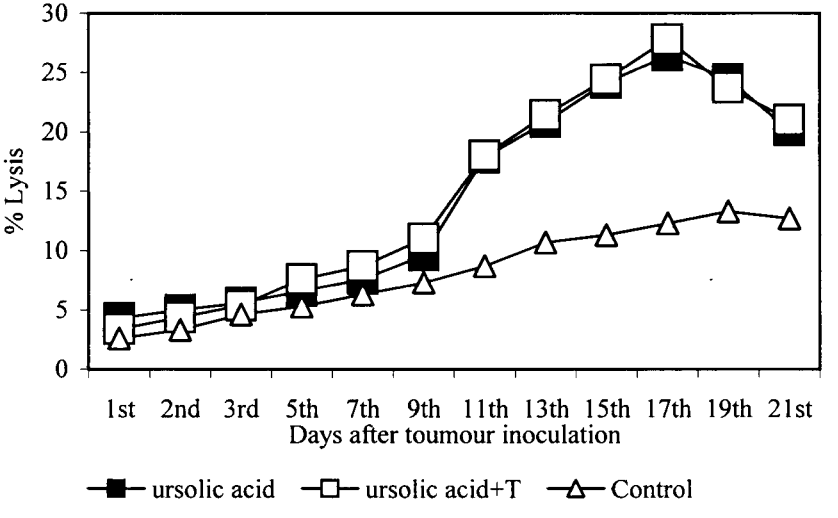


Fig 3 8b. Effect of triterpenoids on ACC









4 DISCUSSION

The immune system is known to be evolved in the etiology as well as pathophysiologic mechanism of many diseases. The use of immunomodulatory drugs both to treat and prevent some diseases have been increasing. The number of immunodefficient patients is growing constantly because of stress, toxic factors, and administration of drugs that may adversely influence the human immunological systems. Immunomodulation is the regulation of immune response whether to suppress them when unwanted or to stimulate them in the prevention of infectious diseases.

Immunomodulatory agents that are free from side effects and which can be administrated for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. There are a variety of chemically derived compounds discovered with immunomodulatory activity but the clinical value of most of these compounds has not been proven completely. The present study demonstrate that unlike other chemically defined compounds, these triterpenoid compounds could activate the immune system without affecting the other parameters of the body

Bone marrow serves as the major source of all blood cells. The majority of all the cell type involved in the immune system is produced from a common hemopoietic stem cells of bone marrow. Bone marrow also provides micro environment for antigen dependent differentiation of B cells. Different cytokines are important for renewal of hematopoietic stem cells and their differentiation into different functionally mature blood cell types. Administration of *Withania somnifera*, plant having some immunopotentiating activity also increase the total bone marrow cells(Davis and Kuttan, 2000). The present study shows an enhancement in bone marrow cellularity and total WBC count by the

administration of various terpenoids indicating their effect on the hemopoiesis. Both innate and adaptive immunity depends upon the activities of WBC. Terpenoids could enhance the total WBC count on the 6^{th} day in a normal range. The innate and adaptive immune systems together provide a remarkably effective defence system.

The mononuclear phagocyte system is composed of bone marrow monocytes precursors, circulating monocytes and macrophages in all tissues including lung liver spleen, adrenal glands, intestine and bone marrow. These cells are highly phagocytic and participate in the eradication of invading micro-organisms and clearance of damaged or scenscent autologus tissues. They also play an active role in antigen processing and prevention to T lymphocytes to generate the primary immune response. Like proliferating bone marrow cells, bone marrow monocyte show non-specific esterase activity (Hayhoe, 1980). Administration of these terpenoid compounds increased the number of α-esterase active bone marrow cells. This indicates the effect of administration of triterpenes on the stem cell proliferation.

The humoral immune response was analysed by the total antibody production and number of antibody producing cells in spleen. The production of antibody producing cells in spleen was increased by terpenoid administration. The circulating antibody titre was also significantly enhanced in the terpenoid treated animals; showing its stimulatory effect on the humoral arm of the immune system.

Delayed hypersensitivity reactions are mediated by specific T cells together with denitritic cells, macrophages, and cytokines especially interleukin 1. The overall effect of these cytokines is to draw macrophages into the area and activate them, promoting increased phagocytic activity and increased concentration of lytic enzymes for more

effective killings (Tami, 1986). As lytic enzyme leakout of the activated macrophages into surrounding tissues localized tissue distruction can be ensured. These non-specific distructions of cells in chronic DTH reaction, characterized by excessive number of macrophages continual release of lytic enzymes are not desirable. Certain plant originated compound have the capacity to inhibit chronic hypersensitivity reaction (Davis and Kuttan, 2000). Administrations of triterpenoids such as glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin have been shown to inhibit the hypersensitivity reaction. Out of these compounds glycyrrhizic acid could inhibit the reaction nearly 95%. Ursolic acid, oleanolic acid and nomilin could produce between 75-85% inhibition of hypersensitivity reaction.

The major objective of the immunotherapy is to modulate immune responses for selected objectives. It include augmentation of cell mediated immunity and cytotoxic antibody. Cell mediated immune responses are mediated by immune cells. Cytotoxic T lymphocytes play major role in this area.

Natural killer cells are large lymphoid cells with prominent intracellular granules. Natural killer cells are large lymphoid cells with prominent intracellular granules. Even though they have no known antigen specific receptors they are able to recognize and kill a limited range of abnormal cells including cancer cells. They produce a number of immunologically important cytokines; they play important role in immune regulation and influence both innate and adaptive immunity. NK cells can also exert a potent barrier to tumour cell growth *in vivo* (Henney et al., 1981; Mandelboim et al., 1999). Activation of NK cells is one of the objectives of tumour immunotherapy. Administration of terpenoids significantly enhanced the NK cell activity and the enhancement was observed much

earlier so that the tumour burden was small for the action of cell mediated immune response.

ADCC is the cooperative interaction of humoral and cell mediated immune effectors. The destruction of antibody coated target cells is called antibody depending cell mediated cytotoxicity. Cytotoxic T lymphocytes and NK cells have Fc receptors that are capable to trigger cytotoxic attack to target cells. Antibody can direct an antigen specific attack by an effector cell lacking specificity for antigen. Administration of monoterpenes enhanced the antibody depending cellular cytotoxicity and the maximum ADCC was obtained in the case of perillic acid treated group.

In addition these terpenoids could enhance the ACC in tumour bearing animals. Complement proteins are responsible for cell lysis and mediators of inflammation, serving to attract phagocytic cells and enhance phagocytosis. It plays a major role in cell mediated immune response. Enhancement of ACC shows the activation of cell mediated immune system by the administration of these terpenes.

The results of blastogenesis assay clearly showed that these terpenoids promoted cell proliferation.. Even the presence of mitogens, terpenoid administration again enhanced the proliferation rate. Con A and PHA are two T cell proliferators and PWM is B cell mitogens. The study clearly indicated that these terpenoids capable of enhancing the proliferation of T and B cells.

The above results indicate that these naturally occurring terpenoids compounds could stimulate the stem cell proliferation and differentiation as well as activated both cell mediated and humoral immune responses in mice. These might play an important role as immunostimulant and could be used as adjuvants during cancer therapy.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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CHAPTER 4

ANTI-TUMOUR AND ANTI-OXIDANT ACTIVITIES OF NATURALLY OCCURRING TERPENOIDS

1 INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role in the causation of several diseases such as liver cirrhosis, arteriosclerosis, diabetes, cancer etc (Player, 1992; Athar, 2002). Mammalian cells have a complex system of antioxidants to destroy ROS produced as a result of normal metabolism. An important component of this system is the antioxidant enzymes that catalytically scavenge free radicals (Player, 1992; Sun, 1990). Exposure to ionizing radiations and many other xenobiotics are also responsible for the generation of free radicals in the body. So the normal level of antioxidant effect is not sufficient for eradication of free radical induced injury. Antioxidant components thus play an important role to protect the human body against damage by reactive oxygen species. Several plant and plant-derived products have been experimentally proved and used as effective protection against free radical induced tissue damage and oxidative stress. (Jain et al., 1967; Anjaly and Manoj, 1995)

The effect of the terpenoids, carvone, limonene, perillic acid, glycyrrhizic acid, ursolic acid, oleanolic acid, and nomilin on the inhibition of free radicals and reactive oxygen species production has not been studied in a systematic way. In the present chapter we report the antitumour and antioxidant activity of triterpenoids, carvone limonene perillic acid, glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin

2 MATERIALS AND METHODS

2.1 Animals

Inbred strains of Swiss albino mice 4-5 weeks old males (20-25g body wt) and C57 BL/6 mice 5-6 weeks old males (20-25g body wt) were used in this study

2.2 Cells

B16F-10 melanoma cells, Dalton's lymphoma ascites cells (DLA) and Ehrlisch ascites tumour cells (EAC) were used in this study

2.3 Chemicals

Nitroblue tetrazolium (NBT). All other chemicals were of analytical reagent grade.

2.4 Terpenoid compounds

Carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin

Drug Adminisyration:-Terpenoids were administered intraperitoneally for 5 consecutive days (carvone and limonene 100μmoles/Kg body wt /dose/animal i.p, perillic acid, ursolic acid, oleanolic acid and glycyrrhizic acid 50 μmoles/Kg body wt /dose/animal i.p and nomilin 10 μmoles/Kg body wt /dose/animal ip.) for antioxidant studies and for 10 consecutive days at the same dosage for anti-tumour studies. All the compounds were suspended in light paraffin oil.

For *in vitro* studies terpenoids were dissolved in minimum volume of DMSO and further diluted in PBS.

2.6 ANITUMOUR STUDIES

2.6a Determination of the effect of naturally occurring terpenoids on the solid tumour development

Inbred strains of Swiss albino and C57 BL/6 mice were used for the study. The animals in each strain were grouped into eight (6 animals/ group). Tumour was induced by injecting $1x10^6$ cells on the right hind limb of all the animals. DLA cells were injected into Swiss Albino mice where as B16F-10 cells were administered to C57 BL/6 mice. Groups I, II, III, IV, V, VI and VII, of each strain were treated with 10

doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin respectively. Group VIII of each strain were kept as untreated controls. Drug administration was started simultaneously with the tumour inoculation and continued for 10 consecutive days. Tumour diameter was taken from the 7^{th} day of tumour inoculation and continued for 30 days. Tumour volume was calculated using the formula - Volume = $4/3\pi\ r_1^2\ r_2$. where r_1 and r_2 are the minor and major diameters (Chapter 2).

2.6b Determination of the effect of naturally occurring terpenoids on survival rate of ascites tumour bearing animals.

Ascites tumour was induced by injecting Ehrlich ascites cells (1 x 10^6 cells/ animal) into peritoneal cavity of Swiss albino mice (3 groups, 6 numbers per group). Groups I, II, III, IV, V, VI and VII were treated with 10 doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin respectively. Group VIII animals were kept as tumour bearing control without any drug treatment. The death pattern of animals due to tumour burden was noted and percentage increase of life span (%ILS) was calculated (Chapter 2).

2.7 ANTIOXIDANT STUDIES

2.7a Determination of the effect of naturally occurring terpenoids in the inhibition of liver lipid peroxidation

Inbred strains of Swiss albino mice were used for this study. Animals were divided into eight groups (6 animals / group). Group I was kept as control with out any drug treatment; groups II, III, IV, V, VI, VII, and VIII of each strain were treated with 5 doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin respectively. Animals were sacrificed 24 hours after the last dose of

drug treatment and liver was excised and homogenized. The lipid peroxidation was measured by the method of Ohkawa et al. (chapter 2)

2.7b *In vitro* antioxidant activity of naturally occurring terpenoids

The terpenoids were dissolved in minimum quantity of DMSO and further diluted in PBS. Various concentrations (ranging from 5 μg/ml to 250 μg/ml) of these terpenoids tested for the antioxidant activity. The superoxide scavenging activity was measured by the method of Mc Cord and Fridovich (chapter 2) that depends on the light induced superroxide generation by riboflavin and corresponding reduction by NBT. The hydroxyl radical scavenging activity was measured by the methode of Elizabeth and Rao (chapter 2). The production of nitrite ions which were measured by Griess reaction (chapter 2). Lipid peroxidation was induced in mice liver homogenate by the method described Bishayee and Balasubramonian (chapter 2) in the presence of different concentrations of the test material and estimated by thiobarbituric acid reactive substances by the method Ohkawa et al, as explained in chapter 2 (Ohkawa et al., 1979).

3 RESULTS

3.1 Antitumour activity of naturally occurring triterpenoids

Administration of terpenoids, carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin were found to reduce tumour development induced with DLA cells compared to tumour bearing control animals (Fig 4.1). In glycyrrhizic acid group the maximum tumour volume was observed on 19^{th} day (0.8 cm^3) and the tumour volume reduced up to 0.47 cm^3 on 30^{th} day after tumour inoculation. Administration of limonene, perillic acid and nomilin also gave a remarkable tumour reduction (0.44 cm^3, 0.67 cm^3 and 0.86 cm^3 respectively on 30^{th} day

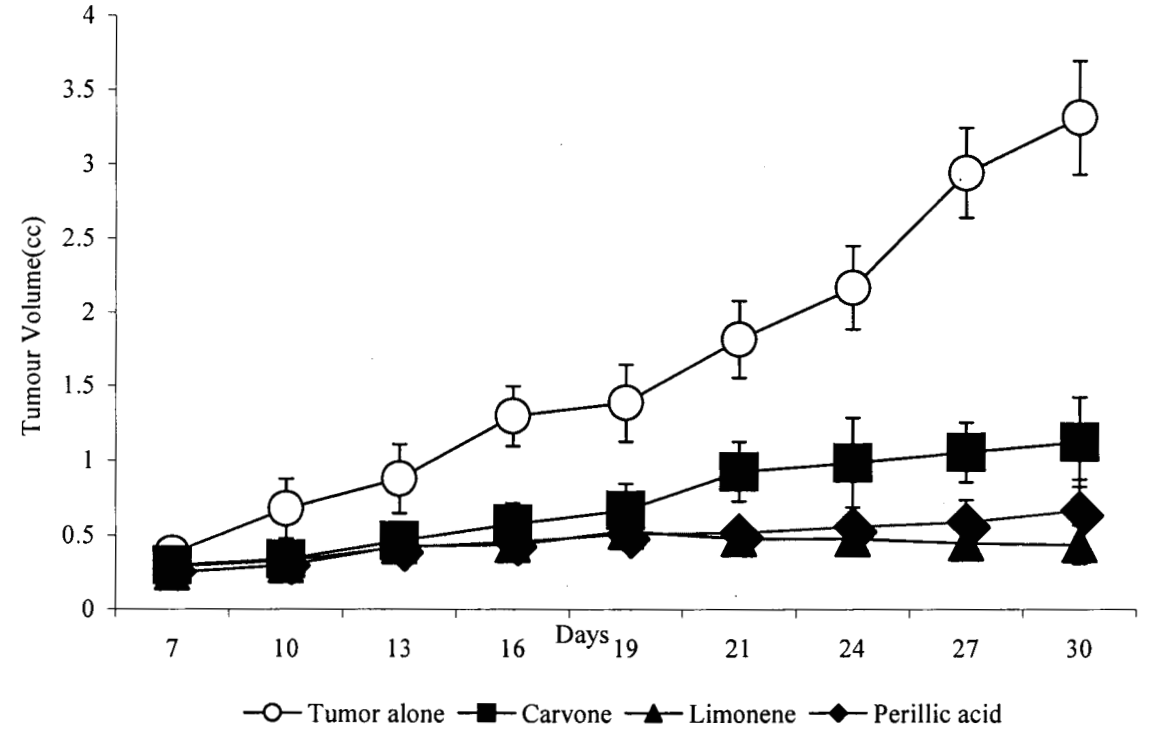
after tumour inoculation). In tumour alone treated control group the tumour volume sharply increased even after the 30 days of study. On the 30th day control tumour volume was 3.31 cm^3.

More or less the same result was observed in B16F10 tumour bearing group. Some of these terpenoids were shown to inhibit the solid tumour development induced by B16F10 melanoma cells (Fig 4.2). In Tumour bearing control animals the tumour volume increased and attained the maximum volume (6.6 cm^3)on 30th day of tumour inoculation. But in limonene and perillic acid treated group tumour volume started to increase only after the 10 th day of inoculation and have reduced tumour growth rate. (1.5 cm^3 and 1.8 cm^3 respectively on 31st day of tumour inoculation) Other terpenoids also showed reduced tumour growth rate (2.4 cm^3, 3.1 cm^3 and 3.7 cm^3 respectively for ursolic acid glycyrrhizic acid and nomilin on 31st day of tumour inoculation)

3.2 Effect of terpenoids on the life span of ascites tumour bearing animals

Terpenoids carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin were investigated for their ability to inhibit the ascites tumour development and the increase life span of tumour bearing animals (Table 4.1). There was 87.5% increase in the life span of ascites tumour bearing mice treated with perillic acid. Administration of glycyrrhizic acid (84.5%), limonene (83.8%) and ursolic acid (56.6%) also increased the life span of tumour bearing animals. But treatment with carvone, nomolin and oleanolic acid, the enhancement in life span was only 30.8%, 21.3% and 17.6% respectively.

Fig 4.1 Effect of terpenes on DLA cell induced solid tumour



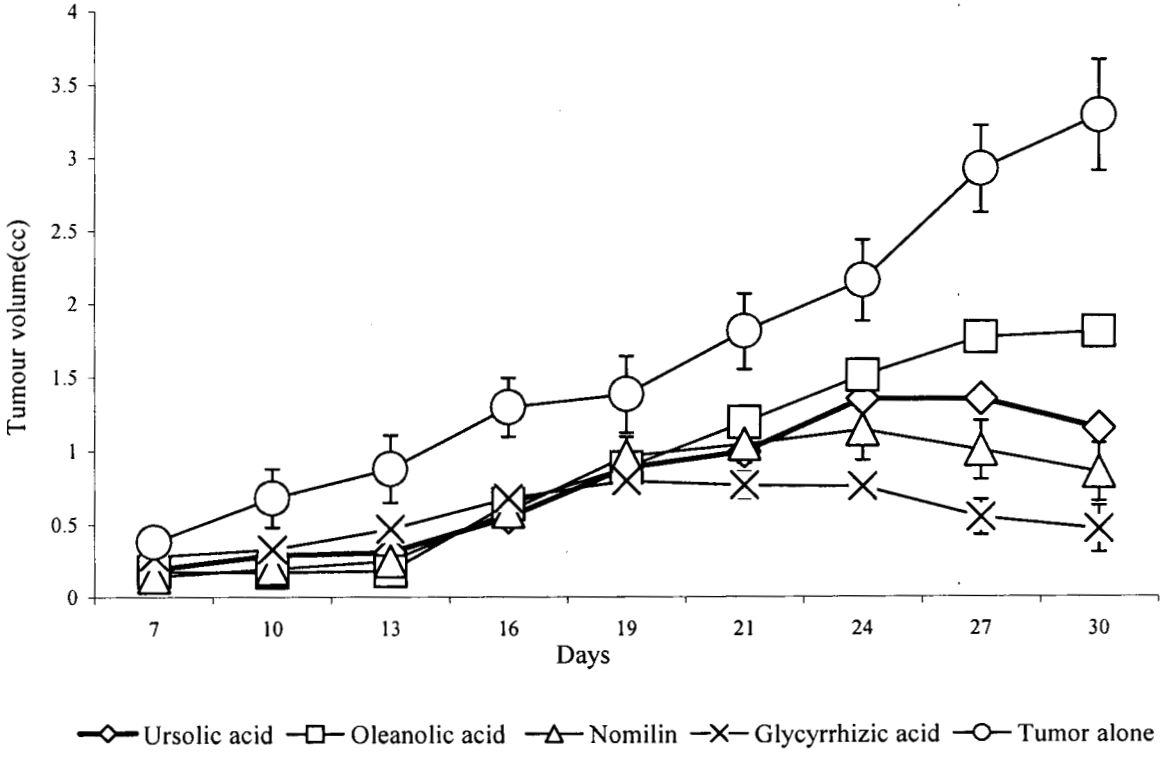


Fig 4.2 Antitumour effect of terpenoids on B16F-10 induced solid tumour

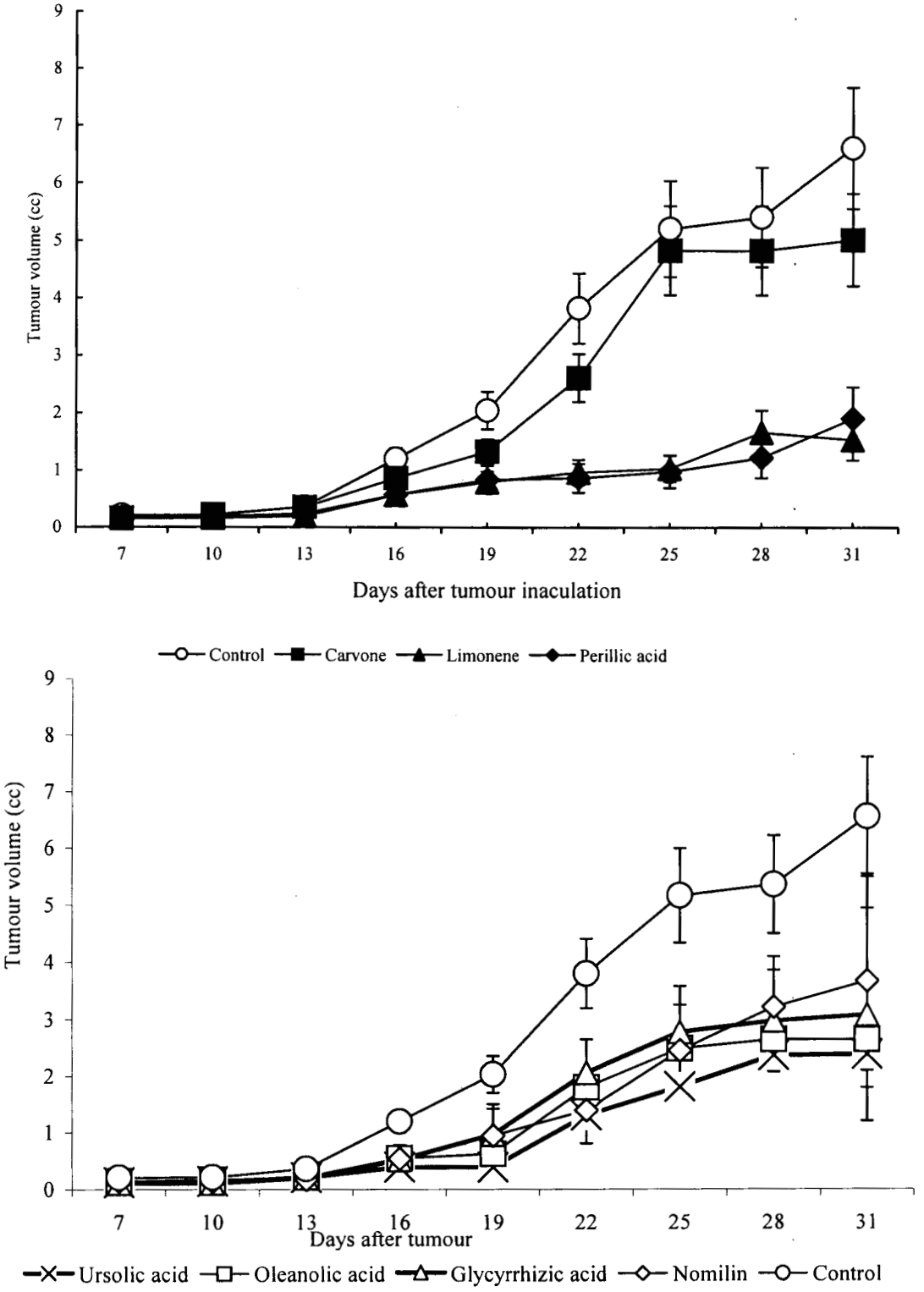


Table 4.1 Effect of naturally occurring terpenoids on the life span of EAC acites tumour bearing animals

Treatment	Average life span	Increase in life span (%)
Tumour alone control	13.6±2.06	
Carvone	17.8±3.9	30.8%
Limonene	25±4.18*	83.8%
Perillic acid	25.5±5.8*	87.5%
Ursolic acid	21.3±4.9**	56.6%
Oleanolic acid	16±1.67*	17.6%
Glycyrrhizic acid	25.1±7.67*	84.5%
Nomilin	16.5±1.04	21.3%

Ascites tumour was induced by injecting Ehrlich ascites cells (1 x 10^6 cells/ animal) into peritoneal cavity of Swiss albino mice. Terpenoid treated groups received 10 doses of various terpenoids. The death pattern of animals due to tumour burden was noted and percentage increase of life span (%ILS) was calculated

(*P<0.05, **P<0.01 Compared with tumour alone treated control animals)

3.3 ANTIOXIDANT STUDIES

3.3a Effect of naturally occurring triterpenoids on the inhibition of lipid peroxidation

The triterpenoids were investigated for their ability to inhibit the ferrous ion induced peroxidation of liver homogenate. Nomilin treated groups (56.9%) showed maximum inhibition in peroxide formation in liver followed by glycyrrhizic acid treated group (43.5%). Limonene and carvone produced 38.8% inhibition of lipid peroxidation while perillic acid was found to produce 39.9 % inhibition of lipid peroxidation (Table 4.2).

3.3b Effect of naturally occurring triterpenoids on the inhibition of *in vitro* oxidant systems

The terpenoids were found to inhibit the production of reactive oxygen species and free radicals. IC_{50} values for each system is shown in table 4.3.

These terpenoids were found to scavenge the superoxide generated by riboflavin photoreduction method. The concentration of terpenoids oleanolic acid and perillic acid needed for 50 % inhibition of superoxide radicals was found to be 8.7 and 9.8 μg/ml respectively. Where as IC_{50} values for ursolic acid and nomilin were 10 μg/ml. Other terpenoids also found to scavenge the superoxide radicals (IC_{50} values for carvone, limonene and glycyrrhizic acid were 13.6,13.8 and 20.1 μg/ml respectively)

All these terpenoid could effectively inhibit the production of nitric oxide radicals generated from sodium nitroprusside. Oxidised terpenoid nomilin and glycyrhizic acid effectively inhibited the production of nitric oxide. (IC_{50} values 10.8 and 10.6 μg/ml respectively for nomilin and glycyrrhizic acid).The terpenoids carvone, limonene, ursolic acid and oleanolic acid also effectively inhibit the NO production.(IC_{50} values 12.5, 15.5, 16.5 and 16.4 μg/ml respectively)

Table 4.2 Effect of naturally occurring terpenoids on the inhibition of liver lipid peroxidation

Treatment	Concentration of MDA nmol/ml	% of inhibition
Control	17.69±1.2	
Carvone	10.83±1.02*	38.7
Limonene	10.82±0.98*	38.8
Perillic acid	10.63±1.51*	39.9
Ursolic acid	11.33±1.07*	35.9
Oleanolicacid	11.17±0.99*	36.8
Glycyrrhizic acid	9.98±1.03*	43.5
Nomilin	7.62±0.94*	56.9

Animals were treated with 5 doses of terpenoids for five consecutive days. Animals were sacrificed 24h after the 5^{th} dose and liver excised and homogenized. The lipid peroxidation was measured by the method of Ohkawa et al (Chapter 2)
(*$P<0.01$ Compared with non treated control)

Table 4.3 Effect of naturally occurring terpenoids on the production of reactive oxygen species and free radicals

Terpenoid	IC 50 values in microgram/ml			
	Superoxide	Hydroxyradical	Nitric oxide	Lipid peroxidation
Carvone	13.6	156.3	12.5	100.1
Limonene	13.8	227.9	15.5	77.3
Perillic acid	9.8	159.8	27.5	118.7
Ursolic acid	10.0	178.9	16.4	79.4
Oleanolicacid	8.7	158.1	16.5	77.8
Glycyrrhizic acid	20.1	218.9	10.61	120.1
Nomilin	10.0	178.9	10.8	119.8

% of inhibition was calculated by comparing the optical density of the test with that of control.

The terpenoids carvone, limonene perillic acid ursolic acid oleanolic acid glycyrrhizic acid and nomilin could also inhibit the degradation of deoxyribose by hydroxyl radicals generated by Fe3+/ ascorbate/ EDTA/ H_2O_2 system. The concentration needed for 50 % inhibition in the production of hydroxyl radicals by carvone, oleanolic acid and perillic acid were 156.3, 158.1 and 159.8 µg/ml respectively. In the case of ursolic acid and nomilin IC_{50} was 178.9 µg/ml

The concentration needed for 50 % inhibition of lipid peroxide generated by induction of Fe2+/ADP/ ascorbate in liver homogenate was 77.3, 77.8 and 79.4 µg/ml for limonene, oleanolic acid and ursolic acid respectively. Where as the IC_{50} values for carvone perillic acid, nomilin and glycyrrhizic acid were 100.1, 118.7, 119.8 and 120.1 µg/ml respectively.

4 DISCUSSION

Production of reactive oxygen and nitrogen species is quite normal. But this gets enhanced during pathophysiological condition creating oxidative stress. Reactive species are also generated during phagocytosis, a manifestation of innate immunity. Migrations of leucocytes at an inflammatory site result in phagocytosis with the release of enzyme and cytokines from both macrophages and neutrophils. During this phenomenon cellular constituents gets altered resulting in decreased state. This may be effectively neutralized by enhancing cellular defense in the form of antioxidants.

Free radicals of oxygen, hydrogen peroxide and organic peroxides have been identified as agents that contribute to tumour promotion probably by forming oxidized DNA bases, which can act as cancer initiators as well as promoters. Lipid peroxidation mediated membrane damage is one of the deleterious effects of free radicals. The evidence for radical mediation of many events in carcinogenesis is very strong (Pryor,

1987) and radical agents produce damage to lipids, proteins, membranes and DNA. The oxygenation, the growth rate and the metastatic potential of a solid tumour depend on its vascularization and in particular angiogenesis. A therapeutical approach affecting angiogenesis has been suggested as an alternative to conventional ones (Blackburn, 1999; Toyokuni, 1995). Certain antioxidant enzymes play major roles in neovascularisation (Blackburn, 1999; Toyokuni, 1995). Even though a number of compounds have been identified for the treatment of malignancy, most of them produce severe side effects. Good immunostimulants and antioxidants could alleviate toxic nature of conventional radio and chemotherapies were well known. But artificially prepared drugs limited their use due to their toxicity. So we gave much importance to plant derived compounds with fewer side effects.

In the present study we could analyze that these compounds could inhibit ferrous ion (Fe2+) induced lipid peroxidation in mice liver homogenate, superoxide generation hydroxyl radical (OH) generation and nitric oxide production. These radicals are the most reactive chemical species known. OH radical severely damages the bases and sugars of DNA and induces strand breakage. Lipid peroxidation mediated membrane damage is one of the deleterious effects of free radicals. These will ultimately lead to cell death (Hochstein and Ernster, 1963; Poyer and McCay, 1971; Minotti and Aust, 1987). We could also analyze that these compounds could inhibit lipid peroxidation in mice liver (*in vivo*). The percentage of inhibition was maximum in nomilin treated group (56.9%). These terpenoids compounds may induce phase II enzymes and also raised the cellular glutathione levels and such increase in GSH presumably augment cellular antioxidant defenses.

Antitumour activity derived from medicinal plants may produce results via a number of mechanisms including effects on cytoskeltal proteins which play a key role in mitosis, inhibition of activity of topoisomerase enzyme I and II, stimulation of immune system or antiprotease-antioxidant activity (Hande, 1998., John Mann, 2002). In the present study we could analyze the antitumourigenic activity of these terpenoids. All of these terpenoids reduce the tumour growth. The maximum reduction in DLA induced solid tumour development observed in ursolic acid, nomilin and glycyrrhizic acid treated groups. Among the terpenoid treated group the maximum increment in life span of EAC tumour bearing animals was perillic acid treated animals.

Antitumour activity of triterpenoids may be due to the combined action of immunostimulatory and antioxidant activity. Hence the results indicated that terpenoids, carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin could act as non-toxic antitumour agent.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

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(FACULTY OF SCIENCE)

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CHAPTER 5

RADIOPROTECTIVE ACTIVITY OF NATURALLY OCCURRING TERPENOIDS

1. INTRODUCTION

Radiotherapy is one of the widely accepted theraputical approaches for cancer. However whole body radiation with a dose of more than 100 rads cause acute effects including hematopoietic syndrome and gastro intestinal syndromes which involves nausea, vomiting, diarrhea, decreased count of various blood elements such as red blood cells granulocytes lymphocytes and platelets (Manuch et al., 1995). Unfortunately there is no specific drug at present, which can effectively reduce these effects. Development of effective and non toxic radio protectors that are capable of protecting normal tissues without compromising the anti-cancer activity of radiation is still an active area of research (Weiss, 1997).

In the last few decades several compounds that include certain chemicals such as amifostain (Tannehill et al., 1996) natural antioxidants like reduced glutathione (Bump and Brown, 1990), biological response modifiers like cytokine and immunostimulators (Neta, 1986) etc. were found to provide good radioprotection in experimental animal models. But the toxicity produced after repeated administration limited their clinical use.

Natural products are rich source of pharmacologically active compounds in which plant materials deserves an important position. Medicinal plants serve as good source of pharmacological-compounds and the plant based medicinal practices are effectively used in indigenous systems of medicine from time immemorial. Therefore there is an emerging interest during the last several years in plant products for their radioprotective efficacy. Compounds having antioxidant properties are known to provide protection against radiation and their oxidative stress (Agarwal 2002). Two flavonoids, orientin and vicenin, isolated from the leaves of the Indian plant *Ocimum sanctum* were tested for their radio

protective effect in mice (Uma Devi et al., 2000). Both compounds provided protection against death from gastrointestinal syndrome as well as bone marrow syndrome when injected intraperitoneally (i.p.) prior to whole-body exposure to 11 Gy gamma radiation (Uma Devi et al., 2000). Our recent studies shows that commonly used medicinal plants and herbel preparation are good source of radioprotectors as well as in patients receiving radio therapy (Rekha et al., 2000, Praveenkumar et al., 1996)

In the present chapter an attempt to investigate the protective effect of some of the terpenoids against sub lethal dose of gamma radiation induced damage in mice is investigated.

2. MATERIALS AND METHODS

2.1 Animals

Swiss albino mice (4-6 weeks old male, 20-25g body wt) and Balb/C mice (5-6 weeks) were used for this study.

2.2 Terpenoid compounds

Carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin

Drug administration:- The compounds were suspended in light paraffin oil and intraperitoneally administrated at different concentrations (Carvone and limonene, administered at a concentration of 100 μmoles/kg body wt/dose/animal, perillic acid, ursolic acid, oleanolic acid and glycyrrhizic were administered at a concentration of 50 μmoles/kg body wt/dose/animal and nomilin 10 μmoles/kg body wt/dose/animal.)

2.3 Radiation treatment and experimental design

Two sets of Swiss albino mice were taken and each set was divided into nine groups (10mice /group). All animals were treated with a single sub lethal dose of radiation 600 rad (6 Gy). The source of radiation was a ^{60}Co. Theratron phoniz teletherapy unit (Atomic energy Ltd Canada Ltd). Animals were restrained in specially designed well ventilated cages without anesthesia and exposed to whole body radiation at a rate of 1.40 Gy/min in a field size of 25X25cm^2 and at distance of 80 cm from the source. Group I, II, III, IV,V, VI, and VII were treated with 10 doses of carvone, limonene perillic acid acid, oleanolic acid glycyrrhizic acid, and nomilin respectively. Group VIII was treated with 10 doses of paraffine oil (vehicle control) and group IX was kept as radiation treated control Drugs were intraperitonially administrated from the same day of radiation and were continued for ten consecutive days for all the experiments. The first set of animals were used to examine hematological parameters and body weight and the second set of animals were used for analyzing intestinal toxicity and bonemarrow cellularity.

2.4 Determination of the effect of naturally occurring terpenoids on hematological parameters of mice after radiation

The set I animals were treated with the terpenoid compounds as explained above. Blood was collected from tail vein and parameters such as total WBC count, differential count, body weight and Hb content were recorded prior to the radiation exposure and continued every third day for 30 days

2.5 Determination of effect of naturally occurring terpenoids on radiation induced gastro intestinal toxicity

After 48h, 7th day and 10th day of radiation treatment six animals from each group of set II were sacrificed by cervical dislocation. Liver was quickly excised and washed with ice-cold saline and used for the estimation of lipid peroxidation (Ohkawa et al.,1979) GPT (Bergmeyer et al., 1974) and GSH levels (Moronet al., 1979) (as explained in Chapter 2)

A portion of the small intestine was taken, washed with ice-cold saline and used for the biochemical analysis and histopathological examination. Intestinal mucosa was collected and used for the estimation of GSH content. Blood was collected for estimating ALP (Kind and King 1954), GPT (Bergmeyer et al.,1974) and lipid per oxidation levels (Ohkawa et al.,1979) (as explained in Chapter 2)

2.6 Determination of the effect of naturally occurring terpenoids on bone marrow cellularity and α-esterase activity

Bone marrow collected from the animals of the previous experiment was made to single cell suspension and cell number was determined using hemocytometer. The number of α-esterase positive cells were determined by azodye coupling method (Bancroff and Cook 1984) . A smear of bone marrow cells from the above preparation was made on clean glass slide air dried stained with a naphthyl acetate and para rosaniline hydrochloride and counter stained with hematoxiline. The number of α-esterase positive cells were expressed out of 4000 cells (as explained in Chapter 2).

2.7 Histopathological studies of liver and intestine

The liver and intestinal tissue samples from the previous experiment were fixed in10% formlin, dehydrated and embedded in paraffine wax. Sections (4μm) were stained with eosine and hematoxiline.

2.8 Determination of the effect of terpenoids on spleen colony formation in irradiated mice.

Inbred strains of Balb/C mice (4-5 weeks) were used for spleen colony assay. The animals were divided in to 22 groups (6 animals/group). Group I toVII animals intraperitoneally treated with five doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin on five consecutive days respectively. Group VIII kept as untreated. Animals were sacrificed 24h after the last dose and bonemarrow cells isolated and made into single cell suspension

Groups IX to XXII animals were exposed to single whole body radiation (6Gy/ animal as explined above). It is well known that exposure of mice bone marrow to 6 Gy of γ rays kills most of the hematopoietic cells (Praveen Kumar et al, 1996). Group IX to XVI received bone marrow cells ($Ix10^6$ cells/animal) from normal mice (group VII) through caudal vein. Group X, XI, XII, XIII, XIV, XV and XVI animals received five doses of terpenoids carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin respectively. Group IX, which kept as untreated served as control. Group XVII, to XV animals received the bone marrow cells from terpenoid treated groups (Group I to VII) and the corresponding drugs were intraperitoneally administered for five consecutive days. Maximum numbers of spleen colonies are seen by 7-9 days (Robert, 1989). Hence all the animals were sacrificed on day 7 of radiation exposure and the

number of nodular colonies on the surface of spleen was counted. Each colony formed was derived from a single precursor stem cell designated as Colony Forming Unit Spleen (CFU-Spleen).

3 RESULTS

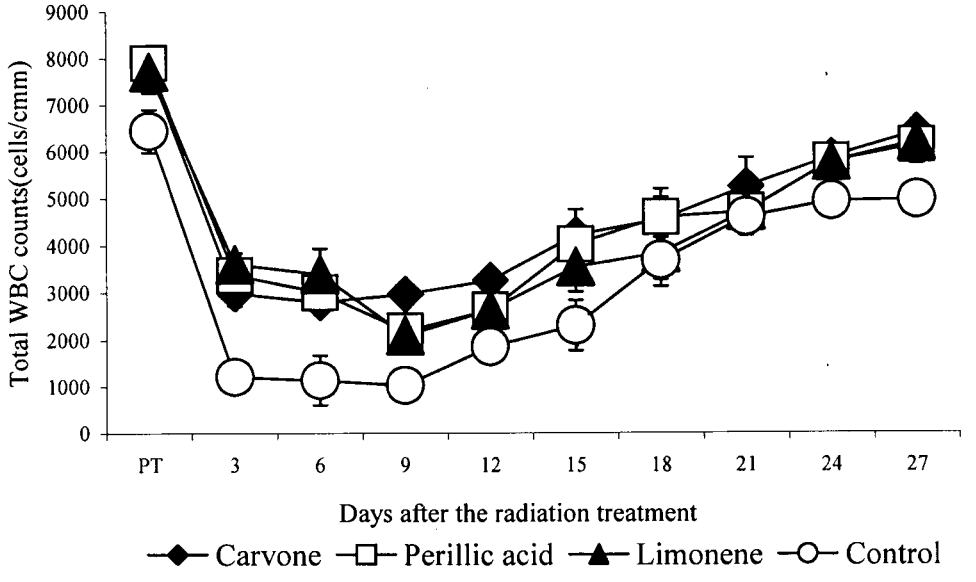
3.1 Effect of naturally occurring terpenoids on hematological parameters

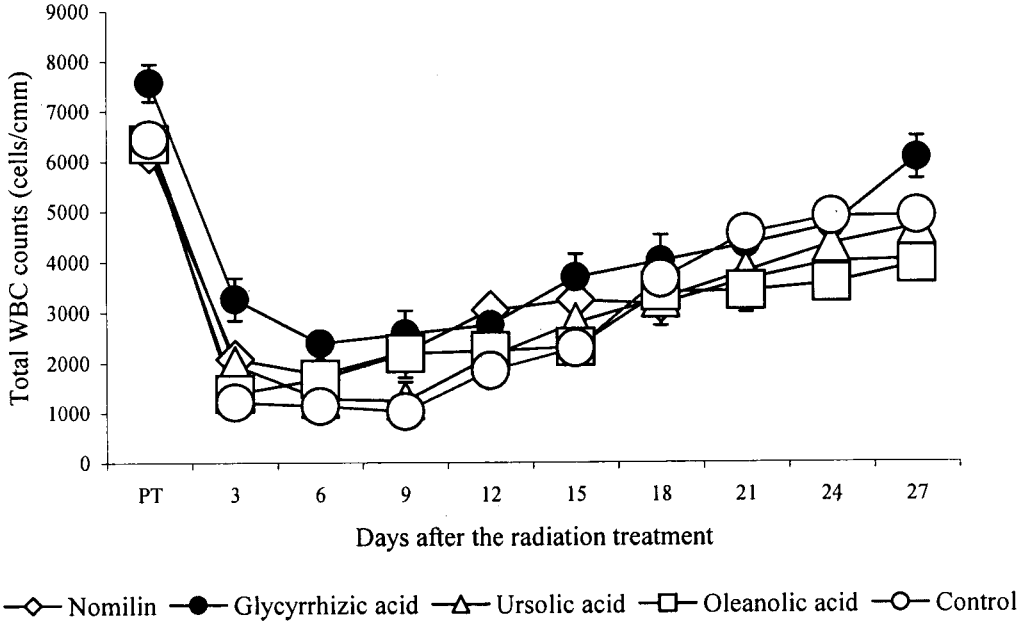
The effect of naturally occurring terpenoids on total WBC count is shown in figure 5.1. The number of WBC in control animals maximally decreased to 1030 cells/cmm after 9th day of radiation treatment and gradually increased up to 4945 cells/cmm by 27 days. On 9th day the total WBC count in carvone, glycyrrhizic acid, oleanolic acid, perillic acid and limonene treated animals was 2970, 2588, 2200, 2188 and 2094 cells/cmm respectively and then these values increased steadily and attained 6381,6177,6100, 5098 and 6100 cells/cmm blood after 30 days. Other terpenoids also increase the total WBC. Where as in the radiation alone treated control group have only the total WBC count was only 4945-cells/cmm even after 30 days. There was no significant change in the differential count of terpenoid treated and untreated control animals. Administration of terpenoid compounds did not have a prominent effect on the Hb levels of radiation-exposed animals.

3.2 Effect of naturally occurring terpenoids on bone marrow cellularity and α-esterase activity in radiation treated mice.

The effect of naturally occurring terpenoid compounds on bone marrow cellularity and α- esterase positive cell is shown in figure 5.2 and 5.3. The number of bone marrow and α esterase positive cells was decreased drastically in the irradiated animals. Administration of the terpenoid compounds could enhance the bone marrow cellularity as

Fig 5.1 Effect ofterpenoids on total WBC counts of radiation treated animals





well as the number of α-esterase positive cells. In control animals after 48th hour of irradiation there was a drastic reduction in the number of bone marrow cells (4X10 ^{6} cells/femur) and α esterase positive cells (35.1 α-esterase positive cells /4000 bone marrow cells) compared to the normal animals. Even 10 days after radiation exposure the bone marrow cells (7X10 ^{6} bone marrow cells/ femur) and α-esterase positive cell number (161 /4000 bone marrow cells) did not reach back to the normal levels (17.9X 10 ^{6} bone marrow cells/femur and 1063 α-esterase positive cells/4000 bone marrow cells). Administration of terpenes could protect the animals from this drastic reduction in the number of bone marrow cells. In the case of carvone, limonene and perillic acid treated animals the bone marrow cellularity was 6.22 X 10 ^{6} cells/femur, 6.76 X 10 ^{6} cells/femur and 8.22 X10 ^{6} cells/femur respectively after 48h of the radiation treatment. On 10th day carvone enhanced bone marrow cell numbers up to 14.6X10 ^{6} cells/femur. By 10th day of radiation exposure carvone treatment could enhance the cell number (14.6X10 ^{6} cells/femur) to normal level. Where as bone marrow cellularity of limonene and perillic acid treated animals on 10th day was 9.5X10 ^{6} cells/femur and 11.8X10 ^{6} cells/femur respectively.

3.3 Effect of naturally occurring terpenoids on gastro intestinal toxicity

The effect of terpenoids in the formation of lipid peroxides in serum and liver of irradiated mice is shown in table 5.1. The whole body radiation elevated the level of lipid peroxides in liver and serum. Administration of terpenoids clearly inhibited the production of lipid peroxides. The maximum inhibition of serum lipid peroxide production after 48h of radiation was obtained in the oleanolic acid and glycyrrhizic acid treated groups (1.8 and 1.9 n mol /ml). Where as after 7th day of radiation was obtained in the perillic acid and

Fig 5.2 Effect of terpenoids on bone marrow cellularity of mice after iradiation

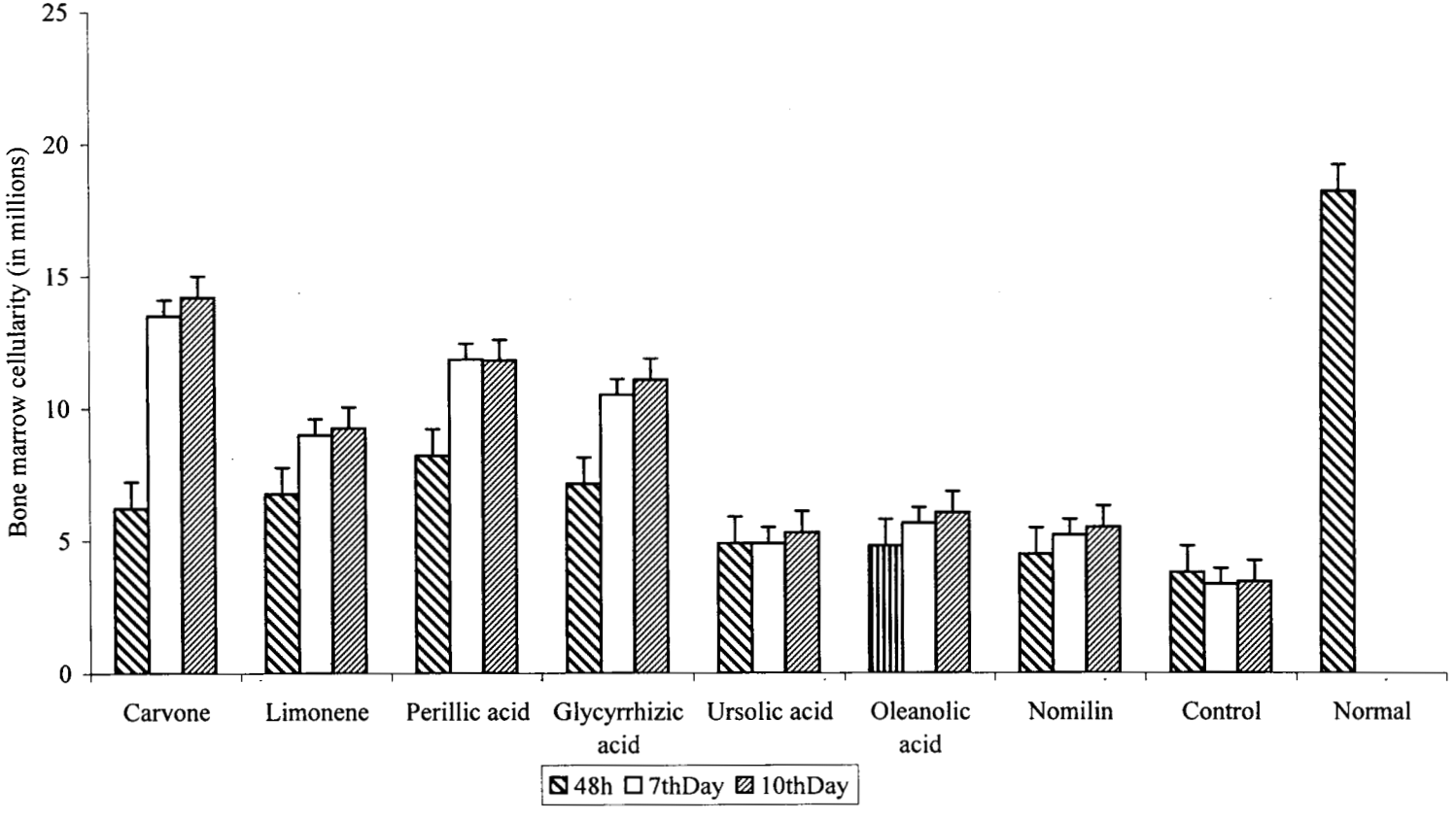


Fig 5.3 Effect of terpenoids on esterase positive cells of mice after iradiation

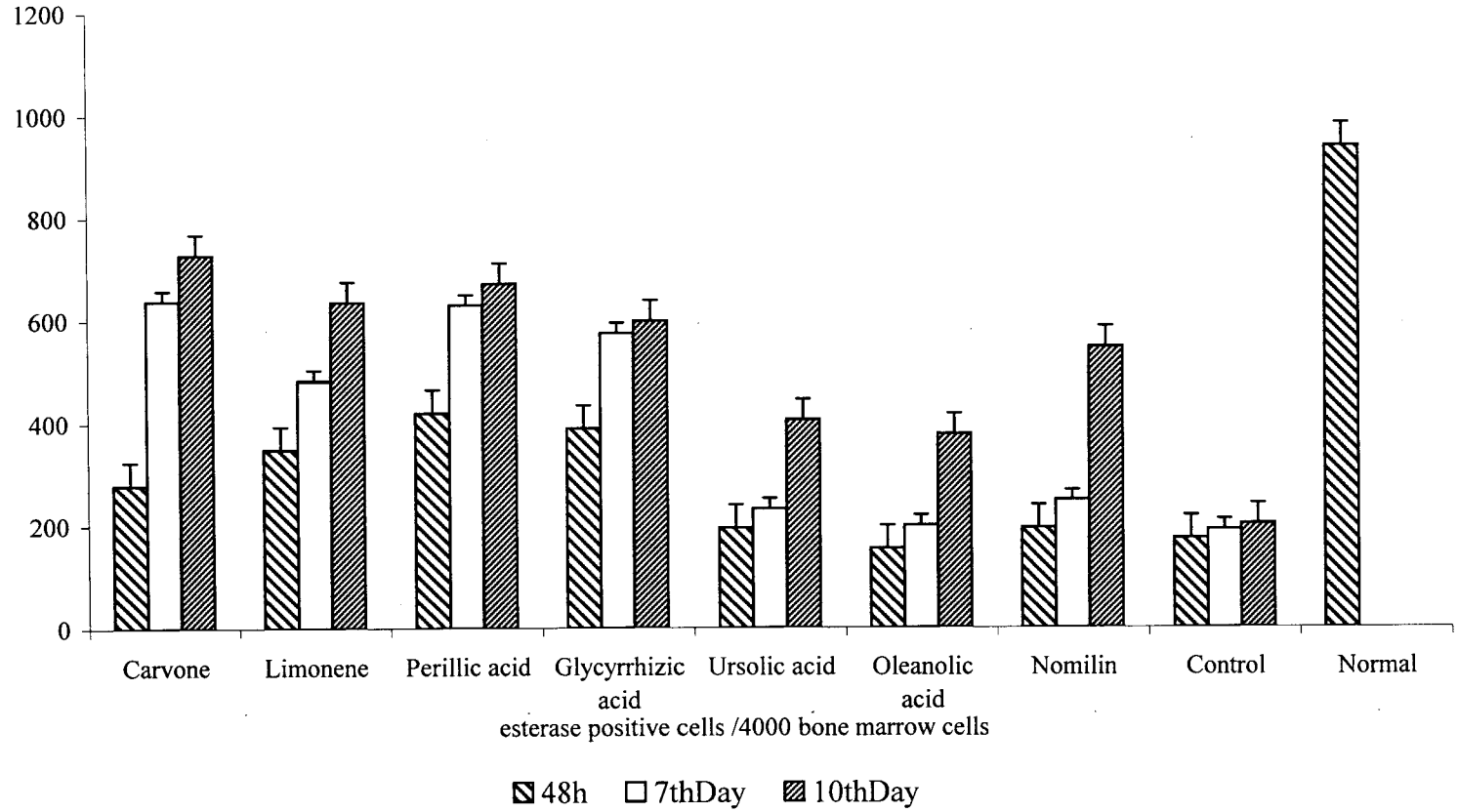


Table 5.1 Effect of terpenoids on the lipid peroxidation in liver and serum of irradiated mice

	Serum lipid (nmol/mg protein)			Liver lipid (nmol/mg protein)		
	48th h	7 th Day	10th Day	48th h	7 th Day	10th Day
Normal	1.5±0.03			0.93±0.05		
Control	2.9±0.08	2.5±0.05	2.3±0.04	3.8±0.2	3.20.±0.1	2.9±0.09
Carvone	2.4±0.01*	2.1±0.06*	1.8±0.08*	2.9±0.2*	2.4±0.05*	2.2±0.1*
Limonene	2.1±0.02*	2.0±0.06*	1.9±0.06*	3.1±0.2*	2.1±0.08*	1.6±0.2*
Perillic acid	2.4±0.09*	1.7±0.04*	1.6±0.05*	2.9±0.08*	2.3±0.09*	1.8±0.11*
Glycyrrhizic acid	1.9±0.08*	1.6±0.05*	1.7±0.1*	3.0±0.07*	2.6±0.09*	2.2±0.09*
Ursolic acid	2.1±0.05*	2.0±0.03*	1.9±0.08*	3.3±0.2*	3.0±0.09*	1.7±0.08*
Oleanolic acid	1.8±0.09*	1.8±0.05*	1.7±0.05*	3.1±0.1*	2.5±0.16*	1.9±0.13*
Nomilin	2.3±0.09*	1.9±0.04*	1.8±0.06*	3.2±0.09*	2.3±0.08*	2.4±0.5*

All animals were treated with a single sub lethal dose of radiation 600 rad (6 Gy). Terpenoids were administrated intraperitoneally to various animal groups. After 48h, 7th day and 10th day of radiation treatment six animals from each group were sacrificed by cervical dislocation. Liver was quickly excised, blood collected by heat pucturing and serum seperated and used for the estimation of lipid peroxidation

(*$P<0.01$ Compared with radiation alone treated control.)

glycyrrhizic acid treated groups (1.7 and 1.6 nmol /ml). 10^{th} after day of radiation treatment all the terpenoid treated groups tend to attain normal value (1.5 n mol /ml) where as radiation alone treated control group shown the peroxide production was 2.3 n mol /ml. Inhibition of liver lipid peroxide production was negligible after 48h of radiation exposure. But 7^{th} day after irradiation limonene treated groups were showed maximum inhibition (2.1 nmol/mg protein) .On the 10^{th} day of irradiation limonene (1.6 nmol/mg protein) ursolic acid (1.7 nmol/mg protein), perillic acid (1.8 nmol/mg protein) and oleanolic acid 1.9 (nmol/mg protein) treated groups were shown the maximum inhibition. Where as in radiation alone treated groups the value was 2.9 nmol/mg protein.

The effect of terpenoids in the formation of alkaline phosphate levels (ALP) after radiation exposure is shown in table 5.2. Administration of various terpenoids lowered the elevated levels of ALP in radiation treated mice. After 48h serum ALP production in radiation alone treated animals was 23.9 KA units where as in carvone, limonene, perillic acid and nomilin treated mice it was only 8.2, 10.1, 11.1 and 10 KA units respectively. Glycyrrhizic acid and ursolic acid treated groups did not show any appreciable inhibition (20.7 and 22.8 respectively). On 10 day after irradiation all the terpenoid treated groups showed normal levels of ALP production (6.1 KA units). After 48h liver ALP production in radiation alone treated animals was enhanced to 17.7 KA units. The maximum inhibition in the production of liver ALP after 48h of radiation exposure was obtained in the case of ursolic acid treated groups (9.7 KA units) followed by limonene (11.5 KA units), glycyrrhizic acid (11.6 KA units), perillic acid (12.2 KA units), nomilin (12.5 KA units) and carvone (12.7 KA units) treated groups. After 10 day of irradiation all the

Table 5.2 Effect of terpenoids on the alkaline phosphatase levels of serum and liver of irradiated mice.

	Serum ALP (KA units)			Liver ALP (KA units)		
Treatment	48th h	7 th Day	10th Day	48th h	7 th Day	10th Day
Normal	6.1±0.4			5.8±0.4		
Control	23.9±1.0	14.4±0.7*	11.2±0.7*	17.7±0.8*	16.7±0.9*	13.3±0.5*
Carvone	8.2±0.1*	10±0.2*	8.1±0.8*	12.7±0.5*	8.2±0.5*	7.2±0.3*
Limonene	10.1±0.4*	8.2±0.5*	7±0.4*	11.5±0.7*	7.8±0.5*	6.8±0.5*
Perillic acid	11.1±0.5*	10±0.5*	9.4±0.5*	12.2±0.7*	7.6±0.6*	6.4±0.6*
Glycyrrhizic acid	20.7±0.8*	11.6±0.4*	7.1±0.3*	11.6±0.8*	7.4±0.3*	6.6±0.4*
Ursolic acid	22.8±0.7**	12±0.6*	6.2±0.4*	9.7±0.7*	7.5±0.3*	6.5±0.6*
Oleanolic acid	18.6±0.8*	13.1±0.7*	6.4±0.4*	13.1±0.5*	6.7±1.0*	6.3±0.7*
Nomilin	10±0.6*	8.2±0.8*	7.5±0.7*	12. 5±0.7*	7.5±0.9*	6.9±0.5*

All animals were treated with a single sub lethal dose of radiation 600 rad (6 Gy). Terpenoids were administrated intraperitoneally to various animal groups. After 48h, 7th day and 10th day of radiation treatment six animals from each group were sacrificed by cervical dislocation. Liver was quickly excised, blood collected by heart pucturing and serum seperated and used for the estimation of alkaline phosphatase levels
(*$P<0.01$, **$P<0.05$ Compared with radiation alone treated control)

treated animal groups showed normal levels of ALP production (5.8 KA units) except radiation alone treated control group.

The effect of terpenoids on serum and liver GPT levels in irradiated mice is shown in table 5.3. The whole body radiation elevated the GPT levels in liver and serum. The maximum inhibition in serum GPT levels, 48^{th} h after irradiation was found to be obtained in nomilin (20.5 U/ml) treated animals followed by ursolic acid (21.2 U/ml), glycyrrhizic acid (21.4 U/ml), perillic acid (22.1 U/ml), oleanolic acid (22.3 U/ml) and limonene (23.9 U/ml). After 48^{th} h of irradiation serum GPT levels of carvone treated group was 27 U/ml where as in radiation alone treated control group the value was 36.6 U/ml. 48^{th} h after irradiation the liver GPT level was highly elevated in radiation alone treated animals (76.3U/ml). Administrations of various terpenoids clearly reduced the elevated liver GPT levels. In glycyrrhizic acid and oleanolic acid treated groups liver GPT levels were only 54.2 U/ml and 55.2 U/ml respectively. Intraperitoneal administration of other terpenoids also reduced the elevated levels of GPT (Carvone (64.6 U/ml), limonene (68.2 U/ml) perillic acid (63.1 U/ml) ursolic acid (65.5 U/ml) and nomilin (58.3 U/ml)). On 10^{th} day after irradiation radiation alone treated control animals alone have elevated levels of liver GPT (69.6 U/ml) where as terpenoid treated groups attained almost normal values (48.9 U/ml)

The change in the GSH levels in liver and intestine is shown in figure 5.4 and 5.5. Administration of terpenoids enhanced the GSH level in liver as well as intestine of radiation exposed animals. In control animals the levels in the intestine and liver were 5.5 and 5.8 n mol/mg protein respectively after 48h of radiation exposure. But in carvone treated animals the GSH levels were enhanced to 7.0 and 5.8 nmol/mg protein in intestine

Table 5.3 Effect of terpenoids on glutamate pyruvate transaminase (GPT) level in serum and liver of irradiated mice.

	Serum GPT (U/ml)			Liver GPT(U/ml)		
Treatment	48^{th} h	7^{th} Day	10^{th} Day	48^{th} h	7^{th} Day	10^{th} Day
Normal	13.5±0.7			48.9±0.4		
Control	36.6±1.4	36.4±0.5	28.5±1.1	76.3±0.2	77.3±0.5	69.6±0.8
Carvone	27±1.1*	28.9±0.9*	20.9±0.6*	64.6±0.9*	52.8±1.1*	50±0.8*
Limonene	23.9±1.2*	25.4±0-.7*	23.1±0.8*	68.2±0.8*	53.8±1.0*	49.4±1.1*
Perillic acid	22.1±0.9*	23.1±0.5*	23.3±0.4*	63.1±1.1*	53.3±0.9*	51±1.0*
Glycyrrhizic acid	21.4±0.9*	17.2±1.1*	13.5±1.2*	54.2±1.2*	50±1.0*	48.9±1.0*
Ursolic acid	21.2±1.5*	21.5±1.5*	20±0.9*	65.5±1.1*	50.2±1.2*	50.2±1.2*
Oleanolic acid	22.3±0.8*	22.5±0.7*	21.6±0.8*	55.2±0.9*	51±0.8*	49.3±1.1*
Nomilin	20.5±0.5*	20.8±0.9*	20±0.9*	58.3±0.8*	53.1±0.6*	49.2±0.9*

All animals were treated with a single sub lethal dose of radiation 600 rad (6 Gy). Terpenoids were administrated intraperitoneally to various animal groups. After 48h, 7th day and 10th day of radiation treatment six animals from each group were sacrificed by cervical dislocation. Liver was quickly excised, blood collected by heat pucturing and serum seperated and used for the estimation of glutamate pyruvate transaminase (GPT) levels

(*$P<0.01$ Compared with radiation alone treated control)

Fig 5.4 Effect of terpenoids in liver GSH production of mice after irradiation

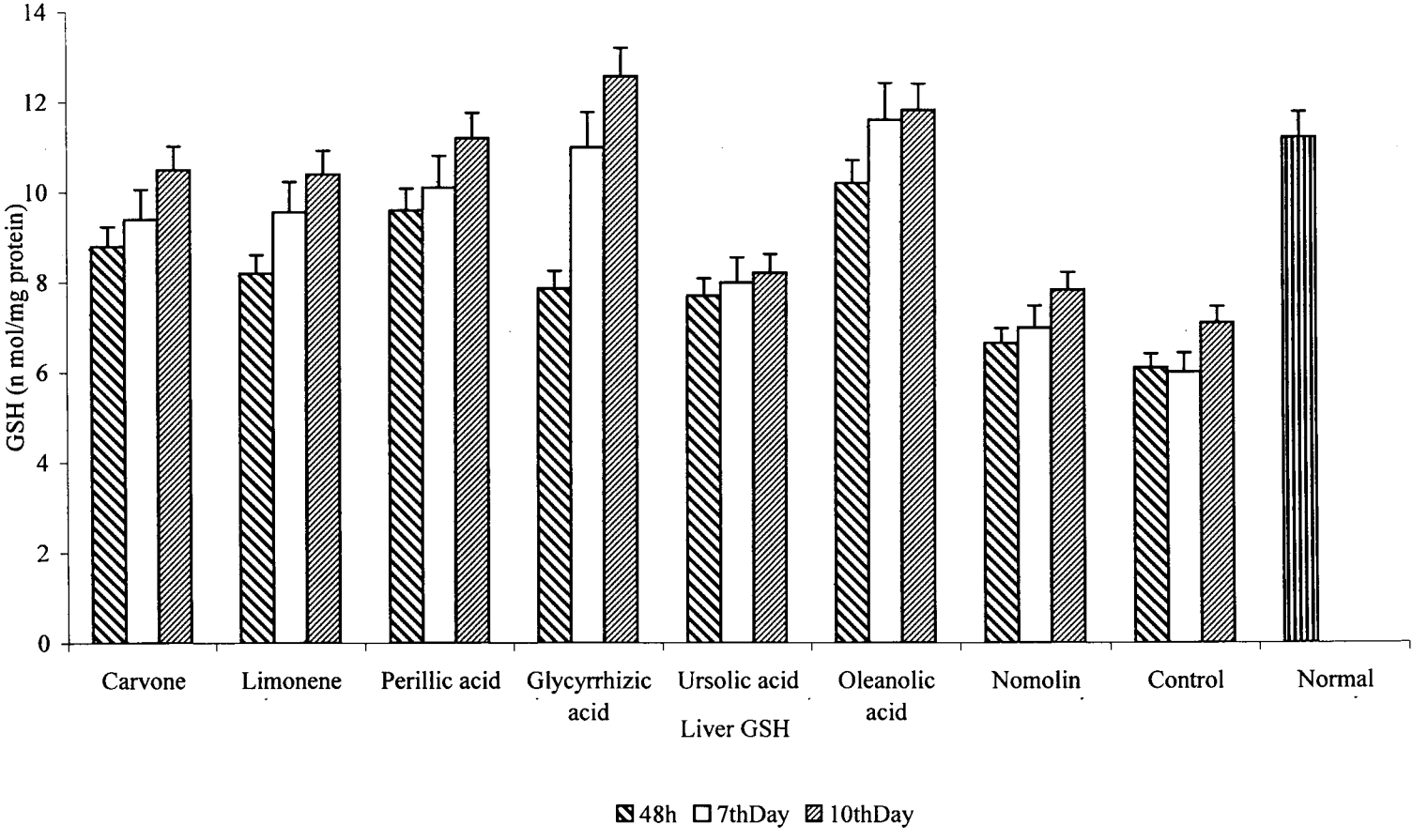
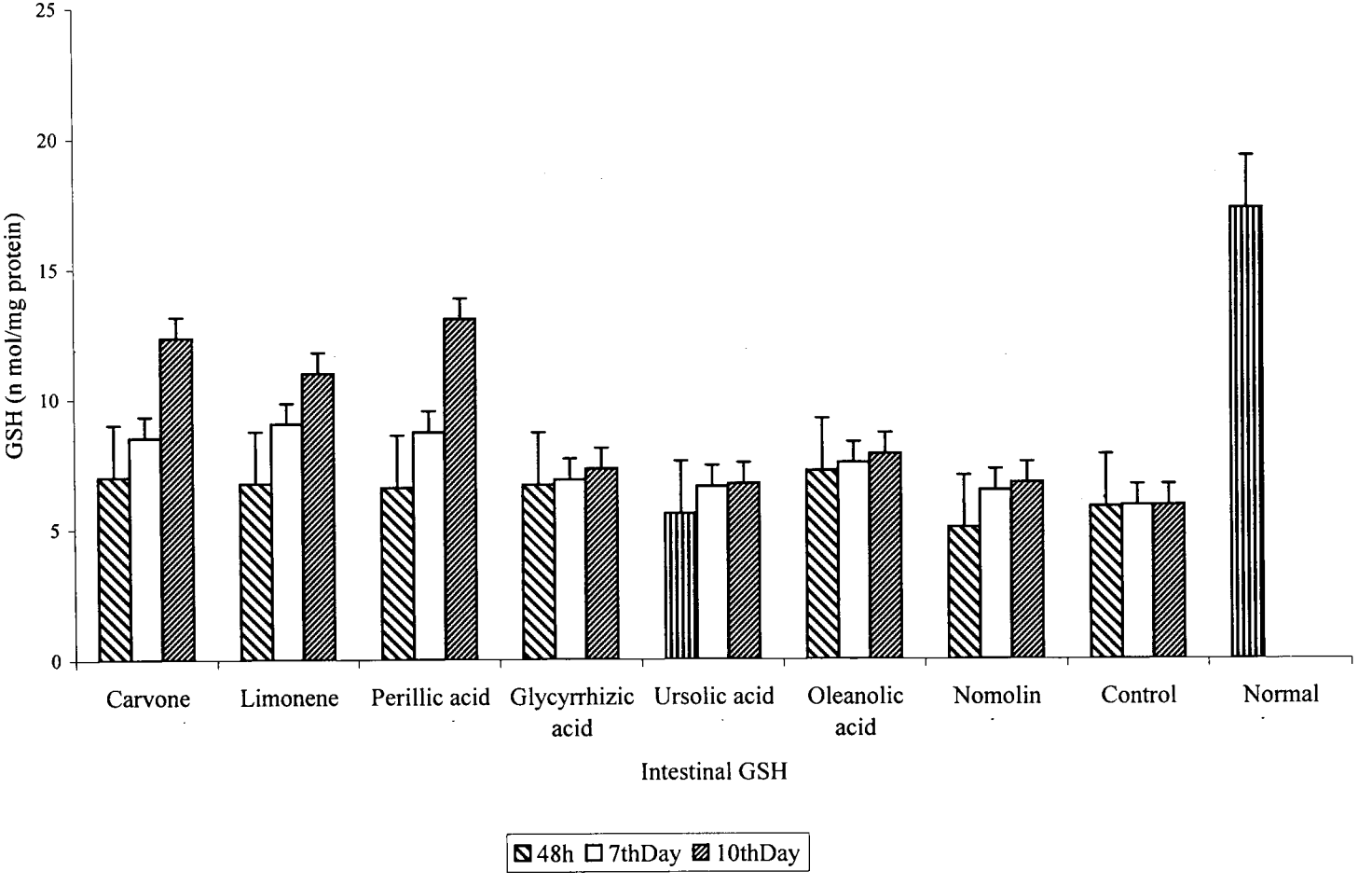


Fig 5.5 Effect of terpenoids in intestinal GSH production of mice after irradiation



and liver respectively. After 48h of radiation exposure liver GSH content of perillic acid and limonene treated animals were 6.6 and 5.8 nmol/mg respectively. On10th day after radiation treatment the GSH values of terpenoid treated animals attained nearly equal to normal values (16.6 and 6.1 n mol /mg protein in intestine and liver respectively) where as in control animals the values were 5.6 and 7.1 n mol /mg protein in intestine and liver respectively.

3.4 Effect of naturally occurring terpenoids on the histopathology of liver and intestine of irradiated mice.

Histopathological studies show damage to the intestine and liver because of its susceptibility to radiation. A severe damage to intestinal villi and crypts could be seen in control animals. Control animals showed maximum damage to intestinal mucosa and maximum damage was seen after 48h of irradiation. The maximum liver toxicity was obtained in control animals after 48h of radiation treatment. The number of globlet and dead cells were also found to be increased in control animals. The increase in the number of globlet cells and dead cells was found to be much less in terpenoid treated group.

3.5 Effect of terpenoids on spleen colony formation in irradiated mice.

Effect of terpenoids compounds on spleen colony formation is shown in figure 5.7. On 7th day after the irradiation, untreated bonemarrow administrated mice have the least number of spleen colonies (the total number of spleen colonies were only 2.5). Animals which received the bonemarrow from terpenoid treated animals after whole body irradiation followed by terpenoid treatment showed maximum number of spleen colonies. Among these groups maximum spleen colonies were observed in perillic acid treated group (13.25 spleen colonies) followed by oleanolic acid (10.5 spleen colonies). The

5.6 Effect of terpenoids on spleen colony formation in irradiated mice

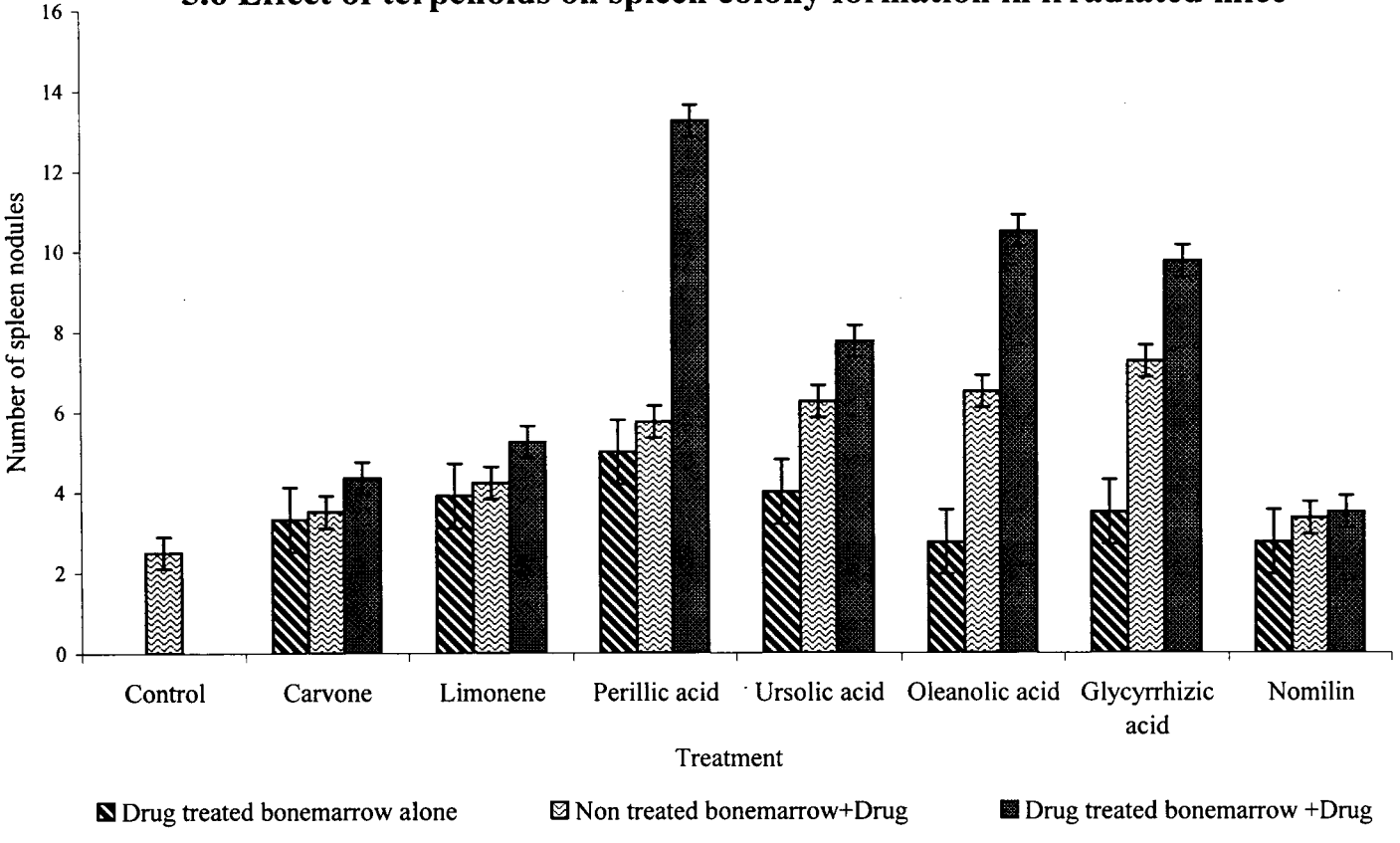


Figure 5.7 Effect of naturally occurring terpenoids on the histopathology of intestine of irradiated mice (48 h)

Figure 5.7a Radiation alone treated control animals

Figure 5.7b Carvone treated

Figure 5.7c Limonene treated

Figure 5.7d Perillic acid treated

Figure 5.7e Ursolic acid treated

Figure 5.7f Oleanolic acid treated

Figure 5.7g Glycyrrhizic acid treated

Figure 5.7h Nomilin treated

Figure 5.7i Normal animal

Fg 5.7 Effect of naturally occurring terpenoids on the histopathology of intestine of irradiated mice.

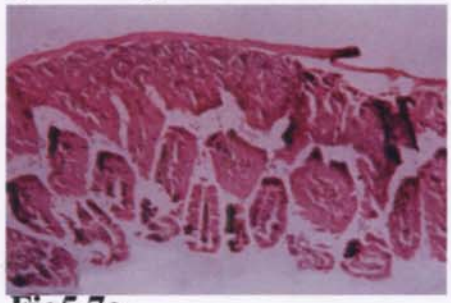


Fig5.7a

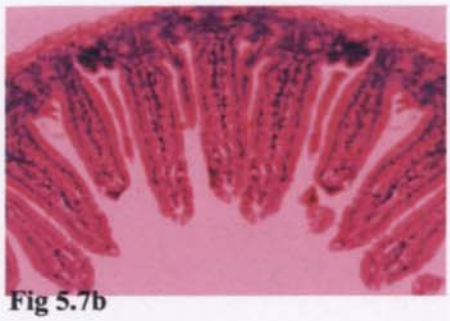


Fig 5.7b

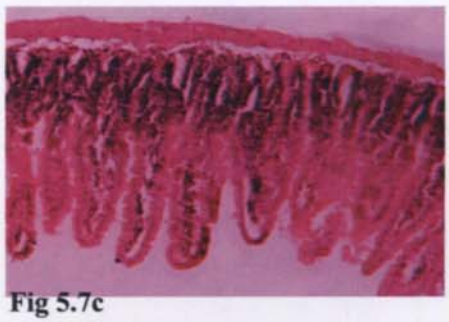


Fig 5.7c

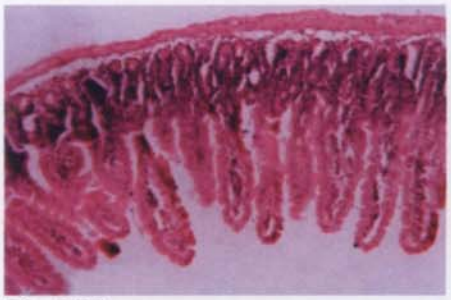


Fig 5.7d

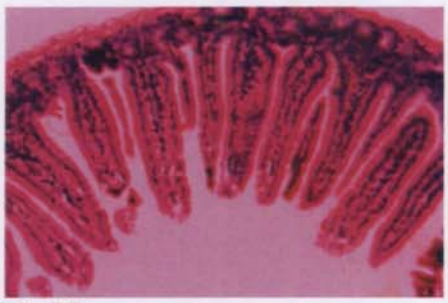


Fig 5.7e

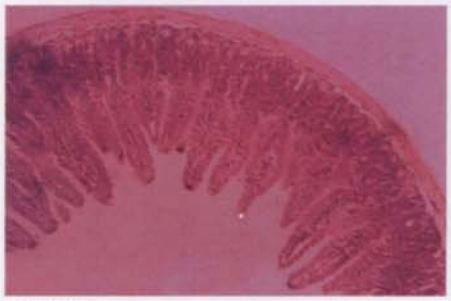


Fig 5.7f

Fig 5.7g

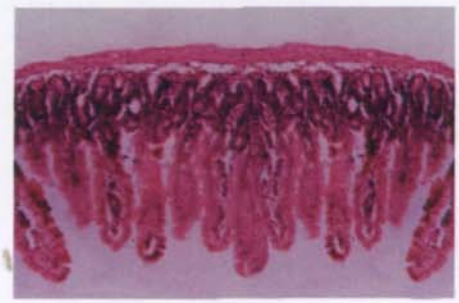


Fig 5.7h

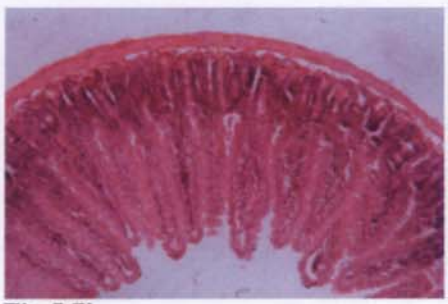


Fig 5.7i

Figure 5.8 Effect of terpenoids on spleen nodule formation in irradiated mice

Figure 5.8a Control animals

Figure 5.8b Carvone treated

Figure 5.8c Limonene treated

Figure 5.8d Perillic acid treated

Figure 5.8e Ursolic acid treated

Figure 5.8f Oleanolic acid treated

Figure 5.8g Glycyrrhizic acid treated

Figure 5.8h Nomilin treated

Figure 5.8i Normal animal

Fig 5.8 Effect of terpenoids on spleen nodule formation in irradiated mice

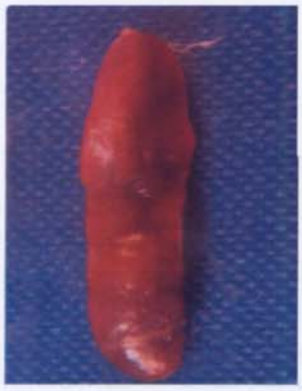


Fig 5.8a

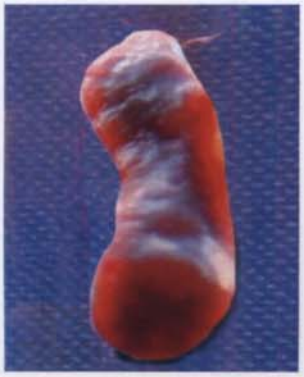


Fig 5.8b

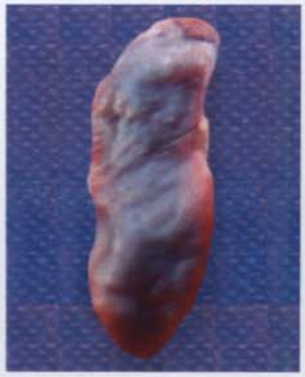


Fig 5.8c

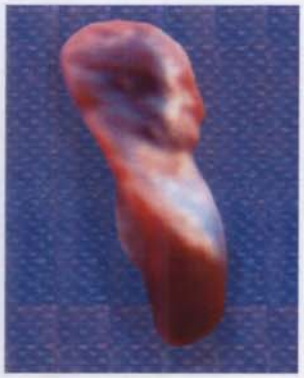


Fig 5.8d

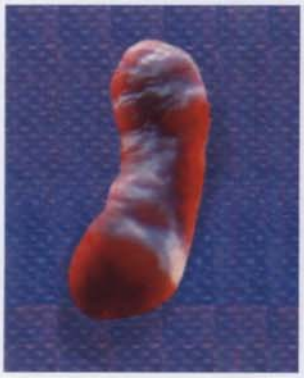


Fig 5.8e

Fig 5.8f

Fig 5.8g

Fig 5.8h

Fig 5.8i

relative spleen weights also assessed. The maximum relative spleen weight was observed in animals which received the bonemarrow from oleanolic acid treated animals after whole body irradiation followed by oleanolic acid treatment (0.47g).

4 DISCUSSION

Ionizing radiation is toxic to organism since it induces deleterious structural changes in essential macromolecules (Bond et al., 1965; Jagetia et al., 2003). Radiation therapy mainly aims the proliferative cells. It also targets normal cells that are in mitotic phase in addition to highly proliferative tumour cells (Shaheen and Hassan,1990). The main side effects of radiation therapy is due to the tissue damage is mainly by cell depletion of target renewal tissues. These effects depend on the balance between cell killing and compensatory replication of stem cell and proliferative cells.

Whole body radiation result in a reduction in total WBC count, bone marrow cellularity and α-esterase positive cell. Bone marrow serves as major source of hemopoetic stem cells. Enhanced number of bone marrow and α-esterase positive cells clearly indicate the effect of these compounds on stem cell proliferation in irradiated mice and its protective activity against radiation induced hemopoetic syndrome.

Ionizing radiation causes damage to living tissue through a series of molecular events depending on the radiation energy. Since human tissue contains 80% water the major radiation damage is due to the aqueous free radical generated by the action of radiation on water. These free radicals react with cellular macromolecule such as DNA, RNA, proteins membrane etc. and cause cell dysfunction and mortality (Moller and Wallin, 1998). Free radicals increase membrane lipid peroxidation, which in turn can alter

the integrity of membrane structure leading to inactivation of membrane, bound enzymes, loss of permeability of membrane and decrease in membrane fluidity. Whole body irradiation increased the level of lipid peroxidation in liver and serum. But administration of terpenoids could effectively inhibit the lipid peroxidation in irradiated mice.

The multiple physiological and metabolic functions of GSH include thiol transfer reactions that protect cell membrane and protein. GSH participate in reaction that destroy hydrogen peroxide, organic peroxide, free radicals and certain compounds (Rana et al., 2002). Administration of terpenoids increased the GSH content in intestine as well as liver in irradiated mice. Intestine is most susceptible to radiation leading to the damage of intestinal villii and crypts. Enhanced level of GSH could prevent the tissue damage in intestine. Higher GSH content in liver of terpenes treated mice could protect the tissue from radiation-induced damage. This observation was also confirmed by histopathological analysis of liver and small intestine. In irradiated mice treated with terpenoid compounds normal liver and intestinal architecture was observed

Serum alkaline phosphates levels also indicate tissue damage. Terpenoid administration showed to decrease the level of ALP levels in irradiated mice, which shows the protective activity of terpenes against radiation induced toxicity.

This study showed that terpenoids could protect damages produced after radiation exposure and also prevent the peroxide damage of cell membrane. These compounds are nontoxic nutritive ingredients present in various fruits and essential oils consumed by man. A detailed clinical study must be carried out to exploit the potential of terpenoids as good radio- protectors.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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CHAPTER 6

CHEMOPROTECTIVE EFFECT OF NATURALLY OCCURRING TERPENOIDS

1. INTRODUCTION

Major draw back of cancer chemotherapeutics agents are their toxic side effects in particular cytotoxicity. The normal tissues affected are those composed of cells undergoing rapid growth such as gastrointestinal tract, hair follicles and bone marrow. Cyclophosphamide (CTX) is a widely used chemotherapeutic agent for antineoplastic therapy. It is a cyclic phosphamide ester of mechlorethamine and function as alkylating agent activated to cytotoxic metabolites 4-OH cyclophosphamide and a phosphoramide mustard by mixed function oxidases in hepatic microsomes (Colvin et al., 1973). CTX is used to treat lymphomas, Hodgkin's disease (De vita, 1991) multiple myeloma (Alexanian et al., 1977), leukemia (Jones et al., 1975), ovarian cancer and breast cancer (Jones et al., 1975). CTX administration causes nausea, vomiting, mucosal ulceration, interstitial pulmonary felorious, hepatic toxicity, lympocytopenia and alopecia (Belli et al., 1967; Bartsch et al., 1982; Glick et al., 1982). Higher doses of CTX administration produce severe urotoxicity with hemorrhagic cystitis on urinary bladder (Hutter et al., 1973). Development of effective and toxic protectors of chemotherapy is essential for reducing the injury to normal cells.

Natural product research continues to explore a variety of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industry. In recent years many natural compounds derived from plants and or crude plant extracts have been proved to have protective effect against toxic effects of many chemicals and to combat a variety of ailments (Rupjyoti Bharali et al., 2003).

Immunosuppression is one of the main toxic side effects of CTX administration. Use of plants as a source of immunomodulators is still in a developing state. Many of them

such as Withania somnifera (Davis and kuttan, 2000), diallyl sulphide and diallyl disulphide (Manesh and Kuttan, 2002) are reported to possess antitumor activity as well as protective activity against CTX induced toxicity.

In this chapter we study the chemo protective activity of naturally occurring terpenoids carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid, and nomilin

2. MATERIALS AND METHODS

2.1 Animals:

Swiss albino mice (6-8 weeks old) weighing 20-25g were used for this study

2.2 Chemicals:

Cyclophosphamide P-rosaniline hydrochloride, α-naphthyl acetate, thiobarbituric acid, Harris Haematoxylin, Glutathione (GSH) and 5-5- dithisobis-2-nitrobenzoic acid (DTNB) (Ellman`s reagent) and total protein analyzing kits were used for this study. All other chemicals used were of analytical reagent grade.

2.3 Terpenoid compounds

Carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin Drug preparation and administration: Terpenes were suspended in light paraffin oil. Five doses of the compounds (carvone and limonene 100μmoles/Kg body wt /dose/animal i.p, perillic acid ursolic acid oleanolic acid and glycyrrhizic acid 50 μmoles/Kg body wt /dose/animal i.p and nomilin 10 μmoles/Kg body wt /dose/animal i.p) were administrated intraperitoneally for all the parameters.

2.4 CTX administration and experimental design

Two sets of Swiss albino mice were taken and each set was divided into nine groups (10mice /group). All the animals were treated with 10 doses of cyclophosphamide (25mg/kg b.wt; ip) on consecutive days.Group I animals were kept as control with out any drug treatment and Group II animals were treated with 10 doses of paraffine oil (vehicle control). Group III, IV, V, VI, VII, VIII and IX animals were treated with 10 consecutive doses of carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin respectively. The first sets of animals were used to examine hematological parameters and body weight and the second set of animals were used for analyzing intestinal toxicity and bonemarrow cellularity.

2.5 Determination of the effect of terpenoids on the haematological parameters in mice after cyclophosphamide treatment

The set I animals were treated with the terpenoid compounds as explained above. Blood was collected from the caudal vein and parameters such as total WBC count (haemocytometer), differential count (Leishman`s stain) Haemoglobin level (cyanmethaemoglobin method) and the weight of the animals were recorded prior to the CTX treatment and continued every third day for 30 days. (Chapter 2)

2.6 Determination of the effect of terpenoids on the bone marrow cellularity and α-esterase activity

The set II animals were treated with the terpenoid compounds as explained above Three mice from each group were sacrificed on 11^{th} and 15^{th} day of CTX treatment and the bone marrow was collected from the femur, and the cell number was determined using a haemocytometer and expressed as total live cells/femur.

A smear using the above bone marrow preparation was made on a clean glass slide and stained with Harris haematoxylin to determine the non specific α-esterase activity according to the method of Bancroft and Cook (Bancroft, 1984), as described in chapter 2

2.7 Determination of the effect of terpenoids on the enzyme levels in serum and liver

The liver and serum of the animals from the previous experiment was used to determine Glutamate pyruvate transaminase (GPT) level by the method of Bergmayer and Bernt (Bergmayer and Bernt, 1974) Alkaline phosphatase activity was determined according to the method of Kind and King (Kind and King, 1954) and lipid peroxidation was determined by the method of Ohkawa et al (Ohkawa et al., 1979) (as explained in Chapter 2).

2.8 Determination of the effect of terpenoids on the GSH levels in intestinal mucosa and liver

A portion of liver and intestinal mucosa of the animals from the previous experiment were used for the estimation of GSH content .The mucosa of small intestine was removed by scarping with a blunt knife and 10% homogenate was prepared in Tris HCl buffer(0.1M, PH 7.4). Liver quickly excised washed with ice cold saline and 10% homogenate prepared in ice cold Tris HCl buffer (0.1M, PH 7.4). GSH was measured by method of Moron et al. (Chapter 2).In this method a portion of tissue homogenate (0.5ml) was precipitated by adding 125µl of 25%TCA (trichloroacetic acid) and the tubes were cooled on ice for 5 minutes. The mixture was further diluted with 0.6ml of 5%TCA, centrifuged at 1000rpm for 10 minutes and supernatant (0.1ml) was made up to 1ml with 0.2M phosphate buffer (pH 8.0). Freshly prepared DTNB solution (2ml) in 0.2M sodium

phosphate buffer was added to the tubes and a yellow colour formed after 10min was measured in a spectrophotometer at 412nm

2.9 Histopathological analysis

Histopathological analysis was carried out by fixing liver and intestine in 10% formaldehyde. After several treatments in different concentrations of alcohol, the dehydrated tissue was embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin and histopathological analysis was carried out.

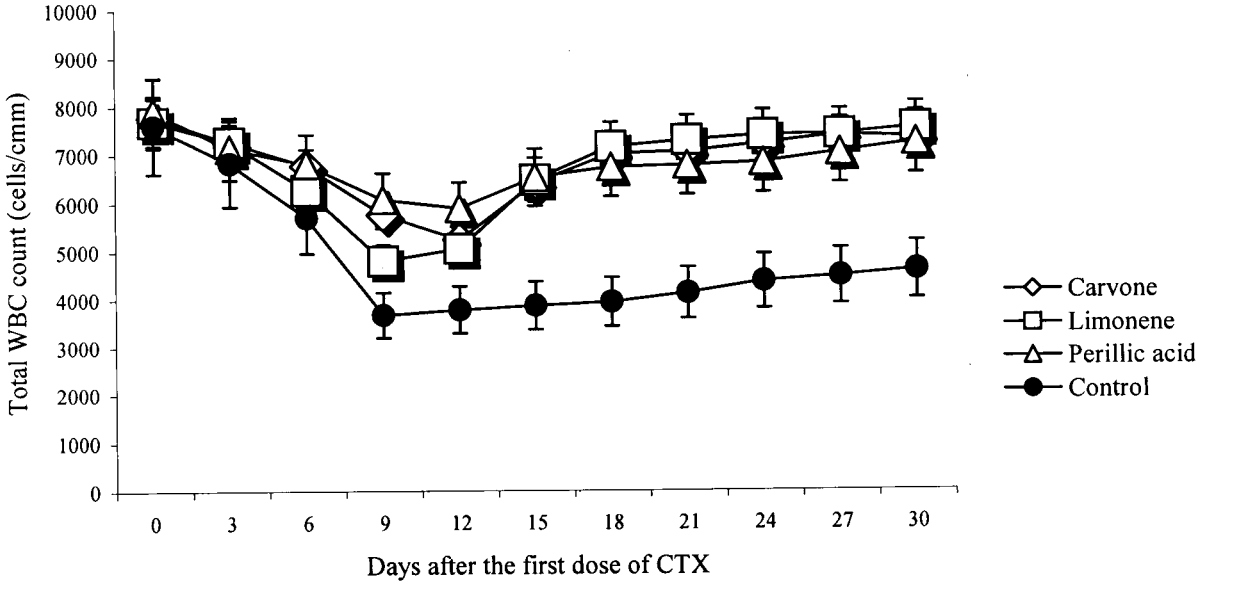
3 RESULTS

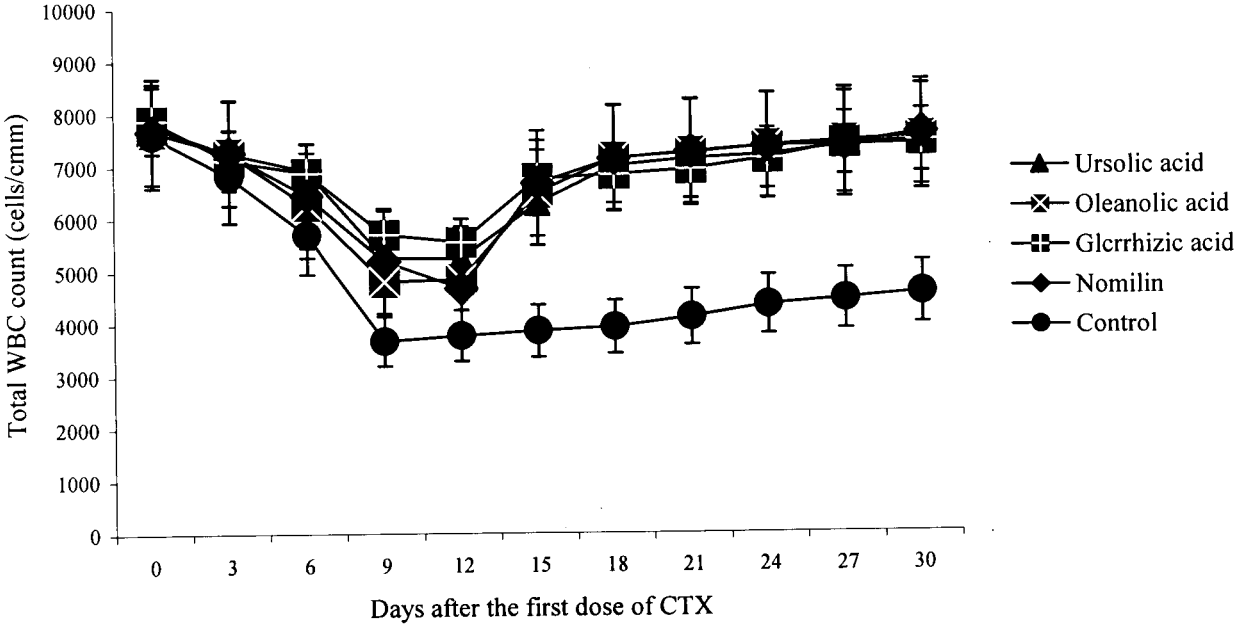
3.1 Effect of terpenoids on hematological parameters of CTX treated animals.

Effect of terpenoids on total WBC count of CTX treated animals is shown in figure 6.1. Administration of CTX reduced the total WBC count to 3668 cell/cmm on 9^{th} day compared to the normal value (7593 cells/cmm before treatment) The reduced total WBC counts were significantly increased by the treatment with perillic acid (6070 cells/cmm) carvone (5760 cells/cmm) and limonene (4800 cells/cmm) on the 9^{th} day. Administration of ursolic acid (5260 cells/cmm), glycyrrhizic acid (5710 cells/cmm) nomilin (5200cells/cmm) and oleanolic acid (4809 cells/cmm) treated groups also showed a remarkable increase in total WBC count (fig 1b). The total WBC count of CTX alone treated animals was only 4593 cells/cmm on 30^{th} day after the administration of CTX.. But in terpenoid treated group the total WBC counts significantly enhanced and regain the normal values.

No change in lymphocyte: neutrophil ratio was observed in terpenoid treated animals compared with CTX alone treated control.

Fig 6.1 Effect of terpenoids on total WBC count of CTX treated animals



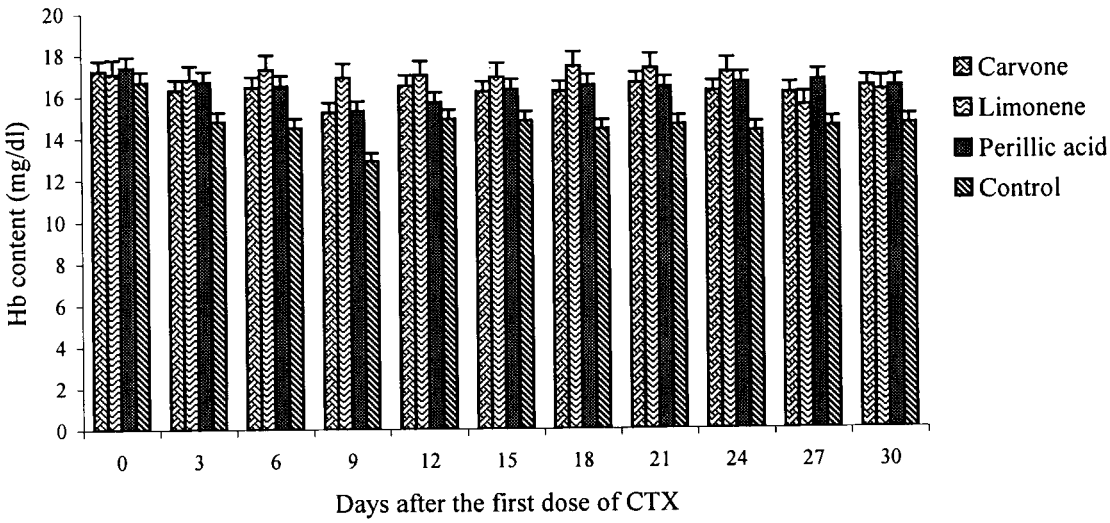


Hb content of CTX alone treated animals also reduced to 12.9 mg/dl on 9th day of CTX administration compared to the normal value (16.7 mg/dl before CTX administration) But administration of terpenoid compounds enhanced the Hb content levels (Fig 6.2).on the 9th day the maximum enhancement was observed in glycyrrhizic acid and limonene treated groups (16.9 mg/dl) and followed by oleanolic acid (16 mg/dl), nomilin (15.4 mg/dl), perillic acid (15.3 mg/dl) and carvone (15.24 mg/dl).On 30th day of CTX administration Hb content of CTX alone treated animals were only 14.6 mg/dl. But Hb content of the terpenoid treated animals regain the normal values on 24 day.

3.2 Effect of terpenoids on bone marrow cellularity and α esterase positive cells in CTX treated animals

Effect of terpenoids on CTX induced reduction in bone marrow cellularity and α esterase positive cells is shown in table 6.1. The bone marrow cellularity in CTX alone treated group was only 5.5x10^6 cells/femur on 11th day after the CTX administration compared to normal animals (18.4 x10^{6}cells/femur). Administrations of various terpenoids inhibit CTX induced reduction in bone marrow cellularity. The maximum enhancement in bone marrow cellularity on 11th day after the CTX administration was observed in perillic acid (10.4 x10^{6}cells/femur) treated animals and followed by glycyrrhizic acid (9.9x10^{6}cells/femur) and limonene (8.8 x10^{6}cells/femur) treated animals. After 15th day of CTX administration CTX alone treated group have only 8.9x10^6 bonemarrow cells/femur. Where as in carvone (16.9 x10^6 cells/femur), perillic acid (15.8x10^6 cells/femur)and glycyrrhizic acid (15.4 x10^6 cells/femur) treated group the bone marrow cellularity enhanced to the normal value (18.4x10^6 cells/femur). In limonene and ursolic acid treated group the bone marrow cell number have shown to be 11.2x10^6

Fig 6.2 Effect of terpenoids on hemoglobin content



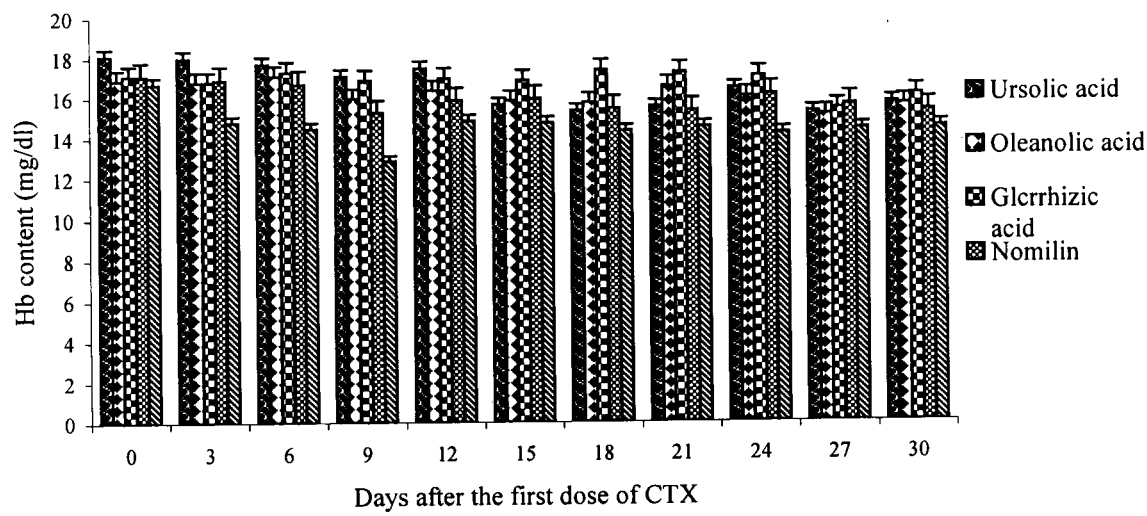


Table 6.1 Effect of terpenoids on bone marrow cellularity and α-esterase activity in CTX treated animals

	Bone marrow cellarity(10^6 cells/femur)		esterase positive cells (cells /4000cells)	
	11^{th}	15^{th}	11^{t}	15^{th}
Normal	18.4±0.92		938±46.9	
Control	5.5±0.275	8.9±0.445	208±10.4	399±15.96
Carvone	8.9±0.42*	16.9±0.5*	389±18.4*	825±15.4*
Limonene	8.8±0.25*	11.2±0.2*	456±20.4*	890±18.9*
Perillic acid	10.4±0.7*	15.5±0.25*	508±10.8*	910±32.1*
Glycyrrhizic acid	9.9±0.53*	15.4±0.27*	406±17.9*	715±24.5*
Ursolic acid	7.4±0.5*	10.9±0.52*	208±42.4	621±27.6*
Oleanolic acid	7.1±0.2*	9.9±0.33*	199±18.7	718±18.4*
Nomilin	6.9±0.17*	9.8±0.31*	205±11.4	783±19.5*

Eight groups of Swiss albino mice (8 mice / group; 20-25g) were used for this study. All the animals were treated with 10 doses of cyclophosphamide (25mg/kg b.wt; ip) on consecutive days. Terpenoids were administrated intraperitoneally to various animal groups.Three mice from each group were sacrificed on 11^{th} and 15^{th} day of CTX treatment and the bone marrow was collected from the femur, and the cell number was determined using a haemocytometer and expressed as total live cells/femur. A smear using the above bone marrow preparation was made on a clean glass slide and stained with Harris haematoxylin to determined the non specific α-esterase activity according to the method of Bancroft and Cook

(*$P<0.01$ Compared with CTX alone treated control)

BM cells/femur and 10.9 $x10^6$ cells/femur respectively on 15^{th} day after CTX administration

Administration of 10 doses of CTX remarkably reduced the α-esterase positive cell number in bone marrow (208 cells/4000 BM cells, on 11^{th} day of CTX administration). The number of α-esterase positive cells clearly increased in CTX treated group by the administration of terpenoids. On 11^{th} day after the CTX administration a clear enhancement in α-esterase positive cells were found to obtain in carvone (389 cells/4000 BM cells), limonene (456 cells/4000 BM cells), perillic acid (508 cells/4000 BM cells) and glycyrrhizic acid (406 cells/4000 BM cells) treated animals. On 15^{th} day after the CTX administration in CTX alone treated control animals the α-esterase positive cell number was only 399 cells/4000 BM cells. The maximum enhancement in α-esterase positive cell number obtained in perillic acid treated group (910 cells/4000 BM cells) followed by limonene (890cells/4000 BM cells), carvone (825 cells/4000 BM cells) nomilin (783 cells/4000 BM cells), oleanolic acid (718 cells/4000 BM cells), glycyrrhizic acid (715 cells/4000 BM cells) and ursolic acid (621 cells/4000 BM cells)

3.3 Effect of terpenoids on enzyme levels in serum and liver of CTX treated animals.

The effect of terpenoids on the GPT levels in serum and liver of CTX treated animals is shown in table 6.2. CTX administration elevated the GPT levels on 11^{th} day in serum (30.48U/ml) and liver (73.38U/mg), which was clearly inhibited by the treatment with various terpenoids. The maximum inhibition in liver GPT levels was observed in perillic acid (63.5 U/mg) and ursolic acid (63.67 U/mg) followed by glycyrrhizic acid (64.05 U/mg) treated groups. On 15^{th} day of CTX administration terpenoids treated groups have a clear inhibition of GPT production in lever compared to CTX alone treated control

Table 6.2. Effect of terpenoids on GPT levels in serum and liver of CTX treated animals

	SGPT(U/ml)		Liver GPT(U/ml)	
	11^{th}	15^{th}	11^{th}	15^{th}
Normal	13.50±0.70		48.90±0.40	
Control	30.48±0.28	31.83±0.21	73.38±2.47	78.81±0.57
Carvone	29.87±3.36	29.95±1.02*	72.36±2.53	63.72±0.69*
Limonene	28.04±1.42	28.46±1.30*	67.44±0.78*	66.61±1.27*
Perillic acid	26.54±0.55*	28.96±0.57*	63.59±0.29*	62.52±0.33*
Glycyrrhizic acid	31.60±1.74	29.61±0.21*	64.05±0.40*	64.48±0.91*
Ursolic acid	26.15±1.43*	25.11±1.33*	63.67±4.50*	69.90±8.81*
Oleanolic acid	23.20±3.27*	21.80±1.97*	66.63±1.76*	63.23±5.66*
Nomilin	18.61±0.97*	19.21±0.90*	68.48±6.87**	65.07±5.16*

Eight groups of Swiss albino mice (8 mice / group; 20-25g) were used for this study. All the animals were treated with 10 doses of cyclophosphamide (25mg/kg b.wt; ip) on consecutive days. Terpenoids were administrated intraperitoneally to various animal groups. Three mice from each group were sacrificed on 11^{th} and 15^{th} day of CTX treatment and liver and blood of the animals collected. The liver and serum of the animals were used to determined Glutamate pyruvate transaminase (GPT) level by the method of Bergmayer and Bernt (Chapter 2)

(*P<0.01, **P<0.05 Compared with CTX alone treated control)

animals (liver 78.8U/mg). SGPT levels also inhibited by the terpenoid administration. The maximum inhibition on 11th day SGPT levels is shown to obtained in nomilin treated group. On the 15th day SGPT levels of nomilin and perillic acid treated animals were enhanced to 19.21 U/ml and 28.96 U/ml respectively. Where as in ursolic acid, glycyrrhizic acid and oleanolic acid the SGPT levels decreased to 25.11 U/ml, 29.61 U/ml and 21.8 U/ml respectively.

CTX administration increased the production of lipid peroxides in serum (3.24 n mol/ml) and liver (3.66 n mol/ml) on 11th day (Table 6.3). Administration of terpenoids inhibited the production of peroxides. After 15th day of CTX administration the liver lipid peroxide production was enhanced to 3.65nmol/mg protein (in CTX alone treated control animals) were as in glycyrrhizic acid (1.51 n mol/ml), limonene (1.85 n mol/ml) and perillic acid (1.1 n mol/ml) treated group the liver lipid peroxide levels tend to attain the normal level (1.1 n mol/mg respectively). Animals treated with carvone (2.4 n mol/mg), ursolic acid (2.62 n mol/mg), oleanolic acid 2.35 (n mol/mg) and nomilin 2.39 (n mol/mg) were also showed clear reduction in TBRS formation in liver. In limonene treated group liver lipid peroxide level was 1.8 n mol/mg on 15th day of CTX administration. Administrations of various terpenoids also reduced the enhanced levels of serum lipid peroxidation. Ursolic acid (1.46 n mol/ml), oleanolic acid (1.67 n mol/ml) and nomilin (1.59 n mol/ml) administrations showed remarkable reduction in the formation of serum TBRS formation on 11th day. Carvone (2.91 n mol/ml), limonene (2.93 n mol/ml) and glycyrrhizic acid (2.71 n mol/ml) were also showed inhibition in the TBARS formation in serum. Ursolic acid (1.46 n mol/mg), oleanolic acid (1.67 n mol/ml) and nomilin (1.59 n mol/ml) administrations showed remarkable inhibition in serum TBRS formation on 11th

Table 6.3 Effect of terpenoids on lipid peroxidation in serum and liver of CTX treated animals

	Serum TBRS(n mol/ml)		Liver TBARS(n mol/mg)	
	11^{th}	15^{th}	11^{th}	15^{th}
Normal	1.3±0.09		1.1±0.03	
Control	3.24±0.50	3.53±0.13	3.66±0.18	3.65±0.26
Carvone	2.91±0.29	2.70±0.28*	2.72±0.37*	2.40±0.20*
Limonene	2.93±0.13	2.69±0.09*	2.63±0.07*	1.85±0.44*
Perillic acid	3.24±0.12	2.89±0.17*	2.10±0.45*	1.10±0.12*
Glycyrrhizic acid	2.71±0.25*	2.37±0.09*	1.90±0.07*	1.51±0.03*
Ursolic acid	1.46±0.05*	1.31±0.11*	2.57±0.69*	2.62±0.43*
Oleanolic acid	1.67±0.37*	1.47±0.22*	2.33±0.39*	2.35±0.58*
Nomilin	1.59±0.26*	1.37±0.07*	1.20±0.14*	2.39±0.11*

Eight groups of Swiss albino mice (8 mice / group; 20-25g) were used for this study. All the animals were treated with 10 doses of cyclophosphamide (25mg/kg b.wt; ip) on consecutive days.Terpenoids were administrated intraperitoneally to various animal groups. Three mice from each group were sacrificed on 11^{th} and 15^{th} day of CTX treatment and liver and blood of the animals collected. The liver and serum of the animals were used to determined lipid peroxidation by the method of Ohkawa et al (Ohkawa, 1979)

(*$P<0.01$ Compared with CTX alone treated control.)

day. On 15th day of CTX administration serum TBARS formation in CTX alone treated animals was enhanced to 3.53 n mol/ml where as in Ursolic acid (1.31 n mol/ml), oleanolic acid (1.47 n mol/ml) and nomilin (1.37 n mol/ml) groups the value was in the normal range (1.3 n mol/ml)

Alkaline phosphate levels in serum and liver also enhanced by the CTX administration (Table 6.4). Administration of the terpenoids reduced the enhanced serum and liver ALP levels. On 11th day of CTX administration almost all the animals showed the enhanced serum ALP levels. But on 15th day glycyrrhizic acid treated groups showed maximum inhibition in serum ALP levels (12.10 KA units) followed by oleanolic acid (12.95 KA units) and perillic acid (13.6 KA units) compared to CTX alone treated control animals (21.4 KA units). Administration of carvone (15.4 KA units), limonene (14.5 KA units), ursolic acid (19.2 KA units) and nomilin (15.59 KA units) were also reduced the serum ALP levels. But in the production of liver ALP levels, a remarkable inhibition in terpenoid treated animals can be seen on 11th day. Maximum inhibition can be seen in the case of nomilin (6.1 KA units), ursolic acid (6.2 KA units) and oleanolic acid (6.7 KA units) treated animals. Administration of limonene (8.2 KA units), perillic acid (11.6 KA units) and glycyrrhizic acid (12.6 KA units) were also inhibited the production of ALP on 11th day. On th 15th day of CTX administration the maximum inhibition was observed in nomilin treated group, (6.92 KA units) followed by limonene (7.35 KA units) and oleanolic acid (7.65 KA units) compared to the control (11.7 KA units).

3.4 Effect of terpenoids on GSH levels.

Figures 6.3 and 6.4 shows the change of GSH levels in liver and intestine after CTX administration. Administration of terpenoids enhanced the GSH levels in liver as well

Table 6.4 Effect of terpenoids on ALP levels in serum and liver of CTX treated animals

	Serum ALP (KA units)		Liver ALP (KA units)	
	11^{th}	15th	11^{th}	15th
Normal	6.1±0.2			
Control	23.29±1.14	21.44±0.54	15.39±0.44	11.76±1.45
Carvone	20.2±0.10*	15.4±0.01*	13.53±2.2**	7.35±0.09*
Limonene	19.9±0.12*	14.5±0.11*	8.2±1.24*	7.35±0.09*
Perillic acid	18.9±0.43*	13.60±0.08*	11.06±1.1*	9.78±0.38**
Glycyrrhizic acid	18.8±0.12*	12.1±0.15*	12.62±1.05*	10.67±2.37
Ursolic acid	19.4±1.07*	19.21±0.08	6.21±0.51*	9.60±1.78*
Oleanolic acid	20.88±0.21*	12.95±3.50*	6.70±1.15*	7.65±0.74*
Nomilin	20.64±0.73*	15.59±4.53*	6.1±0.76*	6.92±0.65*

Eight groups of Swiss albino mice (8 mice / group; 20-25g) were used for this study. All the animals were treated with 10 doses of cyclophosphamide (25mg/kg b.wt; ip) on consecutive days. Terpenoids were administrated intraperitoneally to various animal groups. Three mice from each group were sacrificed on 11^{th} and 15^{th} day of CTX treatment and liver and blood of the animals collected. Alkaline phosphatase activity was determined according to the method of Kind and King

(*P<0.01, **P<0.05 Compared wiyh CTX alone treated controls)

as intestine of CTX treated animals. In control animals GSH levels in intestine and liver were 5:3 and 4.6 nmol/mg protein respectively after 11th day of CTX administration. But in glycyrrhizic acid treated group the GSH levels were enhanced to 11.1 and 7.74 nmol/mg in intestine and liver respectively after the 11th day of CTX administration. Limonene treated group also showed similar enhancement in intestine and liver GSH levels (11.34 and 7.3 nmol/mg in intestine and liver respectively) after the 11th day of CTX administration. Administration of carvone (6.3 nmol/mg protein), oleanolic acid (6.2 nmol/mg protein) and perillic acid (6.32 nmol/mg protein) treated group also showed enhancement in GSH levels in liver as well as intestinal mucosa (9.2, 6.9 and 8.2 nmol/mg protein respectively for carvone, oleanolic acid and perillic acid) after the 11th day of CTX administration.

On 15th day of CTX administration the GSH levels was enhanced in all the terpenoid treated groups. The maximum liver GSH levels were obtained in the case of nomilin (7.8 nmol/mg protein), ursolic acid (8 nmol/mg protein), limonene (7.6 nmol/mg protein), oleanolic acid (7.5 nmol/mg protein) and perillic acid (7.2 nmol/mg protein) treated animals. Carvone and glycyrrhizic acid treated animals were also showed the enhanced liver GSH levels compared to the CTX alone treated control animals. GSH levels in intestine are also enhanced by the terpenoid administration. The maximum production of intestinal mucosa were obtained in the case of glycyrrhizic acid (12 nmol/mg protein) and nomilin (12.1 nmol/mg protein) treated animals followed by oleanolic acid (10.9 nmol/mg protein), ursolic acid (10.75 nmol/mg protein) and limonene (11.8 nmol/mg protein) treated animals. Carvone (9.6 nmol/mg protein) and perillic acid (9.3 nmol/mg protein) treated animals also showed enhaced intestinal GSH levels compared to CTX alone treated control animals (8.8 nmol/mg protein)

Fig 6.3 Effect of terpenoids on GSH content of liver of CTX treated animals

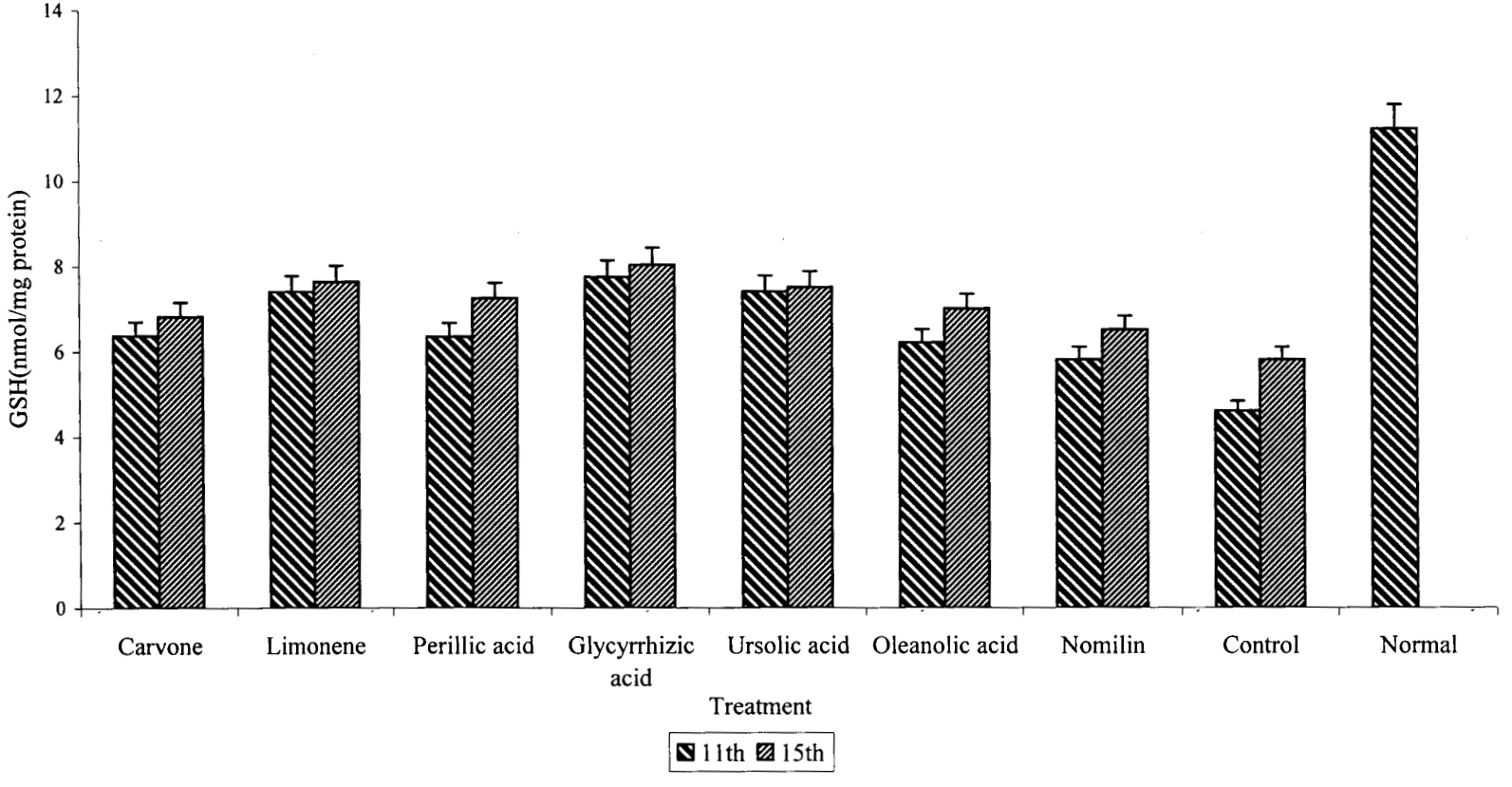
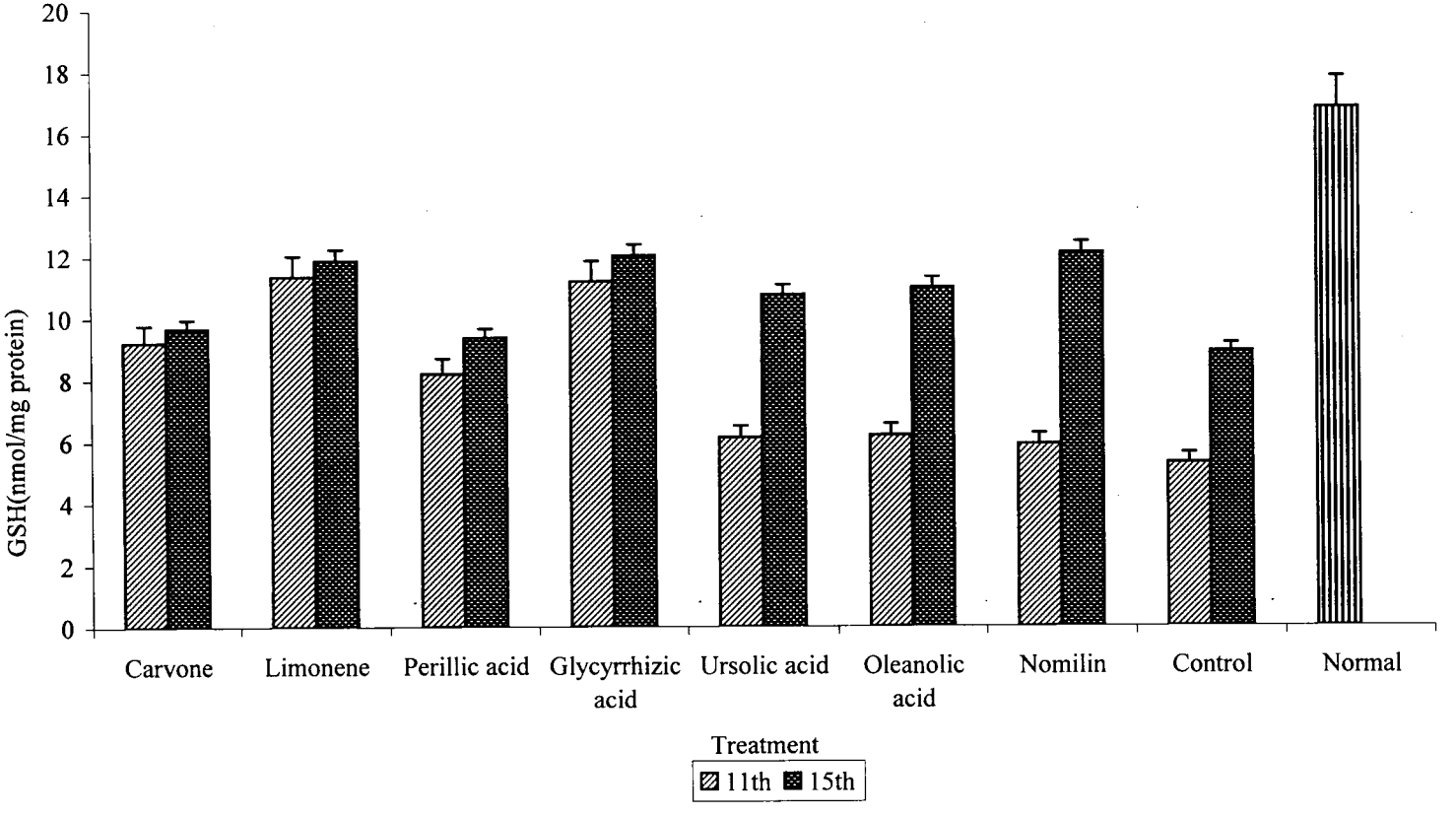


Fig 6.4 Effect of terpenoids on GSH content of intestinal mucosa of CTX treated animals



3.5 Histopathological studies

Histopathological studies on liver and intestine of CTX treated animals showed severe damages to the tissues because of its susceptibility to CTX. A severe damage was observed in CTX alone treated groups. But treatment of terpenoid compounds could prevent the intestinal tissue damage and maintain the intestinal tissue architecture. The number of globlet and dead cells were found to be increased in CTX alone treated liver samples. But in test terpenoids treated group, number of globlet cell and dead cell were found to be much less. This study clearly proved that all these terpenoids protect animals from CTX induced tissue damage.

4 DISCUSSION:

Interest in plants and plant originated compounds for the treatment and prevention of cancer has gained momentum in recent years. This is because of plant and plant products are effective when given orally; have low preparation cost; are nontoxic and can be administered for long periods of time. In this study we evaluate the protective activity of naturally occurring terpenoids on CTX induced toxicity.

Leukopenia is one of the major side effects of CTX treatment. In this study the administration of terpenoids increase the total WBC count in CTX treated animals. In terpenoid treated groups increased WBC count was obtained from 9^{th} day onwards.

Myelosuppression is another side effect of chemotherapy (Belli et al., 1967, Bast et al., 1982, Glick et al., 1982). Administration of CTX decreases bone marrow cellularity and α esterase positive cell number. Administration of these terpenoids significantly enhanced the decreased bone marrow cellularity and α esterase positive cell number. Bone

Figure 6.5 Histopathological studies on intestine of CTX treated animals (11^{th} day)

Figure 6.5a CTX alone treated control animals

Figure 6.5b Carvone treated

Figure 6.5c Limonene treated

Figure 6.5d Perillic acid treated

Figure 6.5e Ursolic acid treated

Figure 6.5f Oleanolic acid treated

Figure 6.5g Glycyrrhizic acid treated

Figure 6.5h Nomilin treated

Figure 6.5i Normal animal

Fig 6.5 Histopathological studies on intestine of CTX treated animals

Fig 6.5a

Fig 6.5b

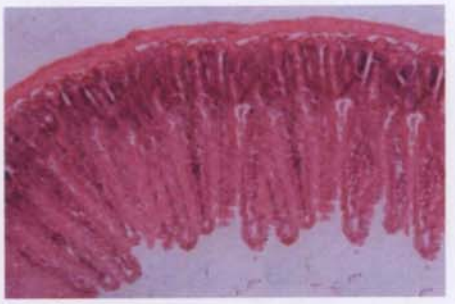


Fig 6.5c

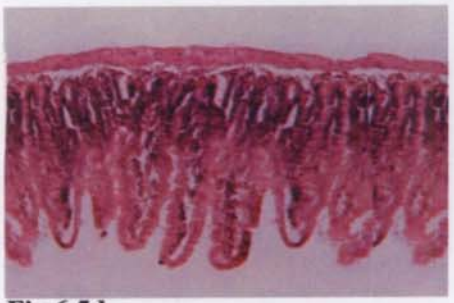


Fig 6.5d

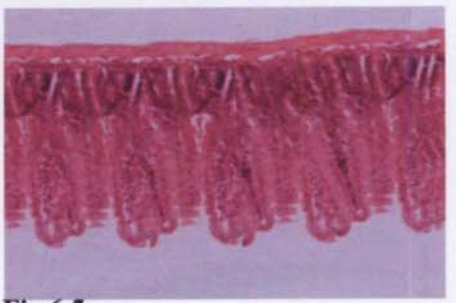


Fig 6.5e

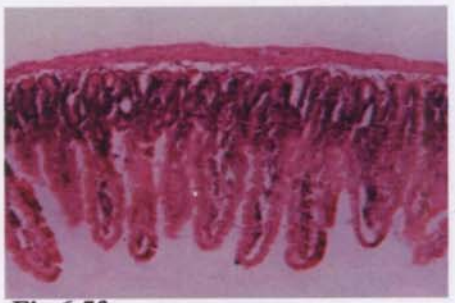


Fig 6.5f

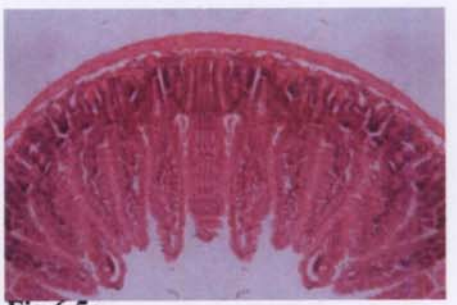


Fig 6.5g

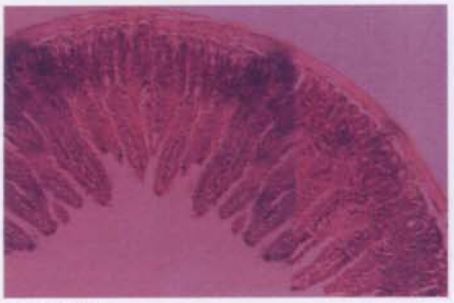


Fig 6.5h

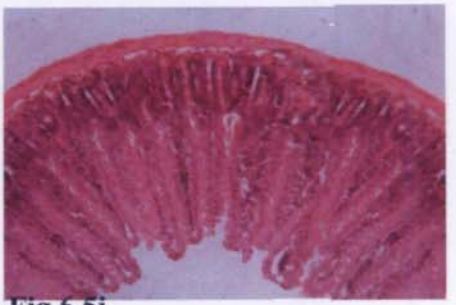


Fig 6.5i

marrow serves as major source of hemopoetic stem cells. Enhanced number of bone marrow cells and α esterase positive cells clearly indicate the effect of these compounds on stem cell proliferation in irradiated mice and its protective activity against CTX induced hemopoietic syndrome.

CTX administration causes damage to living tissue through a series of molecular events. Increased production of free radicals has been shown to be deleterious to the body associated with cellular damage (Kong et al 1998; Ames et al., 1993). Free radicals increases membrane lipid peroxidation which in turn can alter the integrity of membrane structure leading to inactivation of membrane bound enzymes loss of permeability of membrane and decrease in membrane fluidity (Ray and Hussan, 2002). CTX administration therefore increases the lipid peroxidation and enzymes levels. . The serum and liver content of ALP and GPT was highly elevated in CTX alone treated control animals indicating the pathological condition. Administration of terpenoids decreased the enhanced level of lipid peroxidation and enzymes such as ALP and GPT in serum as well as liver.

The multiple physiological and metabolic functions of include thiol transfer reaction that protect cell membrane and protein. Administration of terpenoids increased the GSH content in intestine as well as in liver in CTX treated mice. Higher GSH content in liver of terpenoids-treated mice could protect tissue from CTX induced damage. Major function of GSH is to serve as the reductant of toxic peroxides (Peristeris et al., 1992; Pena et al., 1999; Weijl et al., 1998). GSH depletion altered levels of intracellular calcium, lowered threshold to oxidative stress, caused enhancement of DNA cross-linking and may

cause alteration in DNA repair (Colman et al., 1988). GSH is known to protect against CTX- induced bladder damage. (Arrick and Nathan, 1984; Carmichael and Adams, 1986).

The present study clearly indicated protective role of naturally occurring terpenoids carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin against chemotherapeutic drug toxicity.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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CHAPTER 7

ANTIMETASTATIC ACTIVITY OF NATURALLY OCCURRING TERPENOIDS *IN VIVO* AND *IN VITRO* STUDIES

1 INTRODUCTION

Metastasis of cancer cells is a threat to the available cancer therapy and is the major reason for treatment failure (Weiss, 1985., Mareel et al., 1993).This most malignant aspect of cancer has been shown to depend on a number of interrelated steps known as the metastatic cascade. (Fidler et al., 1974). This complex sequential process requires the cells to enter circulation, arrest at the distant vascular bed and proliferate as secondary tumour colony. Several evidences exists for each of these sequential steps of metastasis and disruption of any of the events in the cascade restrain the establishment of disseminated cancer (Sugar and Ketchem, 1977)

One approach to treating cancer metastasis is by directing the body's natural immune defense mechanism against the tumour cells. Tumour cells encounter with immune systems in the circulation. Any agent or immune cell itself can interfere with any of these steps and can significantly reduce the metastatic potential. Involvement of natural killer cells in this regard are well known (Hill et al., 1994). So boosting up of immune system is also significant in reducing metastasis.

There are several plant originated compounds such as catechin and curcumin (Menon et al., 1995) which are found to produce artificial cross links with collagen (Menon et al., 1998) and thereby inhibit the action of proteolytic enzyme and thus the process of metastasis. Naturally occurring sulphur compounds namely allyl sulphide, diallyl sulphide and allyl methyl sulphide have shown to inhibit the lung metastasis by B16F-10 melanoma cells in C57BL/6 mice (Kuttan and Kuttan ,1999). *Viscum album* shows significant inhibition in lung metastasis (Antony et al., 1997).

In this chapter the effect of naturally occurring terpenoid compounds carvone, limonene, perillic acid, ursolic acid, oleanolic acid, nomilin and glycyrrhizic acid in the inhibition of lung metastasis induced by B16F-10 melanoma cells in C57 BL/6 mice was analyzed.

2 MATERIALS AND METHODS

2.1 Animals

C57BL/6 mice (20-25g body wt, 6-8 weeks old males)

2.2 Cells

B16F-10 melanoma, a highly metastatic cell line

2.3 Terpenoid compounds

Carvone, limonene, perillic acid, ursolic acid, oleanolic acid glycyrrhizic acid, and nomilin

Drug administration: - The compounds were suspended in light paraffin oil. Carvone and limonene, administered at a concentration of 100 μmoles/kg body wt/dose/animal, perillic acid, ursolic acid, oleanolic acid and glycyrrhizic were administered at a concentration of 50 μmoles/kg body wt/dose/animal and nomilin 10 μmoles/kg body wt/dose/animal. The compounds were intraperitonially administered from the same day of tumour inoculation and were continued for 10 consecutive days.

2.4 Determination of the effect of terpenoids on metastatic lung tumour nodule formation

C57BL/6 mice (20-25g body wt, 6-8 weeks old males) were grouped into nine (8 nos/group). All the animals were injected with BI6F-10 melanoma cells (10^6 cells/animal) through lateral tail vein. Groups 1, II, Ill,IV,V,VI,and VII were treated with 10 doses of

carvone, limonene, perillic acid ursolic acid, oleanolic acid glycyrrhizic acid and nomilin respectively as explained above. Group VIII was vehicle control and treated with 10 doses of paraffin oil and group IX was kept as untreated tumour bearing control. The animals were sacrificed on 21st day after the tumour implantation, lungs were excised and serum was collected. The lungs were used for morphological examinations of metastatic tumour nodules appearing on the surface and for the estimation of collagen hydroxyproline (Bergman and Loxley, 1940) hexosamine (Elson and Morgan, 1933) and uronic acid (Schiller et al., 1961). Serum was used for determining sialic acid (Skoza and Mohos, 1976) and gamma glutamyl transpeptidase (γGT) (Tate and Meister, 1974) levels (as explained in chapter 2)

2.5 Determination of the effect of terpenoids on the survival rate of metastatic tumour bearing animals.

A similar set of experiment was conducted and the death of animals due to tumour burden was recorded. The mortality of the animals was observed and the percentage increase in life span was calculated. (Chapter 2)

2.6 Histopathological analysis

Lungs were fixed in 10% formalin, dehydrated and embedded in paraffin wax, sections (4μm) were stained with hematoxylin and eosin.

2.7 *IN VITRO* ANTIMETASTATIC STUDIES

2.7a Tumour cell adhesion assay

Tumour cell adhesion assay was carried out by the method of Inokuchi et al (Inokuchi et al., 1990) as described in Chapter 2. Briefly, B16F-10 melanoma cells were seeded on collagen type I coated wells of flat-bottomed titre plates, in the presence and

absence of terpenoids compounds. (5μM to 50μM) and incubated at 37^0c for 5 hrs. After cells were washed, the adhering cells were fixed and stained. Cells were then counted under a microscope. Each experiment was done in triplicate.

2.7b Collagen matrix invasion assay

The invasion assay was carried out in Boyden chambers as described by Albini et al (Albini et al., 1987)(Chapter 2). Briefly, the lower compartment of the chamber was filled with serum free DMEM and a type-I collagen coated poly carbonate filter membrane was placed on it. B16F-10 cells were then seeded on to the upper chamber.

To test the effect of terpenoids on the invasion of B16F-10 melanoma cells, the compounds were added along with the cells to the upper chamber at non-toxic concentration (5 to 50 μM) . All experiments were performed in triplicate and the results are expressed as percentage inhibition of invasion.

2.7c Tumour cell motility assay

Tumour cell motility assay was performed in the same manner as the invasion assay except that polycarbonate filters were collagen free. Terpenoid compounds (5 to 50 μM concentration) were added along with B16F-10 melanoma cells to the upper compartment of the Boyden chamber. After incubation at 37^0C for 24h, the number of cells migrating to the lower chamber was determined using a haemocytometer. The results are expressed as percentage motility.

2.7d Gelatin Zymography

Gelatin Zymography was performed according to the procedure of Billings et al. (Billings et al., 1991) with some modifications as described in Chapter-2. After determining the protein concentration, supernatant containing the proteases were subjected

to zymographic analysis with or without trypsin activation and the effect of various compounds were evaluated. Gels were fixed stained and clear bands were visualized against a dark background

3 RESULTS

3.1 Effect of terpenoids on the metastatic lung tumour nodule formation and survival rate.

The effect of terpenoids on the inhibition of pulmonary tumour nodule is shown in table 7.1. Untreated control animals developed massive number of tumour nodules and is given an arbitrary number of 250 (Laurie et al., 1994). Administration of terpenoids limonene, perillic acid, ursolic acid, glycyrrhizic acid and nomilin could remarkably inhibit the tumour nodule formation (65%, 67%, 55%, 71% and 58% respectively). In the vehicle alone treated group of animals the tumour nodule formation was similar to that of the untreated controls. But carvone and oleanolic acid administration did not have any effect of the lung tumour nodule formation

The effect of terpenoid compounds on the survival rate of tumour bearing mice is shown in table 7.1. The increase in life span was maximum in the case of Glycyrrhizic acid treated animals (70%) followed by perillic acid (52%) limonene (50.7%) ursolic acid (45%) and nomilin (43%) treated animals. Carvone and oleanolic acid administration did not increase lifespan of tumour bearing animals

Table 7.1 Effect of terpenoids on lung tumour colony formation and survival rate

Treatment	Inhibition on lung colony formation	% of increase in the life span
Tumour alone	0%	0%
Vehicle	0%	0%
Carvone	0%	11%
Limonene	65%	50.7%
Perillic acid	67%	52%
Ursolic acid treated	55%	45%
Oleanolic acid	2%	13%
Nomilin treated	58%	43%
Glycyrrhizic acid treated	71%	70%

B16F-10 melanoma cells ($1x10^6$) were injected to each animal via lateral tail vein. Terpenoid compounds were administered intraperitoneally for 10 days. Control animals were treated with vehicle paraffin oil. Animals were sacrificed on 21st day and lung tumour nodules counted. For survival study death due to tumour burden was recorded, and the life span was calculated

Figure 7.1 Effect of terpenoids on lung tumour nodule formation

Figure 7.1a Tumour alone treated control animals

Figure 7.1b Limonene treated

Figure 7.1c Carvone treated

Figure 7.1d Perillic acid treated

Figure 7.1e Ursolic acid treated

Figure 7.1f Glycyrrhizic acid treated

Figure 7.1g Oleanolic acid treated

Figure 7.1h Nomilin treated

Figure 7.1i Normal animal

Fig 7.1 Effect of terpenoids on lung tumour nodule formation

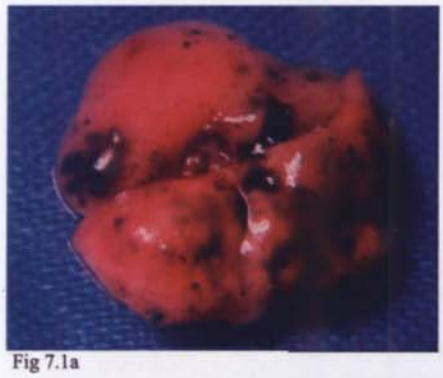

Fig 7.1a

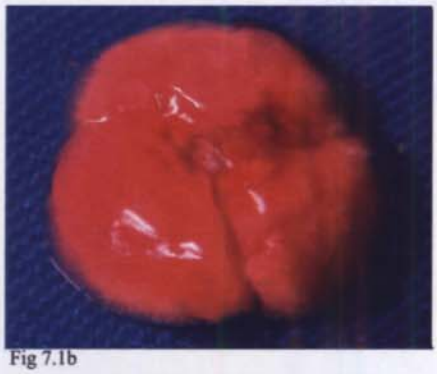

Fig 7.1b

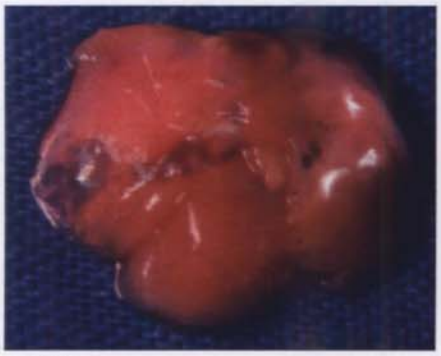

Fig 7.1c

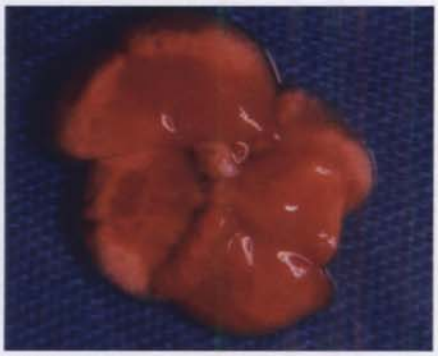

Fig 7.1d

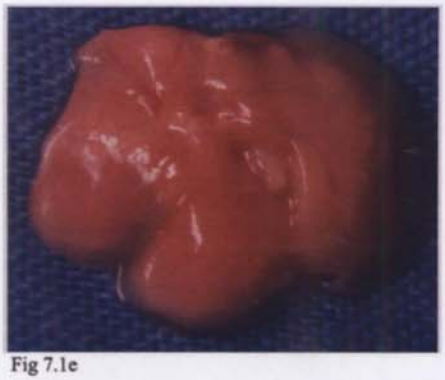

Fig 7.1e

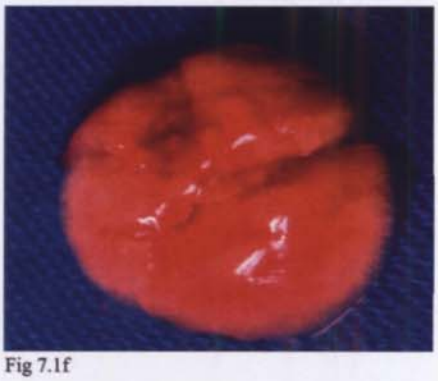

Fig 7.1f

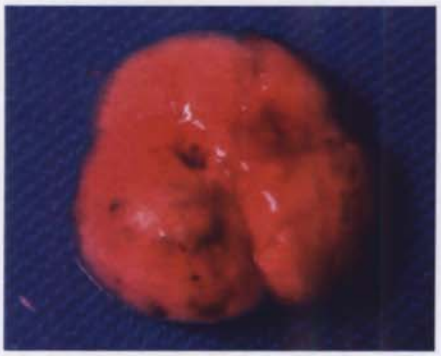

Fig 7.1g

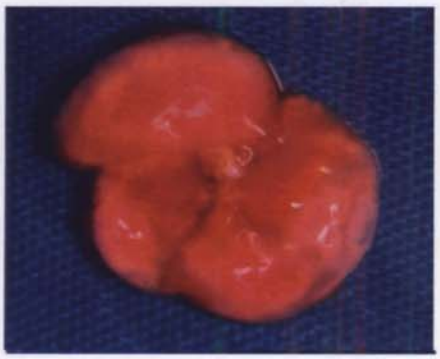

Fig 7.1h

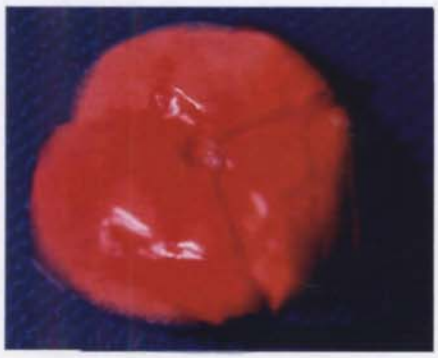

Fig 7.1i

3.2 Effect of terpenoids on the lung collagen hydroxyproline, hexosamine and uronic acid contents.

The effect of terpenoid compounds on lung collagen hydroxyproline, hexosamine and uronic acid contents in metastatic tumour bearing animals is shown in table 7.2. In normal animals the levels of lung collagen hydroxyproline, hexosamine and uronic acid contents were 1.9 μg/mg protein, 0.4 mg/100mg tissue and 25.4 μg/100mg tissue respectively. Control animals showed a drastically increased level of lung collagen hydroxyproline (20.9 μg/mg protein), which was significantly reduced in animals treated with glycyrrhizic acid (6.9 μg/mg protein), perillic acid (6.9 μg/mg protein), limonene (7.5 μg/mg protein), nomilin (9.2 μg/mg protein) and ursolic acid (9 .13 μg/mg protein) .Lung collagen hydroxyproline content in carvone (18.01 μg/mg protein) and oleanolic acid (19.3 μg/mg protein) treated groups were nearly equal to that of the control animals.

Enhanced uronic acid levels in tumour alone treated control animals (326.23 μg/100 mg tissue) was significantly lowered in glycyrrhizic acid (154 μg/l00 mg tissue), limonene (164 μg/l00 mg tissue) and perillic acid (185 μg/l00 mg tissue) treated groups and followed by ursolic acid (186 μg/100 mg tissue) and nomilin (196 μg/100 mg tissue) treated animals

The effect of terpenoids on hexosamine levels in the lungs is given in table 7.2. The hexosamine content was also lowered maximally in glycyrrhizic acid (0.54 μg/100 mg tissue) treated animals compared to the control (2.2 μg/100 mg tissue). In animals treated with limonene, perillic acid, ursolic acid and nomilin the hexosamine levels lowered to 0.88, 1.06, 1.42 and 1.53 μg/100 mg tissue respectively. In animals treated with carvone

Table 7.2 Effect of triterpenoids on lung collagen hydroxyproline, hexosamine and uronic acid levels of metastatic tumour bearing animal

Treatment	Lung collagen hydroxy- proline (µg/mg protein	Hexosamine (mg/100 mg tissue)	Uronic acid (ug/100 mg tissue)
Normal	1.9 ± 0.1	0.4 ± 0.01	25.4± 2.6
Tumour alone	20.9±1.8	2.2±0.25	326.23±56.1
Vehicle	20.58 ± 4.7	2.2±0.26	307.1±50.8
Carvone	18.01 ± 2.16	1.87 ± 0.12	280.1 ± 28.8
Limonene	7.5 ± 1.1*	0.88 ± 0.17*	164.5 ± 13.1*
Perillic acid	6.9 ± 1*	1.06 ± 0.08*	185 ± 13.9*
Ursolic acid	9.13±1.1*	1.42±0.07*	186±14.5*
Oleanolic acid	19.5± 4.4	2.18±0.25	303.1±52.1
Nomilin	9.2±1*	1.53±0.09*	196±24.9*
Glycyrrhizic acid	6.9±1.4*	0.54±0.09*	154±11.4*

B16F-10 melanoma cells ($1x10^6$) were injected into each animal via lateral tail vein. Terpenoid compound were administered intraperitoneally for 10 days. Control animals were treated with vehicle paraffin oil. Animals were sacrificed on 21st day and lung excised. Student t- test was used to compare the mean hydroxyproline, hexosamine and uronic acid levels with corresponding mean tumour alone values.

(* $P < 0.01$Compared with tumour alone treated control)

and oleanolic acid did not lower hexosamine levels (1.87 and 2.1 μg/100 mg tissue respectively).

3.3 Effect of terpenoids on serum sialic acid levels

The effect of terpenoids on the serum sialic acid level of metastatic tumour bearing animals is given in table 7.3. The serum sialic acid levels was high in the case of control animals (126.8 μg/ml serum) which was significantly lowered to 71.2 μg/ml serum, 33.8 μg/ml serum and 64 μg/ml serum by the administration of ursolic acid, glycyrrhizic acid and nomilin respectively. Administration of monoterpenoids also decreased the enhanced level of sialic acid (limonene and perillic acid 49.3 μg/ml serum and 53.6 μg/ml serum respectively).Carvone and oleanolic acid administrations did not show any effect in reducing the elevated levels of serum sialic acid (116.9 and 118 μg/ml serum respectively). In normal animal the serum sialic acid level was only 21.3 μg/ml serum.

3.4 Effect of terpenoids on serum gamma glutamyl transpeptidase (γGT) levels

The effect of various terpenoids on serum γGT level is shown in table 7.3. The drastically elevated levels of γGT in control animals (115.8 nmol p-nitroaniline/ml serum) was significantly reduced to 31.6 nmol p-nitroaniline/ml serum, 31.7 nmol p-nitroaniline/ml serum, and 63.5 nmole p-nitroaniline/ml serum by the administration of ursolic acid, glycyrrhizic acid and nomilin respectively. Administration of the monoterpenoids limonene and perillic acid also reduced the elevated levels of γGT (49.3 nmol p-nitroaniline/ml serum and 31.6 nmol p-nitroaniline/ml serum respectively). γGT level in the serum of normal animals is 24 nmol p-nitroaniline/ml serum.

Table 7.3 Effect of terpenoids on serum sialic acid and serum γGT levels of metastatic tumour bearing animal

Treatment	Serum sialic acid (μg/ml serum)	γGT (10^{-9} mol p-nitraniline/ ml serum)
Normal	21.3±0.5	24±0.17
Tumor alone	126.8±0.3	115.8±7.16
Vehicle	109±4.1	131.66 ±20
Carvone	116.9 ±1.6**	120±6.7**
Limonene	49.3 ±4.7*	49.3±4.7*
Perillic acid	53.6±5.7*	31.62±0.3*
Ursolic acid	71.2±6.7*	38.67±0.11*
Oleanolic acid	118±3.1**	103±14.3**
Nomilin	64±2.1*	63.5±20.2*
Glycyrrhizic acid	33.8±3 *	31.7±0.17*

Experimental design is as given in table 7.1. Animals were sacrificed on 21st day blood was collected by heart puncture and serum separated..

(* P < 0.01, **P<0.05 Compared with tumour alone treated control)

3.5 Effect of naturally occurring triterpenoids on the lung architecture

Lungs in the control animals (Fig.7.2a) showed infiltration of the neoplastic cells around the main bronchioles extended to the pleura. Metastatic tumour bearing animals treated with terpenoid compounds showed a significant reduction in tumour mass. Alveoli and pleura were almost tumour cell free in the case of limonene (Fig.7.2c), perillic acid (Fig.7.2d), glycyrrhizic acid (Fig.7.2e), ursolic acid (Fig.7.2g) and nomilin (Fig.7.2h) treated groups. But carvone (Fig.7.2b) and oleanolic acid (Fig.7.2f) treated group the lung architecture was similar to tumour alone treated control group.

3.6 *IN VITRO* ANTIMETASTATIC STUDIES

3.6a Effect of terpenoid compounds on the adhesion of B16F10 melanoma cells to the collagen matrix

The effect of terpenoids on the adhesion of B16F10 cells to collagen matrix is given in table 7.4. There was a dose dependent inhibition of tumour cell adhesion by these compounds. Maximum inhibition was obtained at a concentration of 50μM for all the compounds. Glycyrrhizic acid could inhibit the tumour cell adhesion by 38.4%.In limonene, perillic acid, ursolic acid and nomilin the percentage inhibition of tumour cell adhesion was 15.9, 18.5,19.2 and 14.7% respectively.

3.6b Effect of terpenoids on the invasion of B16F-10 melanoma cells

Effect of terpenoids on the invasion of B16F-10 melanoma cells is shown in table 7.5. All these compounds could inhibit the invasion of B16F-10 melanoma cells in a dose dependent manner. At a concentration of 50μM glycyrrhizic acid could significantly

Figure 7.2 Effect of naturally occurring terpenoids on the lung architecture of metastatic tumour bearing animals

Figure 7.2a Tumour alone treated control animal

Figure 7.2b Carvone treated

Figure 7.2c Limonene treated

Figure 7.2d Perillic acid treated

Figure 7.2e Glycyrrhizic acid treated

Figure 7.2f Oleanolic acid treated

Figure 7.2g Ursolic acid treated

Figure 7.2h Nomilin treated

Figure 7.2i Normal animal

Fig 7.2 Histopathological analysis of lungs

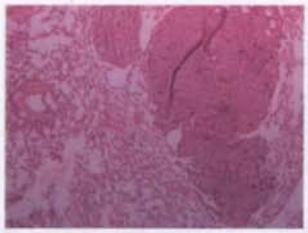

Fig 7.2a

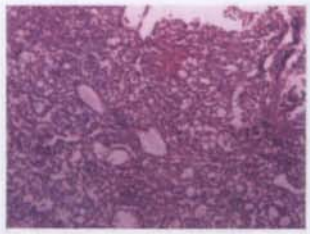

Fig 7.2b

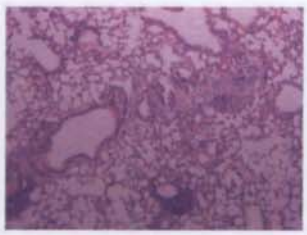

Fig 7.2c

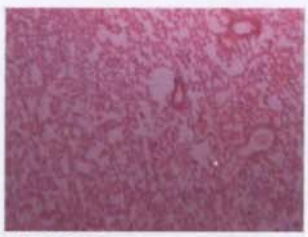

Fig 7.2d

Fig 7.2e

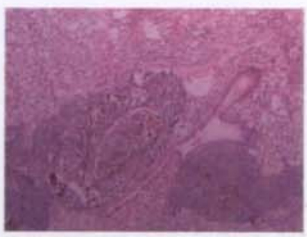

Fig 7.2f

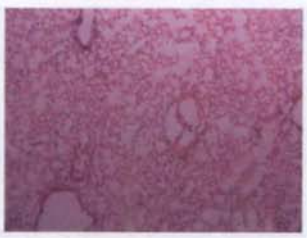

Fig 7.2g

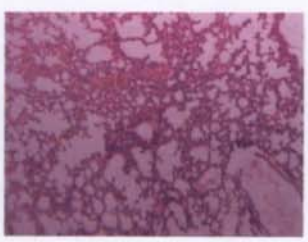

Fig 7.2h

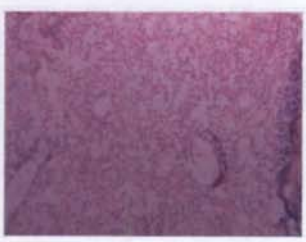

Fig 7.2i

Table 7.4 Effect of naturally occurring terpenoids on the Adhesion of B16F-10 melanoma cells to the collagen matrix

Treatment	% inhibition of Adhesion		
	50μM	10 μM	5 μM
Carvone	6.1%	0%	0%
Limonene	15.9%	6.8%	0%
Perillic acid	18.5%	9.2%	0%
Ursolic acid	19.2%	8.5%	0%
Oleanolic acid	8.1%	2%	0%
Glycyrrhizic acid	38.4%	15.9%	0%
Nomilin	14.7%	10.1%	0%

B16F-10 melanoma cells ($1x10^5$ cells /ml DMEM) were seeded into collagen Type-I coated wells of Flat bottomed titre plates and incubated in presence of terpenoids for 4h at 37^0C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.

Table7.5 Effect of naturally occurring terpenoids on the Invasion of B16F-10 melanoma cells through the collagen matrix

Treatment	% inhibition of Invasion		
	50μM	10 μM	5 μM
Carvone	16.3%	10.2%	8.8%
Limonene	29.5%	16.4%	9.1%
Perillic acid	24.1%	15.2%	7.8%
Ursolic acid	25%	16.9%	7.7%
Oleanolic acid	15.8%	9.1%	4.9%
Glycyrrhizic acid	55.1%	35.2%	5.9%
Nomilin	29.3%	14.9%	6.8%

B16F-10 melanoma cells ($1x10^5$ cells /150μl DMEM) were seeded into collagen Type-I coated polycarbonate membrane on the upper compartment of the chamber. The lower compartment was filled with DMEM. Cells were incubated in presence of terpenoids for 10h at 37^0C. Membranes were removed, fixed, stained and the cells that had migrated in the test and control were counted.

inhibit the invasion of collagen matrix by the tumour cells followed by limonene (29.5%), nomilin (29.3%), ursolic acid (25%) and perillic acid (24.1%).

3.6c Effect of terpenoids on the motility of B16F-10 melanoma cells

Inhibition of tumour cell motility by terpenoids is given in table 7.6. Glycyrrhizic acid could inhibit the motility of B16F-10 melanoma cells across the polycarbonate filters by 29.6%. Other terpenoids did not have any significant effect on the motility of B16F-10 melanoma cells.

3.6d Gelatin Zymographic analysis

Zymographic analysis of the trypsin activated B16F-10 melanoma cell culture supernatant showed digested clear areas at 92KDa and 72KDa. Gels loaded with tumour cell culture supernatant without trypsin activation did not show any clear areas indicating the inactive form of the enzyme collagenase. Trypsin activated tumour cell culture supernatant loaded gels, after incubation with 10mM EDTA did not show clear areas which indicate that EDTA inhibited enzyme activation and the enzyme is metalloproteinase. The conditioned media of limonene, perillic acid, nomilin, ursolic acid and glycyrrhizic acid pretreated cells, which were then activated by trypsin, did not show any clear band indicating the inhibition of expression or activity of gelatinases by the treatment. The conditioned media of all the other compounds pretreated cells did not show any inhibition of expression or activity of gelatinases by the treatment.

When different concentrations of drugs (5 to 50 μM concentration) were added to the incubation buffer, containing 0.1MTris-HCl, 10mM $CaCl_2$ (PH 7.8), along with

Table 7.6 Effect of naturally occurring terpenoids on the migration of B16F-10 melanoma cells through the polycarbonate membrane

Treatment	% Inhibition of Motility		
	50μM	10 μM	5 μM
Carvone	6.5%	0%	0%
Limonene	10.2%	2.1%	0%
Perillic acid	12.1%	3.2%	0%
Ursolic acid	11.8%	4.7%	0%
Oleanolic acid	8.2%	0%	0%
Glycyrrhizic acid	29.6%	14.2%	0%
Nomilin	15.2%	5.8%	0%

B16F-10 melanoma cells ($1x10^5$ cells /150μl DMEM) were seeded into collagen free polycarbonate membranes on the upper compartment of the chamber. The lower compartment was filled with DMEM. Cells were incubated in presence of terpenoids 24 h at 37^0C. After incubation the number of cells migrating to the lower chamber was determined using haemocytometer

Figure 7.3 Gelatin Zymographic analysis

Tumour cell culture supernatant

A with out trypsin activation

B with 10m M EDTA

C with trypsin activation (Control)

D Limonene (50μM pretreated cells)

E Perillic acid (50μM pretreated cells)

F Carvone (50μM pretreated cells)

G Nomilin(50μM pretreated cells)

H Glycyrrhizic acid (50μM pretreated cells)

I Oleanolic acid (50μM pretreated cells)

J Ursolic acid (50μM pretreated cells)

Fig 7.3 Gelatin Zymographic analysis

trypsin- activated tumour cell supernatant loaded gels, clear bands were observed for all gels indicating that these drugs could not inhibit the enzyme once it gets activated.

4 DISCUSSION

In the present study we analysed the effect of terpenoid compounds carvone, limonene, perillic acid, ursolic acid, oleanolic acid, glycyrrhizic acid and nomilin in the inhibition of lung metastasis induced by B16 F-10 melanoma cells. Introducing highly metastatic B16 F-10 melanoma cells into C57BL/6 mice through lateral tail vein can produce metastatic tumour colonies in lungs. In the present study, intra-peritoneal administration of terpenoid compound such as limonene, perillic acid, ursolic acid glycyrrhizic acid and nomilin resulted in marked reduction in the metastases by B16F-10 melanoma

During lung fibrosis collagen gets deposited in lungs. The characteristic of this defect is a increased accumulation of extra cellular matrix protein in the alveolar walls specifically resulting in a reduction in pulmonary function. The increased lung fibrosis was evaluated by estimating lung collagen hydroxyproline content. Lung collagen hydroxyproline content was very high in the case of tumour alone treated group. But there was a reduction in terpenoid treated group. The maximum reduction in the lung collagen hydroxyproline was obtained in the glycyrrhizic acid treated group. A reduction in the number of lung tumour nodules also correlated with these results.

The acidic and basic modifications of monosaccharides yield uronic acids (glucuronic acid) and aminosugars (hexosamines). These form a vital part in many structural polysaccharides and glycosaminoglycans (GAG) found in the ground substance

of ECM. Tumour cells can induce the host stromal cells to supplement the matrix components necessary for the growing tumour (McKinnell et al., 1998). Hyaluronicacids (HA) is a GAG made of repeated disaccharide units of D-glucuronicacid and N-acetyl D-glucosamine (Tammi et al., 2002; Delpech et al., 1997). It is a well-known promoter of metastasis and elevated level is seen in several types of tumour regardless of tumour grade (Hautmann et al., 2000; Setala et al., 1999; Lipponen et al., 2001) and promotes metastasis by opening up spaces for tumour cells to migrate through ECM, by interacting with cell surface receptors for HA (Tammi et al., 2002; Delpech et al., 1997; Turley et al., 2002). The degradation of HA by HAase (Hyaluronidase) liberates disaccharide unitsthat are good promoters of angiogenesis as well by modulating the proliferation, adhesion and migration of endothelial cells (Roden et al., 1989; West et al., 1985). An elevated level of the structural monosaccharidesof HA is seen in the metastasis induced control animals, positively contributing to the elevation of HA in the tumour microenvironment. This in turn will enhance the possibility of metastasis and tumour directed angiogenesis. But treatment of terpenoids reduced the levels of these structural monosaccharides significantly thereby indicating a decrease in the metastatic potential of B16F-10 melanoma. The reduction of these sugars along with the inhibition of gelatinase activity will also negatively contribute to tumour-angiogenesis, which is essential for the tumour to grow beyond a maximum size.

In melanoma, both secretion of glycoprotein (Bhavanandan, 1981; Bizik, 1985) and shedding of gangliosides have been demonstrated ((Bizik et al., 1985). Sialic acid is a family of acylated derivates of neuraminic acid, usually occurs as a terminal component of carbohydrate chains of glycoproteins and glycolipids. Neoplasms often have an increased

concentration of sialic acid on the tumour cell surface. Therefore, total sialic acid levels in serum has been recognized as a valuable non-specific marker of tumour burden in various diseases (Portoukalian et al., 1978; Sela et al., 1989)including melanoma (Portoukalian et al., 1978; Sela et al., 1989). Serum sialic acid level was drastically elevated in metastatic tumour bearing animals. However, this elevated level of serum sialic acid was significantly reduced by simultaneous administration of terpenoid compounds.

A close association between metastatic ability of B16 melanoma sublines and expression of membrane associated gamma glutamyl transpeptidase (γGT) has been reported previously (Obrador et al., 2002). Serum gamma glutamyl transpeptidase (γGT) is also a marker for neoplastic proliferation (Obrador et al., 2002). Tumour cell proliferation was high in the case of tumour alone animals. Elevated levels of γGT were observed in patients with both primary and secondary neoplasm. The increased serum γGT levels in the control animals were reduced by the treatment with these various terpenoids.

In vitro studies also showed the anti-metastatic activity of the terpenoid compounds especially glycyrrhizic acid. These results demonstrate that glycyrrhizic acid could inhibit different stages of metastasis in a dose dependent manner.

Glycyrrhizic acid, limonene, perillic acid, ursolic acid and nomilin could inhibit the production of metallo-proteanase in B16 F-10 melanoma cell line. Regulation of the TIMP/MMP balance is critical to localization inhibition of matrix breakdown for both physiologic invasion of angiogenesis and malignant invasion of metastasis (Bramhall et al., 1996; Bramhall et al., 1997). MMPs are also involved in the cleavage of number of molecules on the cell surface which may alter the cell cycle check point controls and

conceivably promote genomic instability by affecting cell adhesion may disrupt cell signaling and may foster cancer cells to escape immunoscerveillance.

Tumor cell migration is necessary in the inhibition of metastatic cascade at which time the tumour cell leave the primary tumour and gain access to the circulation and also at the end of invasion, when they are entering the secondary site. Tumour cell have been found to respond to a variety of agents in a motile fashion; including host derived motility and growth factors; ECM component and tumour secreted factors (Pawson and Nash, 2003). Terpenoid compound could inhibited the tumour cell migration as well as invasion.

Adhesion molecules play a vital role during trans-endothelial migration of immune and tumour cells (del Pozo et al., 2000) and have been implicated in potentiating metastasis. Inhibition of tumour cell adhesion by these terpenoids may be due to the down regulation of soluble cell adhesion molecule and this in turn will reduce metastatic potential of B16F-10 melanoma cells.

Among the terpenoid compound carvone and oleanolic acid did not inhibit the production of MMPs, cell adhesion, invasion and motility in B16 F-10 melanoma tumours. But glycyrrhizic acid inhibit the production of MMP, cell adhesion motility and invasion and there by inhibit the metastatic tumour progression.

Therefore the above results suggest that these terpenoid compounds except carvone and oleanolic acid could effectively reduce the metastatic potential of B16F-10 melanoma cells.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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CHAPTER 8

EFFECT OF NATURALLY OCCURRING TERPENOIDS ON THE CELL MEDIATED IMMUNE RESPONSES OF METASTATIC TUMOUR BEARING ANIMALS

1 INTRODUCTION

Immunosuppression and dose-limiting toxicities are the major problems for the success of available cancer therapies (Ratain and Relling, 2001). In addition, for advanced tumours developed from epithelial tissues such as lung, colon, breast, prostate and pancreas, conventional cytoreductive therapies are less successful. Therefore, standard chemotherapy and radiotherapy might negate or reduce the therapeutic benefits. Thus combination of chemotherapy or radiotherapy with immunomodulating agents may provide a strategy for overcoming the immunosuppressive effects of chemotherapy or radiotherapy.

Progressive tumour growth in human and animal models is frequently accompanied by concominent immunosuppression regardless of tumour location and etiology. (Chattopadhyay et al., 1986; Nelson and Nelson, 1987) .One explanation for immunosuppression by tumours is due to decrease in cytotoxic T lymphocytes and natural killer cell activities. (Santin et al., 2000; Tsavaris et al., 2002). There are several evidences for the production of soluble factors by developing neoplasia affecting the function of host cells involved in the immunity (Nelson and Nelson 1987; Parhar and Lala, 1998). In addition, the production of factors in abnormal amounts by tumour- bearing hosts may alter normal cytokine network and cause a deleterious imbalance of immune system (Handel et al., 1997).

Plant and plant products have been the basis of treatment of human diseases since time immemorial. Many of them such as *Viscum album* (Kuttan and Kuttan, 1992), *Tinospora cordifolia* (Mathew and Kuttan, 1999), *Withania somnifera* (Davis and Kuttan 2000), *Panax ginseng*, (Sing et al, 1984). *Piper longum Linn.* and Piperine (Sunila and

Kuttan, 2004) have found to possess immunomodulatory activity. They are nontoxic compared to other class of immunomodulators and have high interest in the research field. Plant derived immunostimulating drugs can enhance cell-mediated immune responses and natural killer cell (NK cell) activity, facilitating the killing of the tumour cells by the body (Antony et al., 2000; Lala et al., 1985) Cytokines are a unique family of growth factors. Secreted primarily from leukocytes, cytokines stimulate both the humoral and cellular immune responses. Cytokines are important mediators of immune responses and are found to stimulate immune cells. Most biological agents that enhance NK cytotoxicity do so via their common ability to induce IFN γ (Ehrhardt et al., 1997), increase the activity of Tc cells, macrophages and NK cells (Ehrhardt et al., 1997),. IL-2 and GM-CSF are other important cytokines involved in the immune cell activation. (Misawa et al., 2000)

The effect of terpenoid compounds on cell mediated immune system has not been studied in a systematic way. In the present chapter we report the stimulatory activity of triterpenoids ursolic acid and glycyrrhizic acid on cell-mediated immune system of metastatic tumour bearing animals.

2 MATERIALS AND METHODS

2.1 Cell lines

B16F-10 melanoma cells, K562 human leukemic cells and Sheep red blood cells were used for this study.

2.2 Animals: C57BL/6 mice (5 weeks old, 20-25g males) were used for immunological studies.

2.3 Reagents:

RPMI- 1640 medium continig 10% FCS

Radioactive materials: Tritiated thymidine and $Na_2 Cr^{51} O_4$

All other reagents used were of analytical reagent quality.

2.4 Terpenoid compounds:

Different concentrations of limonene, perillic acid, ursolic acid and glycyrrhizic acid suspended in light paraffin oil and intraperitoneally administered. Five doses of limonene administreted at a concentration of 100 μmoles/Kg body wt/dose/animal. Perillic acid, ursolic acid and glycyrrhizic acid were intraperitoneally administered at a concentration of 50 μmoles/Kg body wt for 5 consecutive days.

2.5 Determination of the effect of terpenoids on natural killer cell (NK cell) activity, antibody dependent cell mediated cytotoxicity (ADCC) and antibody dependent complement-mediated cytotoxicity (ACC) in metastatic tumour bearing animals.

C57BL/6 mice were grouped into five (12 nos./group). All the animals were induced metastasis by injecting B16F-10 cells (10^6 cells/animals) intravenously. Group I, II, III and IV animals were treated with 5 doses of limonene, perillic acid, ursolic acid and glycyrrhizic acid respectively. Group V animals were kept as untreated tumour bearing controls. Animals were sacrificed at different time periods after the tumour inoculation and spleen and blood was collected. Spleen cells were processed to single cell suspension and used as effectors for NK and ADCC. Serum was separated from the blood, heat inactivated and used for the ACC assay.

2.5a Determination of natural killer cell activity

Natural killer cell activity was determined by 4h chromium assay as explained in chapter 2 (Kim et al., 1980). Cr^{51} labeled K 562 cells were used as targets and spleen cells from the metastatic tumour bearing animals were used as effector cells

2.5b Determination of antibody dependent cellular cytotoxicity (ADCC)

ADCC was determined by 4 h chromium release assay as described in chapter 2 (Kim et al., 1980). Chromium labelled SRBC was used as target cells and spleen cells from animals were used as effector cells. Anti SRBC antibody was raised in rabbit and was used as the source of antibody in ADCC assay.

2.5c Determination of antibody dependent complement mediated cytotoxicity (ACC)

Serum from the above animals was used for the determination of ACC activity explained in chapter 2. Serum samples were incubated along with fresh rabbit serum as a source of complement and EAC cells as target cells (10^6) at 37^0C for 3h and percentage cell death was determined by trypan blue exclusion method (Chapter 2).

2.6 Determination of the effect of terpenoids on cytokine production by metastatic tumour bearing animals

C57BL/6 mice were grouped into five (9 nos./group) and all the animals were injected with B16F-10 cells (10^6 cells/animal) intravenously. Group I, II, III and IV animals were treated with 5 doses of limonene, perillic acid, ursolic acid and glycyrrhizic acid respectively. Group V animals were kept as untreated tumour bearing controls. Blood was collected by tail bleeding on 7^{th} and 21^{st} day after tumour inoculation. Serum separated and used for the estimation of cytokines such as IL-1β, IL-2, IL-6, GM-CSF and TNF-α using respective Elisa kits.

3 RESULTS

3.1 Effect of naturally occurring terpenoids on natural killer cell activity of metastatic tumor bearing mice

The effect of terpenoids on the NK cell activity of metastatic tumour bearing animals is shown in figure 8.1. There was a significant enhancement of the NK cell activity in terpenoids treated metastatic tumour bearing animals. In terpenoids treated group maximum cell lysis was obtained on 4^{th} day (60.2%, 43%, 50.3% and 44.7% cell lysis respectively for glycyrrhizic acid, ursolic acid, limonene and perillic acid) after the tumour inoculation. In control animals the maximum cell lysis (25% cell lysis) was obtained only on 16^{th} day.

3.2 Effect of terpenoids on ADCC of metastatic tumour bearing animals

The effect of naturally occurring terpenoids on ADCC activity is given in figure 8.2. Intraperitoneal administration of terpenoids clearly enhanced the ADCC activity in metastatic tumour bearing animals. In tumour alone treated control animals maximum cell lysis was obtained only on 16^{th} day (20 % cell lysis). But in the case of terpenoids treated metastatic tumour bearing animals, the maximum lysis was obtained on 12^{th} day and it was 47%, 44.7%, 33.9% and 32.5% cell lysis respectively for glycyrrhizic acid limonene, perillic acid and ursolic acid treated groups.

3.4 Effect of terpenoids on ACC activity in metastatic tumour bearing animals

ACC was also enhanced by the terpenoids treatment. Maximum cell lysis was observed on 17^{th} day for all the compounds. The maximum ACC activity was obtained in glycyrrhizic acid treated metastatic tumour bearing animals (26.52%).Where as in ursolic

Fig 8.1 Effect of terpenoids on NK cell of metastatic tumour bearing animals

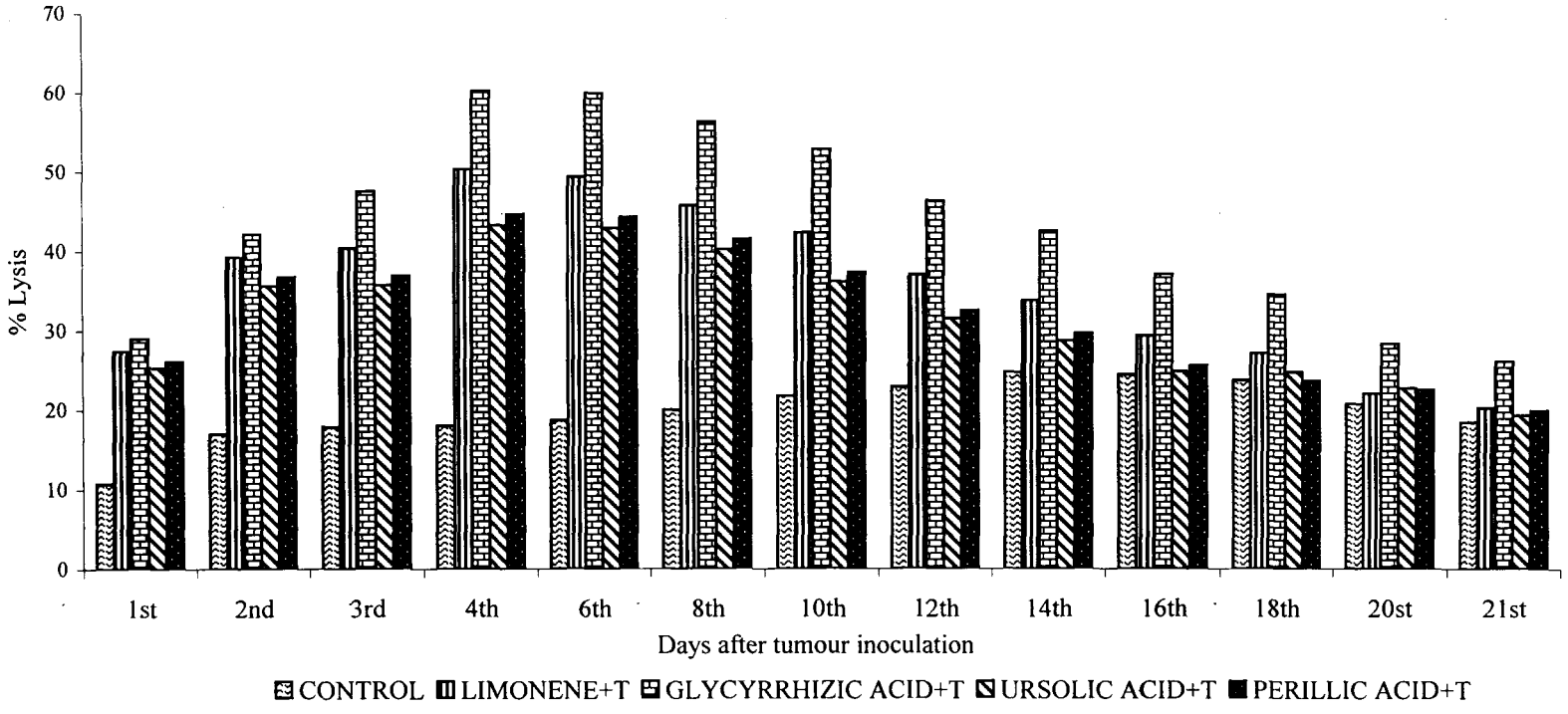


Fig 8.2 Effect of terpenoids on ADCC

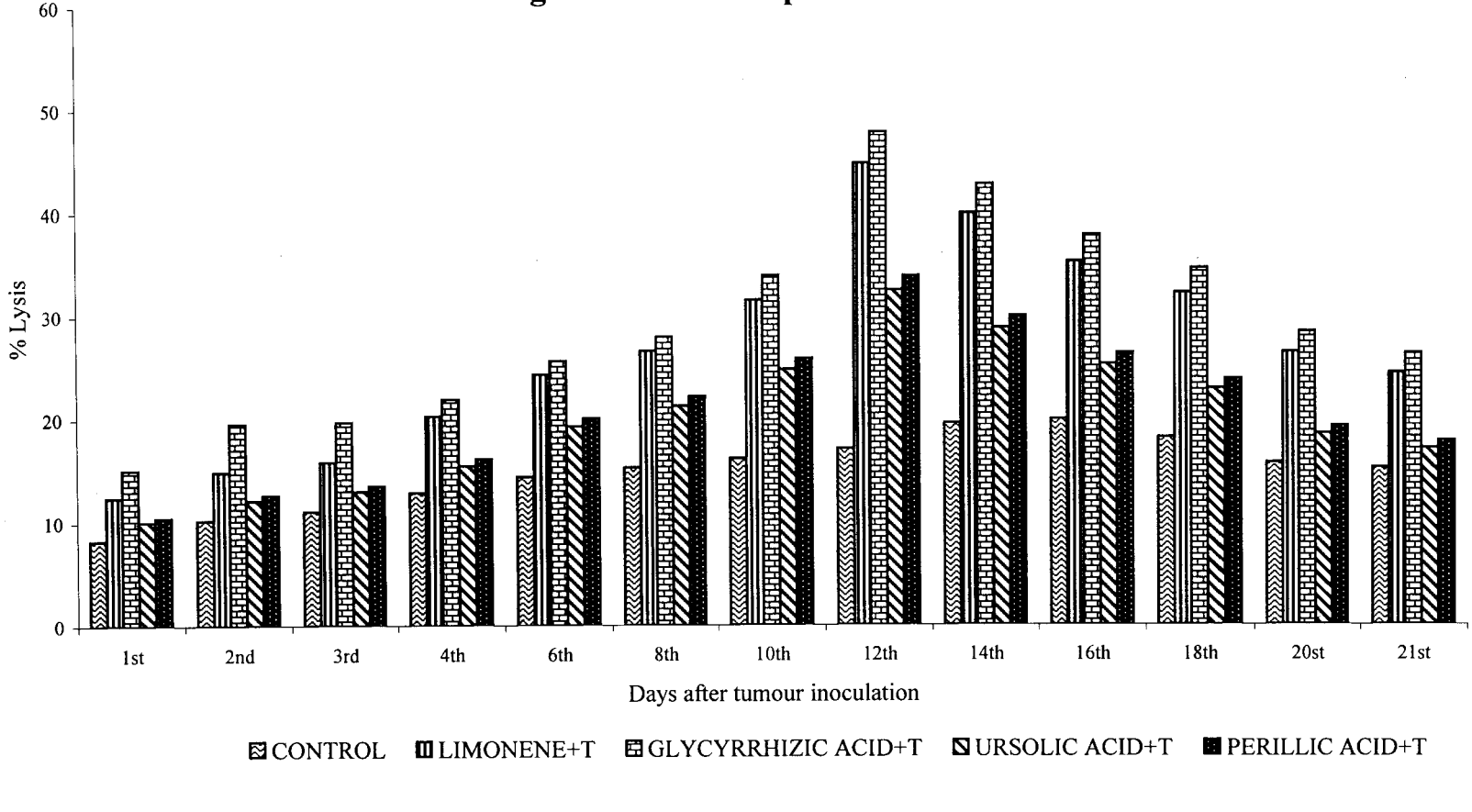
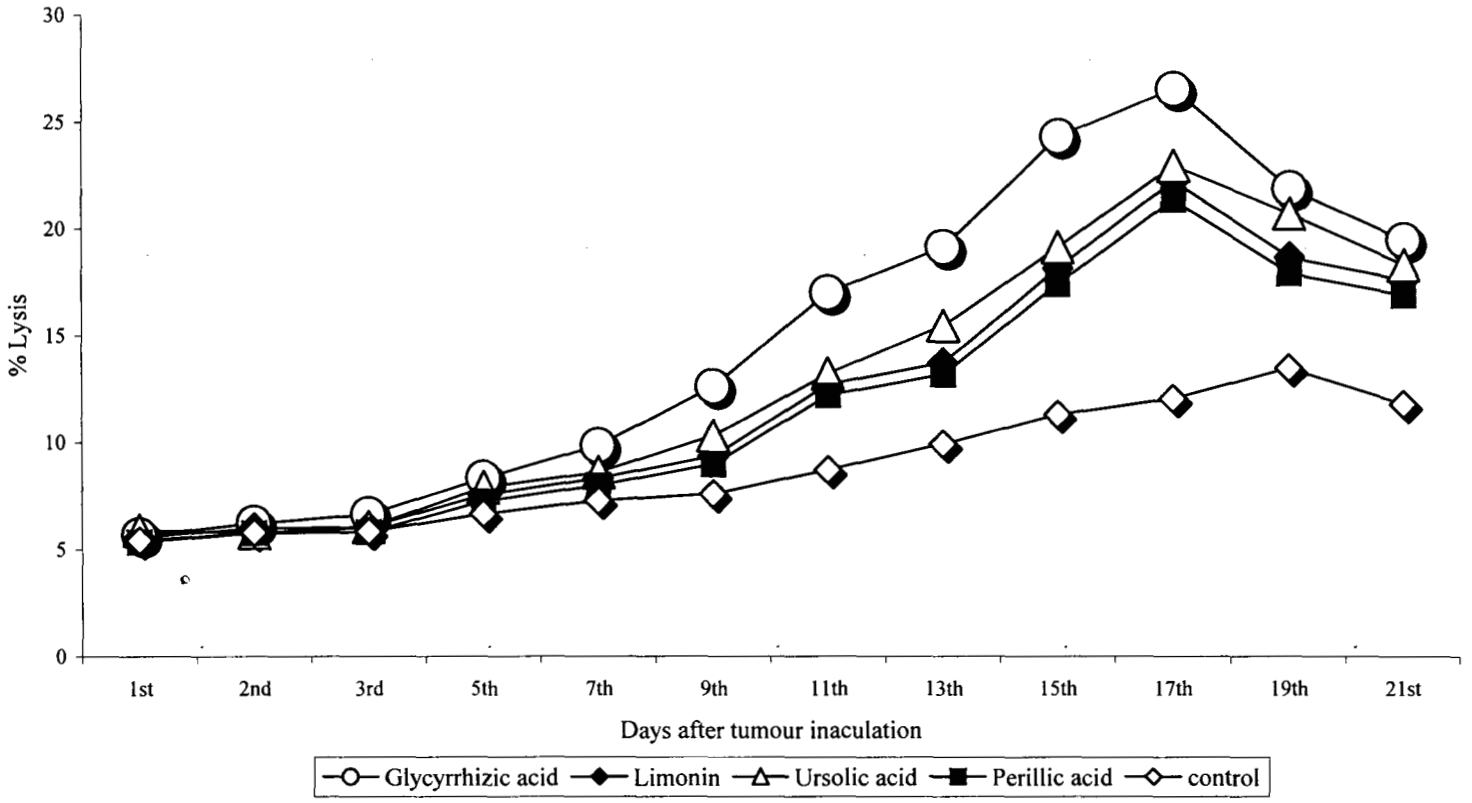


Fig 8.3 Effect of terpenoids on ACC



acid, perillic acid and limonene treated groups maximum cell lysis were 22.9%, 21.3% and 22.2% respectively. But in metastatic tumour bearing control animals, the maximum cell lysis was observed on 19th day and it was only 13.4% (Fig.8.3).

3.4 Effect of terpenoids on the cytokine production by metastatic tumour bearing animals

As shown in table 8.1, 7th day after tumour inoculation, TNF-α level of metastatic tumour bearing control animals was drastically elevated to 241.5 pg/ml which was significantly reduced by the administration of glycyrrhizic acid (102.6 pg/ml), perillic acid (117.6 pg/ml), limonene (124 pg/ml) and ursolic acid (142.3 pg/ml). The level of GM-CSF was also reduced by terpenoids treatment. The maximum inhibition of serum GM-CSF levels was obtained in limonene treated (10.02 pg/ml) metastatic tumour bearing animals and followed by in the treatment with perillic acid (18.7 pg/ml), glycyrrhizic acid (20.3 pg/ml) and ursolic acid (22.5 pg/ml) treated metastatic tumour bearing animals compared to control animals (37.9 ± 1.1 pg/ml). The highly elevated level of IL-6 (370.1 pg/ml) in control animals was reduced by the treatment of glycyrrhizic acid (313 pg/ml) ursolic acid (299 pg/ml) limonene (314.4 pg/ml) and perillic acid (318 pg/ml). The lowered level of IL-2 in the untreated control animals (24.9 pg/ml) was enhanced by the treatment with imonene (39.1 pg/ml) glycyrrhizic acid (37.9 pg/ml) ursolic acid (35.9 pg/ml) and perillic acid (32.6 pg/ml).

On 21st day after tumour inoculation, the enhanced level of TNF-α in metastatic tumour bearing animals was further enhanced to 334.9pg/ml, which was significantly reduced by the treatment of glycyrrhizic acid (96.4 pg/ml) ursolic acid (84.7 pg/ml) perillic acid (98.6 pg/ml) and limonene (108.6 pg/ml). The enhanced level of IL-1β was also

Table 8.1 Effect of terpenoids on serum cytokine levels in metastatic tumour bearing animals

Treatment	Serum cytokine level (pg/ml)									
	7th day					21st day				
	IL-2	IL-Iβ	TNF-α	IL-6	GM-CSF	IL-2	IL-Iβ	TNF-α	IL-6	GM-CSF
Normal	27.4±1.8	16.0±2.1	20.0±3.5	35.0±3.	7.8±1.1					
Control	24.9±0.6	44.8±0.6	241.5±3.5	370.1±7.4	37.9±1.1	7.4±0.9	60.3±1.3	334.9±27.5	559.8±46.9	21.8±4.4*
Limonene	38.1±0.8*	34.2±2.1*	124±10.8*	314.44±12.4*	10.02±0.8*	32.9±1.1*	20.3±1.1*	108.6±4.5*	412.3±31.8*	7.9±1.2*
Perillic acid	32.6±2.1*	33.6±1.6*	117.6±11.3*	318±11.8*	18.7±1.4*	31.4±1.5*	19.8±0.8*	98.6±3.2*	398.5±28.9*	10.9±0.7*
Ursolic acid	35.98±3.2*	36.4±3.1*	142.3±12.8*	299±12.7*	22.5±1.5*	30.5±1.4*	21.2±2.4*	84.7±1.3*	386.9±28.4*	14.7±0.9*
Glycyrrhizic acid	37.9±2.3*	33.4±1.1*	102.60±20.1*	313.32±9.4*	20.03±2.3*	24.5±1.8*	20.1±1.8*	96.4±2.4*	411.8±19.9*	12.6±1.1*

Animals were injected with B16f-10 cells (10^6) intravenously. Animals were treated with 5 consecutive doses of limonene, perllic acid ursolic acid and glycyrrhizic acid. Blood was collected by tail bleeding on 7^{th} and 21^{st} day after tumour inoculation. Serum separated and used for assays.

(*P< 0.01 Compared with tumour alone treated control)

effectively reduced by the treatment of glycyrrhizic acid (20.1 pg/ml), ursolic acid (21.2 pg/ml) limonene (20.3 pg/ml) and perillic acid (19.8 pg/ml) compared to control animals (60.3 pg/ml). The elevated level of GM-CSF in control animals (21.8 pg/ml) after 21 days was reduced by the treatment of limonene (7.9 pg/ml), perillic acid (10.9 pg/ml), glycyrrhizic acid (12.6 pg/ml) and ursolic acid (22.5 pg/ml). The level of IL-6 was highly elevated in metastatic tumour bearing animals (559.8 pg/ml). Treatment with glycyrrhizic acid (411 pg/ml), limonene (412.3 pg/ml), perillic acid (398.5 pg/ml) and ursolic acid (386.9 pg/ml) could effectively reduce the same. Drastically lowered level of IL-2 in control animals (7.4 pg/ml) was significantly enhanced by the treatment of glycyrrhizic acid (24.5 pg/ml), ursolic acid (30.5 pg/ml), perillic acid (31.4 pg/ml) and limonene (32.9 pg/ml).

4 DISCUSSION

Tumour development, out growth and metastasis are under the surveillance of the immune system. The fate of the host- tumour interactions depends on the balance between the intrinsic metastatic potential of the tumour and strength of the host immune response (Cooper et al., 2001). One of the major objectives of immunotherapy is to modulate immune response for selected objectives. Cell-mediated immunity is the component of the immune system most responsible for destruction of infected cells and tumour. Macrophages are critical in presenting antigens to helper T-cells (CD-4) cells via MHC II. This activates the helper T-cell to further activate cytotoxic (killer) T-cells and natural killer cells creating a robust immune response. The cells of the cell-mediated immune system depend on signals to communicate with each other in order to mount an aggressive

and orchestrated attack. These signals are transmitted using cytokines, lymphokines, interferons, and other chemical messengers. In this study we evaluated the cell mediated immune response against B16 F10 metastatic tumour in mice by the activation of NK cell, ADCC, ACC and the production of various cytokines by the administration of terpenoid compounds.

The role of natural killer (NK) cells in the induction and regulation of immune responses has been the focus of many investigations. Due to the peculiar immunoregulatory characteristics of NK cells, it may be a potential target for novel immunotherapeutic interventions aimed at the prevention or treatment of infectious disease, cancer and autoimmune disease (Henney,1981; Mandelboim, 1999). Even though they have no known antigen specific receptors they are able to recognize and kill a limited range of abnormal cells including cancer cells. Activation of NK cells is one of the objectives of tumour immunotherapy. NKcells also produce cytokines, particularly IFN-γ (Kobayashi et al., 1989; Ehrhardt, 1997). The lymphokine IL-2, which was identified as T cell growth factor (Misawa et al., 2000), alone or in combination with IFNs, also promote the lytic activity of NK cells (Caligiuri, 1993) which in turn produces a variety of immunoregulatory molecules that could synergize with IFNs or IL-2 for the induction of antitumour responses (Henney,1981). Treatment with these compounds has stimulated host defense response, as demonstrated by the enhanced level of IL-2.

ADCC is the cooperative interaction of humoral and cell mediated immune effectors. A number of cells that have cytotoxic potential express membrane receptor for the Fc region of the antibody molecule. When antibody is specifically bound to a target cell these receptor bearing cells can bind to the Fc region of the antibody and thus to the

target cells and subsequently cause lysis of the target cells. Cytotoxic T lymphocytes and NK cells have Fc receptors that are capable to trigger cytotoxic attack to target cells (Henney,1981; Mandelboim,1999). Intraperitoneal administration of terpenoids elevated NK cell activity resulted in significant enhancement of ADCC in both tumour bearing as well as normal animals where as untreated tumour bearing animals with low NK cell activity showed decreased ADCC activity.

Complement is a system of plasma proteins that can be activated by antibody, leading to a cascade of reactions that occurs on the surface of pathogens and generate active components with various effector functions. Administration of these terpenoids ursolic acid and glycyrrhizic acid could enhance the ACC activity in tumour bearing animals. Complement proteins are also responsible for cell lysis and mediation of inflammation, serving to attract phagocytic cells and enhance phagocytosis. It plays a major role in cell mediated immune response. Enhancement of ACC shows the activation of cell mediated immune system by the administration of terpenoids.

IL-6 is a key inflammatory mediator produced by many cell types. It is the major inducers of the acute-phase response and fever. Blocking IL-6 may alleviate rheumatoid arthritis and may also be effective in other autoimmune, inflammatory, and bone-erosive diseases. In mice, IL-6 is required for development of oil-induced plasmacytomas (Hilbert et al,. 1995) and is involved in tumor cachexia (Strassmann, 1995). In humans, IL-6 is a growth factor for myelomas, (Licastro et al., 1993). In this study administration of terpenoids were shown to reduce the IL-6 production in the metastatic tumour bearing animals. TNF-alpha and IL-1β are also the principle cytokines that mediates acute inflammation. In excessive amounts TNF-alpha also is the principal cause of systemic

complications such as the shock cascade. Interleukin-1s (IL-1) are secreted by stimulated macrophages and induce the synthesis of collagen and collagenase. Blocking IL-1 activity via receptor antagonist, soluble receptors, or newly tailored drugs shows promise in controlling inflammatory diseases, such as rheumatoid arthritis and septic shock. IL-1, probably most effectively if combined with blockade of other inflammatory cytokines, such as TNF and IL-6. GM-CSF binds to receptors on neutrophils, eosinophils, and monocytes, it activates these cells and inhibits apoptosis. Administration of terpenoids decreased the elevated levels of this cytokines. Lymphokine IL-2, stimulates cytotoxic activity of NK and T cells and acting as a cofactor in activating macrophages and B cells. Administration of these terpenoids reduced the enhanced level of TNF, IL-6, GM-CSF and IL-1β and also increases the production of IL-2 in metastatic tumour bearing animals.

The above study revealed immunostimulatory effects of glycyrrhizic acid and ursolic acid on metastatic tumour bearing animals, and these activities may be due to the stimulation of IL-2 production and there by enhanced cell mediated immune responses.

INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

Thesis submitted to the University of Calicut for the Degree of

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY

(FACULTY OF SCIENCE)

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SUMMARY AND CONCLUSION

Therapeutic treatment modalities of chemotherapy, radiotherapy, and surgery, are effective only for some patients with solid malignancies. They cannot be successfully employed for many other patients who expire from the direct or indirect effect of the progressive out growth of metastatic foci or from the adverse and toxic effect associated with some standard therapeutic modalities (Goldfarb and Brunson 1982).Some standard therapeutic treatments may be immunosuppressive and lead to infectious disease related mortality (Goldfarb and Brunson 1982; Nicolson and Filder 1988).

The field of tumour immunology has undergone explosive growth and has presented the oncology community the practical applicability of immunological approaches for treatment of cancer. In recent years there has been a renewed optimism based on the detailed characterization of tumour associated antigens and their relationship to the MHC, the role of antibodies and cellular immunity in cancer, detailed evolution of tumour infiltrating lymphocytes, and encouraging approaches with tumour vaccines, adaptive immunotherapy, gene therapy and chemoimmunotherapy for treatment of cancer in both animal model and in the clinic (Goldfarb and Whiteside, 1994).

Biologic therapy is cancer treatment that produces antitumour effects primarily through the action of natural host defense mechanisms or the administration of natural mammalian substances. Biologic therapy has emerged, as an important fourth modality for the treatment of cancer the question of how to best augment and sustain antitumor responses during cancer progression has been a focus of biotherapeutic approaches for a long time.

In the present study we have evaluated the immunomodulatory and antimetastatic activity of some naturally occurring terpenoid compounds carvone, limonene, perillic

acid, ursolic acid, oleanolic acid, nomilin and glycyrrhizic acid in experimental animals. We have also tried to elucidate the mechanism of action of these drugs in the inhibition of metastasis using *in vitro* models. The immunomodulatory, chemo protective and radio protective activities of these compounds were also investigated.

Immunosuppression is one of the major problems for the success of available cancer therapies (Ratain, and Relling2001).The main objectives of immunotherapy is to modulate immune response for selected objectives. Immunomodulation is the regulation of immune response whether to suppress them when unwanted or to stimulate them in the prevention of diseases. There are a variety of naturally and chemically derived compounds discovered with immunomodulatory activity such as levamasole, glucan, IL-2, IFN etc. are used in combination with cisplatin, adiramycin, 5-flurourasil etc. against many types of carcinomas. But most of these compounds have side effects namely fever, myalgias fatigue etc Immunomodulatory agents that are free from side effects and which can be administrated for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. The present study demonstrate that unlike other chemically defined compounds, these terpenoid compounds modulate the immune system without affecting the other parameters of the body. The humoral immune response was analysed by the specific antibody production and number of antibody producing cells in spleen. The production of antibody producing cells in spleen was increased by terpenoid administration. The circulating antibody titre was also significantly enhanced in the terpenoid treated animals; showing its stimulatory effect on the humoral arm of the immune system

Cell-mediated immunity is the component of the immune system most responsible for destruction of infected cells and tumour. Macrophages are critical in presenting antigens to helper T-cells (CD-4) cells via MHC II. This activates the helper T-cell to further activate cytotoxic (killer) T-cells and natural killer cells creating a robust immune response. The cells of the cell-mediated immune system depend on signals to communicate with each other in order to mount an aggressive and orchestrated attack. These signals are transmitted using cytokines, lymphokines, interferons, and other chemical messengers. The role of natural killer (NK) cells in the induction and regulation of immune responses has been the focus of many investigations. Due to the peculiar immunoregulatory characteristics of NK cells, it may be a potential target for novel immunotherapeutic interventions aimed at the prevention or treatment of infectious disease, cancer and autoimmune disease (Henney,1981; Mandelboim, 1999). Even though they have no known antigen specific receptors they are able to recognize and kill a limited range of abnormal cells including cancer cells. Activation of NK cells is one of the objectives of tumour immunotherapy. NK cells also produce cytokines, particularly IFN-γ (Kobayashi et al., 1989, Ehrhardt, 1997). Administration of all of the terpenoids used in this were shown to enhance the NK cell activity in normal as well as tumour bearing animals. Treatment with these terpenoid compounds has stimulated host defense response, as demonstrated by the enhanced level of IL-2. IL-6 is a key inflammatory mediator produced by many cell types. It is the major inducers of the acute-phase response and fever. Blocking IL-6 may alleviate R arthritics and may also be effective in other autoimmune, inflammatory, and bone-erosive diseases. In mice, IL-6 is required for development of oil-induced plasmacytomas (Hilbert et al,. 1995) and is involved in tumor

cachexia (Strassmann, 1995). In humans, IL-6 is a growth factor for myelomas, (Licastro et al., 1993). TNF-alpha and IL-1β are also the principle cytokines that mediates acute inflammation. In excessive amounts TNF-alpha also is the principal cause of systemic complications such as the shock cascade. Interleukin-1s (IL-1) are secreted by stimulated macrophages and induce the synthesis of collagen and collagenase. GM-CSF binds to receptors on neutrophils, eosinophils, and monocytes, it activates these cells and inhibits their apoptosis. In this study administration of terpenoids limonene, perillic acid, ursolic acid and glycyrrhizic acid were shown to reduce the elevated levels of these inflamatory cytokines and stimulates cytotoxic activity of NK and T cells and acting as a cofactor in activating macrophages and B cells in tumour bearing animals.

ADCC is the cooperative interaction of humoral and cell mediated immune effectors. The destruction of antibody coated target cells is called antibody depending cell mediated cytotoxicity. Cytotoxic T lymphocytes and NK cells have Fc receptors that can capable to trigger cytotoxic attack to target cells. Antibody can direct an antigen specific attack by an effector cell lacking specificity for antigen. Administration of terpenoids carvone, limonene, perillic acid, ursolic acid, oleanolic acid,nomilin and glycyrrhizic acid enhanced the antibody depending cellular cytotoxicity compared to tumour alone treated control.

Treatment with these terpenoid compounds promoted the proliferation of spleen, thymus and bone marrow cells as is evident from blastogenesis assays and is a potent stimulator of lymphocytes, especially T cells as it can enhance the mitogenic potential of mitogens such as PHA and Con A. PHA and Con A are two T cell proliferators, PWM is a B cell mediated T cell proliferator and LPS is a B cell proliferator. Bone marrow serves

as the major source of all blood cells. The majority of all the cell type involved in the immune system is produced from a common hemopoietic stem cells of bone marrow. Thymus provides micro environment for differentiation of T cells. Intraperitoneal administration of these terpenoid compounds promoted the proliferation of spleen, thymus and bone marrow cells as is also evident from *in vivo* blastogenesis assays .These results show that the terpenoid compounds might play an important role as immunostimulants.

Antimetastatic activities of these terpenoid compounds have been studied both *in vivo* and *in vitro*. Administration of these terpenoid compounds could inhibit the metastatic tumour colony formation in the lungs after 21 days of tumour induction. There was also a corresponding increase in the life span of these compounds treated metastatic tumour bearing animals. Administration of limonene, perillic acid, ursolic acid, glycyrrhizic acid and nomilin when given simultaneously could reduce the hydroxyproline content of the lung indicating the effect of these compounds on the inhibition of lung fibrosis. It was found that tumour cells have changed surface properties from their normal counter parts and these changes are partially due to altered sialoglyco conjugates expressed on the plasma membrane. (Thomas, 1996). Hence there is always an increased expression of surface sialic acid on circulating tumour cells, which facilitate their invasive behavior. In melanoma both secretion of glycoproteins and shedding of gangliosides have been demonstrated (Hersey, 1985). Therefore total serum sialic acid estimation can be used as a non-specific marker of melanoma development both in humans and in animal models (Vedralova and Borovansky, 1994). Serum sialic acid level was drastically elevated in metastatic tumour bearing animals. The elevated level of

serum sialic acid was significantly reduced by simultaneous administration of terpenoid compounds, limonene, perillic acid, ursolic acid, glycyrrhizic acid and nomilin, indicating suppression on the invasive property of the tumour cells. The elevated levels of lung uronic acid and hexosamine contents also significantly reduced by the simultaneous administration of these compounds indicating a reduction in the tissue damage and lung fibrosis. Serum gamma glutamyl transpeptidase, which is a marker of cell proliferation, was found to be increased in metastatic tumour bearing animals. Increase of GGT has been reported during the growth of tumour cells (Haningan et al., 1994). But treatments with terpenoid compounds by simultaneous modality could reduce the same.

Adhesion and invasion are the two major steps in the process of metastatic dissemination. For tumour cells to carryout invasion it has to attach to the extra cellular matrix through cell surface receptors that bind to specific adhesion molecules in the matrix. Adhesion molecules play a vital role in trans-endothelial migration of tumour cells (Burrow et al., 1991) and have been implicated in metastasis. These terpenoid compounds could inhibit the adhesion of B16 F-10 melanoma cells to the collagen matrix in a dose dependent manner and this may be due to the down regulation of soluble cell adhesion molecule and which, in turn will reduce the metastatic potential of B16F-10 melanoma cells. Tumor all migration is necessary in the inhibition of metastatic cascade at which time the tumour cell leave the primary tumour and gain access to the circulation and also at the end of invasion, when they are entering the secondary site. Tumour cell have been found to respond to a variety of agents in a motile fashion; including host derived motility and growth factors; extra cellular matrix component and tumour secreted

factors (Pawson and Nash, 2003). Terpenoid compound glycyrrhizic acid could effectively inhibited the tumour cell migration as well as invasion.

A critical proteolytic event early in the metastatic cascade appears to be the degradation of basement membrane collagen (Powel, 1996). Matrix metalloproteases (MMPs) are a family of neutral metalloenzymes secreted as latent proenzymes. They require activation through proteolytic cleavage of the amino-terminal domain, and their activity depends on the presence of Zn^{++} and Ca^{++}. Increased MMP activity has been detected and shown to correlate with invasion and metastatic potential in a wide range of cancers, including ovary, lung, prostate, breast, and pancreas cancers (Hulboy et al., 2001). Type IV collagen is a critical component of the basement membrane architectural scaffolding, on which laminin, heparan sulfate, proteoglycan, and minor components of the basement membrane are assembled. Among the terpenoid compounds studied carvone and oleanolic acid did not inhibit the production of MMPs, cell adhesion, invasion and motility in B16 F-10 melanoma tumours. But glycyrrhizic acid inhibited the production of MMP, cell adhesion motility and invasion and there by inhibit the metastatic tumour progression

Free radicals of oxygen, hydrogen peroxide and organic peroxides are factors that contribute to tumour promotion by forming oxidized DNA bases. The evidence for radical mediation of many events in carcinogenesis is very strong (Pryor, 1987) and they cause damage to lipids, proteins, membranes and DNA. These terpenoid compounds were found to inhibit nitric oxide (NO) production by peritoneal macrophages and ferrous ion induced lipid peroxidation in mice liver homogenate. *In vitro* studies revealed the hydroxyl radical (OH) scavenging activity of these compounds.

There is a continued interest in and need for the identification and development of non-toxic and effective radio and chemo protective compounds that can reduce the effect of radiation and cytoreductive chemotheraputics. Such compounds could potentially protect humans against the genetic damage, mutation, alteration in the immune system and teratogenic effects of toxic agents which act through the generation of free radicals. Good immunostimulants and antioxidants could alleviate toxic nature of conventional radio and chemotherapies were well known. Depletion of intracellular GSH has been implicated as one of the causes of chemo and radiation-induced tissue damage, while increased levels of intracellular GSH are responsible for the tissue protective action. Treatment with terpenoid compounds was found to enhance GSH levels in intestinal mucosa and liver of irradiated as well as CTX treated animals, which offer protection to tissue damage (Peristeris et al., 1992; Pena et al., 1999; Weijl et al., 1998).

In the present study we could analyze the antitumourigenic activity of these terpenoids. Among the terpenoids we have studied, limonene, perillic acid, ursolic acid, glycyrrhizic acid and nomilin reduced the tumour growth and increased the life span of ascites tumour bearing animals The maximum reduction in DLA induced solid tumour development observed in ursolic acid, nomilin and glycyrrhizic acid treated groups. Antitumour activity of triterpenoids may bedue to the combined action of immunostimulatory and antioxidant activity.

The above results indicate the effectiveness of naturally occurring terpenoid compounds in the inhibition of metastasis and their potency as immunostimulators without toxicity. These immunomodulators of plant origin will be highly useful for the treatment of malignancy because of their radioprotective and chemoprotective effects.

The increase in the total WBC count and bone marrow cells after the administration of terpenoid compounds indicate that these compounds could stimulate haemopoiesis. Treatment with these compounds could stimulate the proliferation of spleen cells, thymocytes and bone marrow cells in the presence of mitogens. These results show that the terpenoid compounds might play an important role as immunostimulant and could be used as adjuvant during cancer therapy.

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INVESTIGATIONS ON IMMUNOMODULATORY AND ANTIMETASTATIC ACTIVITY OF NATURAL TERPENOIDS AND THEIR USEFULNESS IN CANCER THERAPY

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