Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

for the fulfillment of the degree of

Doctor of Philosophy In Immunology

(FACULTY OF SCIENCE)

By

E.S. SUNILA M.Sc

AMALA CANCER RESEARCH CENTRE
AMALA NAGAR, THRISSUR-680555
KERALA, INDIA
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DECLARATION

I, Sunila.E.S 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

29th November 2006

Signature of the candidate

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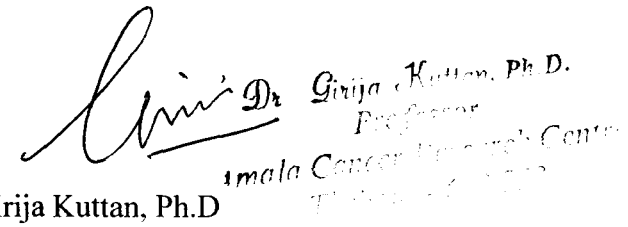
CERTIFICATE

This is to certify that the present report is an authentic account of the work carried out by Ms. Sunila.E.S, under my supervision and guidance and no part thereof has been presented before any other degree.



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29th November 2006

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Introduction

Cancer represents diseases in which one or more cells in the body lose their normal growth-controlling mechanisms and continue to grow, causing the death of the host. In some instances cells within a tissue display functional alterations. Most of these alterations are carried in the form of chromosomal translocations, deletions, amplifications, point mutations or certain oncogenic viral infections or integration of its DNA into the genome. The consequence of these alterations leads to the formation of pathological overgrowth known as a tumor (Bishop, 1991). There are considerable variations occurs among tumors, some show little deviation from normal tissue while others bears no obvious similarity to normal tissues, either structurally or functionally. The former referred to as benign tumors and the latter as malignant tumors or cancers. Malignant tumors are more aggressive and have characteristics of immortalization, transformation and metastasize (Sugarbaker and Ketchem, 1999).

In most cases, primary tumor can be removed by surgery or local irradiation, but cells disseminated in the body may give rise after variable time periods, to metastases formation. However the metastatic cascade is extremely complex, the basic principles and mechanisms of this processes need to be elucidated in order to improve prevention, treatment and cure of metastasis. Formation of tumor metastasis is the major cause of treatment failure in cancer patients and is a principle contributing factor to cancer morbidity and mortality.

A major and frequently limiting toxicity associated with the conventional therapeutic modalities such as chemotherapy, radiotherapy and surgery, which are

currently employed in the clinical management of patients with neoplastic disease, cause profound bone marrow suppression and immunosuppression (Herman and Rodger, 2003). In addition to the immunosuppression induced by the above cancer treatment, impairment of immuno-competence in the host is common among the consequences of established cancer (Almot and Smith, 1995). Lack of the appropriate immune response not only decreases the resistance to infection but may also facilitate progressive tumor growth; several attempts have been made to reduce this complication (Mausch et al, 1995).

Recently there is an increasing interest in the search of potential drugs that are capable of modifying immune responses with less side effects. Those immunomodulatory agents may selectively activate either cell mediated or humoral immunity and the modulation of immune response by using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigation. Some of these medicinal plants and their active components of *Viscum album* (Kuttan and Kuttan, 1992), *Tinospora cordifolia* (Mathew et al, 1999), *Curcuma longa* (Antony et al, 1999) and *Withania somenifera* (Davis et al, 2000) show very potent immunomodulatory activity.

Invasive cancer cells degrade natural tumor barriers ie, the basement membrane and connective tissues (Pater and Clerck, 1982). Specific proteolytic enzymes produced by tumor cells, stromal cells and infiltrating leukocytes mediate this process. Urokinase plasminogen activator, heparanases, cathepsin-B are some of the proteolytic enzymes which degrade the extra cellular matrix. Matrix degrading proteases are of several types, of which matrix

metalloproteinases (MMPs) play a major role in basement membrane degradation (Sun et al, 1997). Among the MMPs reported previously gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are key enzymes for degrading type-IV collagen, which is a major component of basement membrane (Wei et al, 2005).

There are classes of molecules which inhibit the activity of MMPs known as Tissue inhibitors of Metalloprotease (TIMPs). Tissue inhibitors of metalloproteinases (TIMPs) are known as the physiologic inhibitors of the MMPs (Madlener et al, 1998). TIMPS are secreted proteins that complex with individual MMPs and inhibit the activity of MMPs. Together, the MMPs and TIMPs form a complex biological system strictly controlling degradation of extracellular matrix.

Survival of the tumor cells and successful formation of a secondary tumor require the manipulation of environment in and around the tumor cells (Jasti et al, 2005). To grow and metastasize tumors must stimulate the development of new vasculature through a process known as angiogenesis. Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide with biologic effects that include regulation of hematopoetic stem cell development, extracellular matrix remodeling and inflammatory cytokine regeneration. Its expression can up regulate several proangiogenic and prometastatic molecules.

Several substances such as proinflammatory cytokines have been identified to act as prometastatic, proangiogenic and antiapoptotic agents of tumor cells. Pleiotropic cytokines such as IL-Iβ, IL-6, GM-CSF, TNF-α and IL-8 can act as autocrine growth factors for tumor cells. Expression level of these proinflammatory cytokines directly correlates with the metastatic potential of

several human carcinomas (Huang et al, 2000).

Variety of genes such as IL-Iβ, IL-6, IL-8, MMP-2, MMP-9, COX-2 and TNF-α involved in cancer are regulated by transcription factors such as nuclear factor-κB (NF-κB) and activated protein (AP-1) (Huang et al, 1998). These transcription factors play an important role in the survival, proliferation, cell cycle regulation and tumor promotion. Nuclear factor-κB represents a family of transcription factors that participates in the regulation of diverse biological processes including immune and inflammatory responses, cell growth and apoptosis

One of the most extensively studied intracellular signaling cascades involved in proinflammatory responses is the mitogen-activated protein (MAP) kinase pathway. All these proinflammatory, prooxidant and transcriptional factor activation are closely linked to tumor promotion linked with the free radicals and oxidative molecules generated in the signaling cascade.

A wide variety of phenolic substances derived from edible plants have been reported to retain marked anti-oxidant and anti-inflammatory activities which contribute to their chemopreventive potential (Ruby et al, 1995). We are trying to find out a few anticancer agents not only cytotoxic to towards the malignant cells, but also inhibit the metastasis and angiogenesis through the control of proinflammatory or prometastatic or proangiogenic factors mediated by the regulation of transcription factors.

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Chapter 1

Review of Literature

Cancer is a disease of misguided cells, which have high potential of excessive proliferation without apparent relation to the physiological demand of the organ involved (Hanahan and Weinberg, 2000). Cancer is a multi-factorial multi-staged and multi-mechanistic complex process. It involves the interaction of the environmental and host factors in the inception, progression of manifestation (Seth and Ana, 2001). Inherited genetic dispositions contribute significantly to 5-10 percent of breast cancer and 5-13 percent of colon cancer incidences (Emory et al, 2001). In the industrialized nations, roughly 7 percent of cancer deaths are attributable to viral infections; 4 percent to occupational hazards; 2 percent to sunlight; 2 percent to pollutions of air, water, and soil; and less than 1 percent to food additives and industrial products (Balmain et al, 2003).

Etiology of cancer

Many chemical and physical carcinogens can induce one or more of a variety of mutations in cells when given chronically. A good number of cancer causing chemicals are man-made and used either as industrial agents, pesticides, pharmaceutical chemicals or as food additives. Carcinogens are extremely diverse structures and include both natural and synthetic products. Some are direct acting carcinogens (require no chemical transformations to induce carcinogenisity) eg, alkylating agents.The indirect acting carcinogens are referred as procarcinogens, eg, Benzo(a)anthracene, Benzo(a)pyrene etc. All chemical carcinogens are highly reacting electrophiles that react with the electron rich atoms like RNA, DNA and protein.

Metals are known to be carcinogenic to humans. Some of the important

metals such as arsenic and arsenic compounds, chromium, nickel, cadmium and beryllium can induce the development of lung cancer and prostate cancer (Sabine et al, 1987). Physical carcinogens such as x-ray, γ-ray and UV ray may cause the formation of pyrimidine dimmers, apurinic sites with consequent break in DNA and formation of free radicals, which cause break, leading to somatic mutations. A large number of DNA and RNA viruses have proved to be oncogenic in animals, while only a few viruses have been linked with human cancer (Darcel, 1994).

The most life-threatening aspects of the oncogenic process is metastasis. Even though the clinical significance of such expression of the malignant phenotype has been well appreciated, advances in understanding the molecular mechanisms involved in metastasis have lagged behind other developments in the cancer field.

Metastasis

The most deviating aspect of cancer is the probability of malignant cells to spread from primary site to distant target organs. Metastatic dissemination of a neoplasia to secondary sites is the primary cause of death among cancer patients (Fiddler, 1997). The appearance of metastases in latter stages of neoplastic disease results in unfavorable clinical prognostics; metastatic cells become resistant to the majority of known drugs and treatment strategies (Condeelis et al, 2000; Engers et al, 2000). Formation of metastases is a multi-step event that may be arbitrarily divided into four stages.

a) Migratory and adhesive properties of tumor cells

Neoplastic cells that are capable of migration appear at the primary site (primary colony) (Malcolm et al, 2004). These cells exhibit altered cell-to-cell interactions (they lack homotype relations Via E–cadherins) as well as altered interactions with the extracellular matrix (ECM) (Engers et al, 2000). An important role in this altered relation between neoplastic and normal cells as well as the extracellular matrix is ascribed to adhesion molecules such as integrins, selectins, ICAM-1, VCAM-1, NCAM, mucins, glycosphingolipids, CD 44 molecules, Lu- ECAM-1 and others (Peter et al, 2005). The migrating cells are also able to secrete and/or activate proteolytic enzymes (matrix metalloproteases, MMP) involved in cell locomotion across the ECM (Engers et al, 2000).

b) Intravasation of tumor cells

Migrating metastatic cells penetrate into the lumen of blood vessels or, rarely, lymphatic vessels (intravasation). The cells are passively carried with the bloodstream and become arrested in micro vessels of diameter smaller than their own (Chambers et al, 2000). The arrested cells would frequently become deformed. They might attach to blood vessel walls, most likely by adhesive interactions. Alternatively, single metastatic cells could be arrested in vessels with a diameter larger than their own (Stanis et al, 2002). In that case interactions of adhesive nature would definitely be responsible for the cells sticking on to the blood vessel walls.

c) Extravasation of tumor cells

During extravasation cancer cells leave the blood or lymph vessels. Their penetration through the vessel wall doses not cause mechanical damage to the latter, 24hours from the arrest of metastatic cells in microcirculation 80% of them undergo extravasation (Chambers et al, 2000). Metastatic cells adhering to the walls pulmonary vessels do not actually extravasate but begin to proliferate and form micro colonies entirely within the blood vessels, and the vessel wall is destroyed after sometime resulting is the colony growth outside of it (metastasis) (Stanis et al, 2002).

d) Formation of metastases.

The last stage of the metastatic process involves formation of secondary colonies called metastases. Importance of this stage of metastasis may be the chemotactic interactions. Due to the presence of specific receptors cancer cells are belived to migrate towards the source of specific chemokines. These effector molecules are belived to be responsible for the observed preferences in metastatic spread to specific organs; for eg, melanoma metastasizes foremost into lungs while breast cancer into bones, etc. (Muller et al, 2001).

Matrix metalloproteinases (MMPs) in tumor progression

In considering the steps in the process of tumor growth and metastasis tumor cell population must not only be able to invade into surrounding tissues as the tumor mass expands in size, but must also be able to cross two, and in most cases three, basement membranes to successfully complete its journey to a secondary site. The main component of basement membrane is type 1V collagen

with additional components of laminin, entactin and fibronectin. Proteiases of the aspartic, cysteine and serine classes as well as matrix degrading metalloproteinases are candidate proteolytic facilitators of tumor cell invasion and metastasis. Several agents have been developed that block the synthesis of MMPs, prevent them from interacting with the molecules that direct their activities to the cell surface or inhibit their enzymatic activity.

MMPs can be divided into four categories based on substrate preference: collagenases, gelatinases, stromelysins and membrane–associated MMPs. Gelatinase A (MMP-2) and gelatinase–B (MMP-9) are key enzymes for degrading type 1V collagen, which is a major component of the basement membrane (Gijbels et al, 2004). Expression levels of MMP–2 and MMP-9 are associated with tumor metastasis for various human cancers (Hiro-omi et al, 2000). Metastatic tumor expresses high levels of type 1V collagenase activity than the non–metastatic tumors (Stefan et al, 1993). MMPs are also largely involved in the process of neoangiogenesis. During angiogenesis, endothelial cells also produce MMP-1, MMP-2, MMP-3 and membrane type–MMP (MT-MMP) (Kleiner, 1999). The role of tumor associated MMPs seems to be in almost all facets of tumor progression; tumor growth, invasion and metastasis. The matrix metalloproteinases, as the name implies, depend upon a metal ion (Zn+) for activity and have several interesting features that implicate them in the process of metastasis. Common salient features of MMP include a conserved metal binding site in the catalytic domain consisting of the amino acid sequence HEXGHXXGXXHS and a conserved sequence in the pro–region comprised of

the amino acids PRCGVPDV. The latter sequence, responsible for the latency of the enzyme, introduces an additional level of proteolytic activation. The activity of these molecules is thought to be controlled by a mechanism know as cysteine swich (Fu et al, 2001) in which a conserved unpaired cysteine residue in the pro–domain forms a coordinate bond with the zinc ion in the active site, an association which effectively 'close the door' of the enzyme and renders it inactive. MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage (Nagase et al, 1999; Brew et al, 2000). Therefore, the regulation of MMPs occurs at three levels: gene expression, proenzyme processing, and inhibition of enzymatic activity. There are classes of molecules present endogenously known as tissue inhibitors of metalloproteinases which could inhibit the activity of MMPs (TIMPs) (Rainer et al, 2001).

Tissue inhibitors of metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) are the main physiologic inhibitors of the MMPs (Jiang et al, 2002; Baker et al, 2002). TIMPS are secreted proteins that complex with individual MMPs and regulate the activity of individual MMPs. Together, the MMPs and TIMPs form a complex biological system strictly controlling degradation of extracellular matrix. The MMPs and TIMPs have a significant role in facilitating tumor invasion and metastasis, not only through their direct role in degrading extracellular matrix but also by interaction with other biological systems implicated in tumor invasion, including cell adhesion molecules, cytoskeletal proteins and growth factors (Leeman et al, 2003; Egeblad et al, 2002).

TIMP-1

TIMP-1 mRNA expression is up-regulated in many human cancer types and in some cases correlates with more severe clinical outcome eg, colorectal carcinoma, non-small cell lung carcinoma and breast carcinoma (Sternlicht et al, 1999). Studies in experimental mouse models have revealed paradoxically that TIMP-1 can exhibit proneoplastic and antineoplastic effects during primary and metastatic tumor development (Noritake et al, 1999).

TIMP-2

TIMP-2 is a multifunctional inhibitor of angiogenesis, tumor growth and tumor invasion (Hoegy et al, 2001). These processes involve not only tumor cells themselves but also the modulation of complex tumor–host interactions. Because the host response to the tumor microenvironment can act either to facilitate or to inhibit tumor invasion and spread, manipulating these host response elements has become a major focus of novel anticancer strategies (Feldman et al, 2003; Liotta et al, 2001). Although TIMP-2 can block the action of MMPs (Hoegy et al, 2001), it may also rely on MMP-independent mechanisms that modulate tumor–host interactions (Seo et al, 2003). TIMP-2 has a direct role in regulating tyrosine kinase-type growth factor receptor activation.

Angiogenesis

Angiogenesis, the formation of new capillaries, is among the key events in various destructive pathologic processes, such as tumor growth, metastasis, arthritis etc as well as in physiologic processes, like organ growth and development, wound healing and reproduction (Folkman, 1995).Blood vessels

constitute the first organ in the embryo and form the largest network in our body but sadly are also often deadly. When disregulated, the formation of new blood vessels contributes to numerous malignant, ischemic, inflammatory, infectious and immune disorders. Molecular insights into these processes are being generated at rapidly increasing pace, offering new therapeutic opportunities that are currently being evaluated.

Angiogenesis in tumor growth and metastasis.

Angiogenesis is required for invasive tumor growth and metastasis and constitutes an important point in the control of cancer progression. For tumors to develop in size and reach metastatic potential they must make an angiogenic switch through perturbing the local balance of proangiogenic and antiangiogenic factors.

Tumors that have become neovascularized often express increased levels of proangiogenic proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The expression of proangiogenic proteins can be induced by several factors, including hypoxia, activation of oncogenes or inactivation of tumor suppressor genes (Laughner et al, 2001; Ravi et al, 2000; Semenza, 2000). In some tumors, the angiogenic switch is the result of down regulation of antiangiogenic factors (Arbiser et al, 1997). In most adult tissues, the balance between proangiogenic and anti angiogenic signaling favors vasculature. In some cases, however, proangiogenic activities prevail, resulting in the tumor vascularization and metastatic growth.

Two general approaches have been used in the development of

antiangiogenic agents: inhibition of proangiogenic factor and therapy with endogenous inhibitors of angiogenesis (eg: angiostatin, endostatin).

Vascular endothelial growth factor

Solid tumors are multicompartmentalized structures, consisting of three major compartments: cancer and stromal cells, the extracellular matrix (ECM), and the vasculature (Jain et al; 2002). The volumes of each of these components vary depending on the origin and size of the tumor and the organ in which primary tumor develops (Jain et al; 2002). Tumors require vasculature to gain access to oxygen and other nutrients, allowing growth and metastasis (Carmeliet et al, 2000). VEGF (vascular endothelial growth factor) has been shown to be one of the most potent angiogenic factors produced by tumor cells (Ferrara et al, 1997 and, Nicosia; 1998). It binds to endothelial cell surface receptors and activates various functions of the cell, including angiogenesis (Gera et al, 1999; Veikkoln et al, 2000). VEGF, also known as vascular permeability factor (VPF or VEGF-A) is the critical and central regulator of angiogenesis. The other members of the VEGF family, VEGF-B, VEGF-C, VEGF–D and PIGF also play a role in angiogenesis. It can up-regulate expression of adhesion molecules on vascular endothelium (Melder et al, 1996).

Inhibitors of angiogenesis

Purified angiostatin inhibited angiogenesis in both *in vitro* and *in vivo* assay systems and blocked growth of metastases. Sequence analysis of angiostatin revealed that it is a proteolytic fragment of plasminogen. The inhibitory activity of angiostatin is specific to this proteolytic fragment because the intact

plasminogen lacked this activity.

Another endogenous inhibitor of angiogenesis is endostatin, which is also a proteolytic fragment of another protein, collagen XVIII (Reilly et al, 1997). Endostatin was shown to be a more potent inhibitory factor than angiostatin. Systemic application of recombinant endostatin was capable of inhibiting angiogenesis as well as blocking growth of several primary tumors.

Role of hypoxia in angiogenesis

Beyond a certain size, simple diffusion of oxygen to metabolizing tissues becomes inadequate. Tumor development forms, the increasing metabolic demands of the growing mass of cells. Many tumors develop a severely hypoxic microenvironment (Christopher et al, 2003) and secrete angiogenesis-stimulating factors (Daniela et al; 2005) such as induce platelet-derived growth factor (Eunice et al, 2001) and VEGF (Shwciki et al, 1992). In tumors, VEGF expression is enhanced in zones surrounding necrotic foci, suggesting a mechanism by which a hypoxic microenvironment might stimulate tumor angiogenesis (Jin et al, 2006). By activation of the hypoxia-inducible factor (HIF) family of genes, which cod for heterodimeric basic helix-loop-helix proteins composed of ∝ and ß subunits. HIF-1∝ is manufactured in the cytoplasm of cells but is rapidly degraded under normoxia, however, the intracellular content of HIF-1∝ increases immediately after a decrease in oxygen tension. HIF-1∝ is a transcription factor that mediates hypoxic induced responses, including apoptosis and VEGF gene regulation (Carmeliet et al, 1998).

Hence; the oxygen availability is an important regulator of tumor angiogenesis.

Role of immune system in tumor development- immune surveillance

Host provides both humoral and cell mediated immune responses to tumor antigens and proven to be effective in the immune destruction of tumors. A number of tumors have been shown to induce tumor-specific cytotoxic-T lymphocytes (CTLs). The important effectors include natural killer cells, macrophages and tumors specific antibodies.

T-Lymphocytes

CTLs provide effective antitumor immunity in host. CTLs may perform a surveillance function by recognizing and killing potentially malignant cells that express peptides which are derived from mutant cellular or oncogenic viral proteins which are presented in association with class I MHC molecules.

Role of NK cells and macrophages

NK cells can be activated by direct recognition of tumor or as a consequence of cytokines produced by tumor-specific T lymphocytes. Recognition of tumor cells by NK cells is not MCH restricted. In some cases, Fc receptors on NK cells can bind to antibody-coated tumor cells leading to antibody dependent cellular cytotoxicity (ADCC). Numerous observations indicate that activated macrophages also play a significant role in the immune responses to tumors by releasing lysosomal enzymes, reactive oxygen metabolites or by producing TNF-α. Macrophages also express Fc receptors enabling them to mediate ADCC. Activated macrophages secrete TNF-α that has potent antitumor activity.

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Antitumor antibodies

Antibodies are probably less important than T cells in mediating effective antitumor immune responses. But antitumor antibodies of the appropriate subclass are effective in suppressing small number of tumor cells. Binding of antibody to the tumor target cell does not by itself result in suppression or destruction. It serves as a recognition signal for cytotoxic effectors such as complement, macrophages, or K cells to perform the cytotoxic event.

Complement

The complement system comprises a group of more than 30 serum and cell surface proteins most of which are beta globulins with protease activity. The complement system plays a significant role in humoral immune responses. The binding of complement components to the appropriate immunoglobulin sub class initiates a cascade of complement activation and macro nuclear aggregation that results in the release of anaphylatoxins, which cause neutrophil chemotaxis, neutrophil activation, increased vascular permeability. Assembly of the membrane attack complex, which inserts in the lipid bilayer of the target cell membrane forming a 'doughnut' and thus providing for the free exchange of water and electrolytes and consequent osmotic lysis of the cell.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

In ADCC, the target tumor cells, which are coated with 1gG antibodies, are selectively lysed by killer cells, a special type of lymphomonocytic cell (Schulz et al, 1983). Several different leukocyte populations like neutrophils, eosinophils, mononuclear phagocytes and NK cells are capable of lysing the

target cells. Recognition of bound antibody occurs through a low affinity receptor for Fcγ on the leukocyte, called FcγRIII or CD16. The antibody molecule provides the specific recognition signal while the otherwise quiescent and non-specific effector cells are directed to the target cells to provide the cytotoxic event.

Tumor escape mechanism

Malignant tumors may express protein antigens, which are recognized as foreign by the tumor host, and although immunosurveillance may limit the outgrowth of some tumors, it is clear that the immune system often does not prevent the occurrence of human lethal cancers. It may be due to the rapid growth and spread of a tumor overwhelms the effector mechanism of the immune responses. The inability of the host to develop an effective immune response has also been shown in several classes (Dean et al, 2001). The process of tumor escape may be a result of several mechanisms as given below.

a). Class I MHC expression can be down regulated on tumor cells, which is required for CTL recognition.

b). Tumor products may suppress antitumor immune responses (eg, TGF-β).

c). Loss of surface expression of tumor antigens.

d). Tumor surface antigens can be hidden from the immune system.

Cytokines

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They are small, structural proteins

with molecular weights ranging from 8 KD to 40 KD. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokines are endogenous immunostimulatory proteins (Guillot et al, 2002). Cytokines play an important role in tumor metastasis. Some of the cytokines may inhibit tumor growth by interfering with host tumor relationship for example by inhibiting tumor angiogenesis and modulation of extra cellular matrix.

Interleukin-1 (IL-1)

IL-1 is one of the most important immune response-modifying interleukin. The predominant function of IL-1 is to enhance the activation of T-cells in response to antigen. Activation of T-cells, by IL-1, leads to increased T-cell production of IL-2 and of the IL-2 receptor, which in turn augments the activation of the T-cells in an autocrine loop. This effect of T-cell activation by IL-1 is mimicked by TNF-α which is another cytokine secreted by activated macrophages. The most salient and relevant properties of IL-1 in inflammation are the initiation of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). Production of IL-1 by different cell types occurs only in response to cellular stimulation. IL-1 is also an angiogenic factor and plays a role in tumor metastasis and blood vessel supply (Kaoru et al, 2006). IL-1 also stimulates the production of other proinflammatory cytokines like IL-6. In addition to its effects on T-cells, IL-1 can induce proliferation in non-lymphoid

cells.

Interleukin-2 (IL-2)

IL-2, produced and secreted by activated T-cells, is the major interleukin responsible for clonal T-cell proliferation. IL-2 also exerts effects on B-cells, macrophages, and natural killer (NK) cells. The production of IL-2 occurs primarily by CD4+ T-helper cells. Indeed, the IL-2 receptor is not expressed on the surface of resting T-cells and is present only transiently on the surface of T-cells. In contrast to T-helper cells, NK cells constitutively express IL-2 receptors and will secrete TNF-α, IFN-γ and GM-CSF in response to IL-2, which in turn activate macrophages. IL-2 has been used clinically in several ways.

Interleukin-6 (IL-6)

IL-6 is produced by macrophages, fibroblasts, endothelial cells and activated T-helper cells. It is a key inflammatory mediator produced by many cell types. In particular, IL-6 is the primary inducer of the acute-phase response in liver. IL-6 also enhances the differentiation of B-cells and their consequent production of immunoglobulin. Unlike IL-1, IL-2 and TNF-α, IL-6 does not induce cytokine expression; its main effects, therefore, are to augment the responses of immune cells to other cytokines. In humans, IL-6 is a growth factor for myelomas, (Elizabeth et al, 2000) suggesting further applications of IL-6 blockers.

Tumor Necrosis Factor (TNF)

TNF-α was originally identified as a cytokine responsible for endotoxin

induced necrosis (Bazzoni and Beutler, 1995). Several independent groups reported that therapy with recombinant TNF-∝ was effective against several types of murine models of hepatic and pulmonary metastasis (Jan et al, 2001). TNF-∝ and TNF-ß have been shown to exhibit direct antitumor activity, killing some tumor cells and reducing the rate of proliferation of others while sparing normal cells. In the presence of TNF-∝ or TNF-.ß, a tumor undergoes visible hemorrhagic necrosis and tumor regression. TNF-∝ has also been shown to inhibit tumor-induced vascularization (angiogenesis) by damaging the vascular endothelial cells in the vicinity of a tumor, thereby decreasing the flow of blood and oxygen that is necessary for progressive tumor growth (Anita et al., 2004). TNF has potent antitumor activity against large tumor burdens in some murine models. (Flavio et al, 2000). However, Humans can only tolerate 2% of the systemic TNF dose (by weight) required in mice, due to dose limiting hypotension (Dong et al, 2001). High doses of TNF, administered locally via direct tumor injection (Kramer et al, 2001) or isolated limb perfusion can result in dramatic tumor regression in some cancer patients.

Interferon (INF-γ)

Interferons are a family of proteins that are produced by the T-cells in response to viral infections or stimulations with double stranded RNA, antigens, or mitogens (Maryam et al, 2000). IFN- γ is secreted primarily by CD8+ T-cells. Nearly all cells express receptors for IFN-γ and respond to IFN- γ binding by increasing the surface expression of class I MHC proteins, thereby promoting the presentation of antigen to T-helper (CD4+) cells. Interferons have a variety of

biologic properties which include immunomodulatory activities, antiviral activities, the ability to interfere with cell proliferation, inhibition of angiogenesis, regulation of differentiation, and enhancement of the expression of a variety of cell-surface antigens (Kenji et al, 2001).Interferons have antitumor activity against a variety of tumor types, including hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell lymphoma, and Kaposi's Sarcoma (Jiang et al, 1983).

GM-CSF

Colony stimulating growth factors (CSFs) are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults. GM-CSF is a pleotropic cytokine produced by a number of different cell types. GM-CSF is a growth factor for erythroid, megakaryocyte and eosinophil progenitors. IL-3 (secreted primarily from T-cells) is also known as multi-CSF, since it stimulates stem cells to produce all forms of hematopoietic cells. GM-CSF stimulates macrophages for antimicrobial and antitumor effects. GM-CSF is the pivotal mediator of the maturation and function of dendritic cells, the most important cell type for the induction of primary T-cell immune responses. GM-CSF may enhance Ab-dependent cellular cytotoxicity and the generation and cytotoxicity of NKcells. (Jay et al, 2000). GM-CSF is a macrophage activating factor and promotes the differentiation of Langerhans cells into dendritic cells. Recombinant GM-CSF and G-CSF are increasingly used to speed bone marrow recovery after cancer chemotherapy.

Nuclear factor- κB (N F-κB)

Nuclear factor κB (NF-κB) designates a group of transcription factors defined by their ability to bind a specific DNA sequence first identified in the enhancer of immunoglobulin κ light chain gene. NF-κB factors are dimers of Rel family of proteins. There are five members of the NF-κB family of transcription factors: Rel (c- Rel), Rel A (p65), Rel B, NF-κB 1 (p105/p50) and NF-κB 2 (p100/p52). Together, these proteins regulate the expression of genes encoding cytokines, chemokines, adhesion molecules and antimicrobial peptides, thereby orchestrating both innate and adaptive immune responses (Gosh et al, 2002). NF-κB /Rel proteins exist as homo or hetero dimers and possess a conserved N-terminal Rel homology domain (RHD) that mediates dimerization as well as DNA binding.

In most cell types, inactive NF-κB complexes are sequestered in the cytoplasm via their interaction with inhibitory proteins known as Inhibitory kappa B (IκBs). In response to multiple stimuli, including cytokines, viral and bacterial pathogens and stress-inducing agents the latent cytoplasmic NF-κB/IκBa complex is activated by phosphorylation on conserved series residues at the N-terminal portion of IκB; this modification occurs at Ser 32 and Ser 36 in the case of IκBa (Karin et al, 2002; Kumar et al, 2003). Phosphorylation targets IκBa for ubiquitination by the SCF- ubiquitin ligase complex, which leads to degradation of the inhibitory subunit by the 26S proteosome (Karin et al, 2002, Wilkinson, 2003). This process activates NF-κB, which then translocates to the nucleus and binds to its cognate DNA-binding site (5'-GGGRNNYYCC-3') in the promoter or

enhancer regions of specific genes.

The ability of NF-ĸB to suppress apoptosis and to induce expression of proto–oncogenes such as C-myc and cyclin D1, which directly stimulate proliferation, suggest that NF-ĸB may stimulate in many aspects of oncogenesis (Pahl, 1999; Guttridge et al; 1999) NF–ĸB also regulates the expression of various molecules such as cell adhesion proteins, matrix metallo-p oteinases, cycloxygenase-2 (cox-2), iNos, chemokines , and inflammatory cytokines, all of which promote tumor cell invasion and angiogenesis (Bharti et al, 2004). Inhibition of NF-ĸB abrogates tumor cell proliferation (Younes et al, 2003; Bharti et al, 2003; Mukhopadhyay et al, 2001).

Although it is widely accepted that inhibition of NF-ĸB triggers apoptosis in many tumor cell types (Yamamoto et al, 2001), there are a few exception in which NF-ĸB activation blocks malignant growth. NF-ĸB and oncogenic Ras both induce cell-cycle arrest in normal human epidermal cells. The cell cycle arrest in normal human epidermal cells. The cell cycle arrest by oncogenic Ras can be bypassed by inhibition of NF-ĸB through the over expression of IĸB a protein, which results in malignant epidermal tissues resembling squamous cell carcinoma (Van et al, 1999; Seitz et al, 1998). These findings thus suggest that NF-ĸB can play a different role in the regulation of cell growth in tissue–context-dependent manner.

Apoptosis

Cell death is a physiological process which is required for normal development and existence of multicellular organisms. In most cases,

physiological cell death occurs by apoptosis as opposed to necrosis. Abnormalities in this process are implicated as cause or contributing factor in a variety of diseases. Inhibition of apoptosis can promote neoplastic transformation, particularly in combination with disregulated cell cycle control, and can influence the response to tumor cells to anti-cancer therapy. A family of intracellular proteases, the caspases, is responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis. Diverse regulators of the caspases, including activators and inhibitors of cell death proteases are also discovered. It is an essential process in controlling tissue homeostasis in multicellular organisms. Apoptosis is sometimes referred to as programmed cell death (PCD) because it is an integral part of the developmental program and is frequently the end result of temporal course of cellular events.

Apoptosis can be induced by a variety of stimuli such as ionizing radiations, gluco-corticoids chemotherapeutic agents, lymphokines deprivation and various oxidants (Rajeev Goel 1998). Although the stimuli which induce apoptosis vary markedly, the morphological features of the process are however conserved in different cell types. It includes chromatin condensation, nuclear fragmentation, Plasma membrane blebbing, cell shrinkage and formation of apoptotic bodies.

Caspases

A family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases (Cysteine Aspartyl-specific proteases) (Alnemri et al, 1996). These proteases are present as inactive zymogens in

essentially all animal cells. In humans and mice, approximately 14 caspases have been identified. They can be sub grouped according to either their amino acid sequence similarities or their protease specificities. Though most caspases are directly involved in cell death, a few are not, atleast in mammals and higher eukaryotes. A subgroup of caspases, including caspase 1, 4 and 5 in humans, is involved in processing of proinflammatory cytokines such as pro-interleukin-1ß (pro-IL-1ß), pro-IL-18. Many pathways for activating caspases are exist, but only two have been elucidated in detail. One of these centers on tumor necrosis factor (TNF) family receptors, which use caspase activation as a signaling mechanism, thus connecting ligand binding at the cell surface to apoptosis induction (Ferry et al, 2005). The other involves the participation of mitochondria, which release caspase activating proteins into the cytosol, thereby triggering apoptosis (Tomomi et al, 1998). The death receptor and mitochondrial pathways for caspase activation are sometimes referred to as the extrinsic and intrinsic apoptosis pathway respectively. Caspase-8 represents the apical caspase in the TNF family death receptor pathway, whereas caspase-9 serves as the apical caspase of the mitochondrial pathway (Alakananda and Ayako, 2002). In the case of intrinsic pathway, release of cytochrome c from mitochondria triggers caspase activation by binding to the caspase-activating protein Apaf- 1 (Newmeyer et al, 2000). The Apaf- 1 protein normally resides is an inactive conformation in the cytosol, but on binding cytochrome c, an ATP/dATP–binding oligomerization domain within this protein mediates Apaf-1 aggregation (Zhou et al, 1998). The oligomerized complex then binds Pro-caspase-9, and facilitates trans-processing of caspase-9

zymogens via the induced proximity mechanism (Li et al, 1998).

Bcl-2

Apoptosis is an evolutionarily conserved cell suicide process executed by cysteine proteases (caspases) and regulated by the opposing factions of the Bcl- 2 protein family (Suzanne et al, 2003). They are a family of homologous proteins, where some members are proapoptotic and some are antiapoptotic. In humans, 20 members of the Bcl- 2 family genes are actively participating in apoptosis. These genes encode the anti-apoptotic proteins, Bcl-2, Bcl-XL, Mcl-1, Bfl-1(A1), Bcl-W and Boo (Diva) as well as the pro–apoptotic proteins Bax, Bak, Bok (Mtd), Bad, Bid, Bim, Bik, Hrk etc. Some of these proteins may display anti–apoptotic activity in some cellular backgrounds and have pro-apoptotic functions in other cellular contexts (eg, Boo/Diva, Bcl-2, Bax) (Chen et al, 1996). Many Bcl-2 family proteins are constitutively localized to the membranes of mitochondria, whereas others are induced to target these organelles in response to specific stimuli. Caspase-8 mediated activation of Bid represents an important mechanism accounting for cross-talk between the death receptor (extrinsic) and mitochondrial (intrinsic) pathway (Yin et al, 1999). When the Bcl-2 family proteins reach the mitochondria, they regulate the release of cytochrome c from mitochondria, with pro-apoptotic Bcl-2 family proteins inducing or making it easier to induce release of this caspase activating protein and antiapoptotic members of the family suppressing cytochrome c release.

Bcl-2 family proteins have been reported to control the release of other proteins from mitochondria. The proteins include [i] certain caspases (caspase- 2,

3 and 9) which reportedly are sequestered inside mitochondria in some types of cells (Susin et al, 1999) [ii] apoptosis inducing factor (AIF), a flavoprotein implicated in nuclear manifestations of apoptosis via caspase–independent mechanisms (Susin et al, 1999) and [iii] Smac/Diablo, the inhibitor of IAP family proteins (Du et al, 2000; Verhagen et al, 2000). All of these proteins are encoded within the nuclear genome, transported into mitochondria, and stored in the space between the inner and outer membranes and awaiting to release into the cytosol upon breakdown of the outer membrane.

MAP Kinase

The mitogen-activated protein kinase (MAPK) Pathway is one of the primordial signaling systems that nature has used in several permutations to accomplish an amazing variety of tasks. It exists in all eukaryotes, and controls such fundamental cellular processes as proliferation, differentiation, survival and apoptosis. The basic arrangement includes a G-protein working upstream of a core module consisting of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK (Robinson et al, 1997; Schaeffer et al, 1999; Dhanasekaran et al, 1998). Two components of this pathway, Ras and Raf, are proto–oncogenes. The major function of this pathway pertains to growth control in all its facets, including cell proliferation, transformation, differentiation and apoptosis.

A wide variety of hormones, growth factors and differentiation factors as well as tumor promoting substances, employ this pathway. Most of these stimuli activate Ras proteins by inducing the exchange of GDP with GTP, which converts

RAS into its active conformation. The Ras exchange factor, sos (son of sevenless), is towed to the membrane by the growth–factor–receptor–bound protein 2 adapter protein. Activated Ras functions as an adapter that binds to Raf kinases with high affinity and causes their translocation to the cell membrane, where Raf activation takes place. The Raf family of serine/threonine. Specific kinases comprise three members in higher vertebrates, A-Raf, B-Raf and C-Raf or Raf-1, which play a vital role in regulating cell growth, differentiation and apoptosis. They lie at the apex of a highly conserved protein kinas e module which relays extracellular signals to the nucleus (Pearson et al, 2001; Kolch, 2000). In this modules Raf kinases phosphorylates and activates MEK-1/2 which in turn phosphorylates and activates ERK-1/2. Activated ERK-1/2 can then translocate to the nucleus and activate transcription factors by phosphorylation, thus altering the expression of specific genes.

In addition, ERK-1/2 has a number of cytosolic substrates which influence gene expression directly or indirectly. Active ERK may also allow the tumor to develop its own angiogenic support system by inducing the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Eliceiri et al, 1998). Activation of ERK in tumors may allow evasion of apoptosis by inducing cell survival. ERKs are known to play a role in cell survival in many cell systems. In fibroblasts, ERK activation by Raf leads to a selective reduction in expression of the Bim pro-apoptotic member of the Bcl-2 family and B-Raf over expression in fibroblasts has also been shown provide a protection against apoptosis by inactivating caspases after cytochrome C release (Erhardt et al,

1999).

Conventional cancer therapies.

Designing a proper treatment plan for a patient with malignant disease depends upon determining extend of disease spread, together with a knowledge of the natural history and the available therapeutic alternatives or the particular type of cancer. Accurate diagnosis is a critical step in planning appropriate cancer therapy. The application of current treatment techniques (surgery radiation therapy chemotherapy and biological therapy) results in the cure of > 50% of patients diagnosed with cancer

Surgical therapy

Surgery is an effective method to cure patients whose tumors are confined to particular anatomical sites. Surgery has been used as means of cytoreduction when complete excision has not been possible. However, unless such surgical debunking is combined with additional therapy, such as chemotherapy or radiation therapy, it will not much effective.

The selection of patients who will benefit from this approach requires considerable judgment and skill. Overly aggressive surgical intervention in palliative setting may lead to prolonged hospitalizations, unnecessary discomfort and additional financial burden to the patient or family.

Radiation therapy.

Radiation therapy is a local modality used in the treatment of cancer. This word depends to a large extent on the inherent radio sensitivity to the tumor and adjacent normal tissues. Ideally radiation therapy should destroy cancerous tissue

while causing minimal destruction to surrounding normal structures. Isotopes such as Cs^{313}, Ir^{192}, CO^{60} are used in radiation treatment.

Radiation therapy is dependent on the application of ionizing electromagnetic radiation to tumor site. The term x-ray denotes high-energy electromagnetic radiation produced by instruments such as linear acceleration. γ-rays are also electromagnetic radiation but are produced by radioactive isotope decay. Both are used in radiation therapy and there is no inherent difference in their physical characteristics or biologic effects. A given dose of radiation kills a constant percentage of cells. Ionizing radiation generates free radicals and reactive oxygen intermediates that damage cellular substituents including DNA.

Radiation therapy combined with chemotherapy has largely replaced surgery as a curative treatment for carcinoma of the anus. Radiation therapy is also used in the palliative management of many tumors.

Radiation therapy is associated with both acute toxicity and long-term sequel common manifestation include skin reaction with erythematic, desquamation, gastrointestinal toxicity, hair loss with nausea, vomiting, dysphagia or diarrhoea and myelosuppression with leukopenia, thrombocytopenia, and anemia. Rapidly proliferating normal tissues such as intestinal mucosa, bone marrow and skin are particularly susceptible to the radiation-induced cytotoxicity. Radiation therapy associated with an increased risk of developing solid tumors in previously irradiated fields. In view of extreme radio sensitivity of lymphocytes, the immune response is generally depressed following radiotherapy and patients are rendered more susceptible to infection (Yamagata and Green, 1976).

Chemotherapy

Cancer chemotherapy had its roots in the work of Paul Ehrlich, who coined the word chemotherapy. There are four ways chemotherapy is generally used as an induction treatment for advanced disease, as an adjunct to the local methods of treatments, as the primary treatment for patients who present with localized cancer and by direct installation into sanctuaries or by site directed perfusion of specific regions of the body most affected by the cancer.

One of the most important and still evolving roles for systemic chemotherapy is its use in the adjuvant setting. Its purpose is to eliminate undetectable micrometastatic disease. Chemotherapy whether given with curative or palliative intent, usually requires multiple cycles of treatment. Some of the commonly used important chemotherapeutic agents are of different types such as alkylating agents, antimetabolites, natural products, hormone antagonists, miscellaneous agents etc.

Every chemotherapeutic regimen administered in adequate doses will have some deleterious side effects on normal host tissues. Myelosuppression, nausea, vomiting, stomatis and alopecia are most frequently observed complications associated with chemotherapy (Rivera, 2003). Alkylating agents such as cyclophosphamide are potential carcinogens. Another major problem in chemotherapy is the tumor cell resistance to drugs or chemotherapeutic agents because the cancer cell presents a variable and moving target for anticancer drugs.

Biologic or immunotherapy

Biologic therapy is cancer treatment that produces antitumor effects

primarily through the action of natural host defense mechanism. Biologic therapy has emerged as an important fourth modality for the treatment of cancer (Mitchell, 1988). Most applications of biologic therapy for cancer have attempted to stimulate immune defense mechanisms. Many immuno therapies attempted to cause the tumor to appear more 'foreign' compared with normal tissues or tried to magnify relatively weak host immune reaction to growing tumors.

Strategies for the immunotherapy of cancer can be divided into active and passive approaches. Active immunothearpy refers to the immunization of the tumor-bearing host with materials designed to elicit an immune reaction capable of eliminating or retarding tumor growth. Active immunotherapy can be subdivided into nonspecific or specific immunization.

The advent of recombinant cytokines provided a more selective means for stimulating the immune system. Treatment with the interferones (Maier et al, 2003) or with IL-2 is a form of nonspecific active immunotherapy. Cytokines comprise the largest group of biologic therapeutics in clinical trails and include interferons, interleukins and hematopoietic growth factors. Lymphoid cells incubated with IL-2 develop a capacity to lyse fresh tumor cells (Wang et al, 2003).

The development of techniques for generating monoclonal antibodies has greatly improved the ability to obtain preparations with specific reactivity to human tumor-associated antigens. These antibodies are being employed alone or conjugated with toxins or radiolabel in cancer treatment.

Quite often the presence of the cancer itself will associate with

immunosuppression, also the treatment given for the cancer (radiotherapy and chemotherapy) is immunosuppressive. Infections are therefore not uncommon in patients with widespread cancer undergoing therapy. The organism responsible may cause mild symptom in normal individuals but can be severe or fatal in the immunodepressed patients.

Use of immunomodulators.

Immunomodulation is any procedure, which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reaction is named as immuno stimulation and primarily implies stimulation of non-specific system that is stimulation of the function and efficiency of granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may be because of environmental or chemotherapeutic factors. Apart from specific stimulative or suppressive activity certain agents have been shown to posses activity to normalize or modulate the pathophysiological processes in the underlying immune response and hence the terms Immunomodulation or immunomodulatory agents are now used.

Chemical agents as immunomodulators

Most of the chemical agents, which are fairly known to have effect on immune system, are immunosuppressants and cytotoxic agents (Nasrollah, 2004). The immunostimulants are mainly reported from the natural resources.

A wide variety of compounds are capable of potentiating immune

responses. Many of the drugs and hormones particularly the biogenic amines, cholinergic agents, prostaglandin appear to be linked to change in cyclin nucleotide metabolism in lymphocyte and thus it can enhance or suppress various aspects of immune response. Other nonspecific stimulants include BCG, *Corynbacterium parvum* and levamisol. Interleukins-1 is produced by macrophages and can stimulate the growth of thymocytes and T-cells. IL-2 is a protein produced by T-lymphocytes in response to antigenic or mitogenic stimulii. It is useful as a general restorative of immune competence in immuno suppressed individuals (Hamer et al 2004). Among the synthetic, semisynthetic or isolated pharmacological agents azothioprine and penicillamine have been extensively studied. Azathioprine inhibits DNA synthesis and has anti-inflammatory activity by virtue of it effects on PMN and monocyte production. Penicillamine is a currently accepted second line disease modifying agents used in advanced unresponsive rheumatoid arthritis. Despite the beneficial effects obtained with this drug, its usefulness is limited by its toxic side effects, which include rash, taste abnormalities and hematological toxicities (Israeli 1981). It can be assumed that the chemotherapeutic agents available today have mainly immunosuppressant activity. Most of them are cytotoxic and exert variety of side effects. Number of plant extracts have been shown to be immunomodulators and are briefly reviewed here.

Immunomodulators from natural products

A large number of chemotherapeutic agents used in cancer treatment have been discovered from natural products. Similarly, several laboratories through out

the world have directed considerable effort towards discovering new therapeutic agents from natural products. Because listing the entire spectrum of compounds that have been discovered by various groups could be overly cumbersome, we describe some important contributions but not in an exhaustive manner. Traditionally, plants are selected based on its known efficacy for treatment of inflammatory mediated diseases including cancer or related diseases. In our laboratory, interesting studies have been carried out using Iscador, which is an extract of the plant *Viscum album* and we have reported its antimetastatic activity (Antony and Kuttan, 1996), We also reported the antimetastatic as well as antitumor activity of naturally occurring sulfur compounds such as Diallyl sulfide, Diallyl disulfide, Allyl Methyl Sulfide (Manesh and Kuttan, 2003) and polyphenolic compounds such as Curcumin, Catechin and Piperine, an alkaloid (Menon et al, 1999; Pradeep and Kuttan, 2002). It has been reported that the anticarcinogenic activity of *Curcuma longa* and *Embilica officienalis* (Ruby et al 1995; Rajeshkumar et al 2003). It is interesting to note that many of the clinically used antineoplastic drugs such as camptothecin, taxol, vincristine, vinblastine are plant derived products and interestingly several clinical trials on the use of nutritional supplements and phytochemicals to prevent cancer are going on now.

Curcumin from turmeric, capsaicin from chilli-peppers, 6-gingerol from ginger, epigallocatechin-3 gallate from green tea, genistein from soyabean, lyopene from tomatoes, sulforaphane from broccoli, diallyl sulphide from garlic, resveratol from grapes, caffeic acid phenyl esters from honey, indol-3-carbinol from cabbage etc are well studied immunomodulative agents that target different

cellular signaling molecules which regulate proliferation and differentiation The past 10 years studies of signal transduction pathways which has resulted in the development of important new molecular therapeutics for cancer, particularly the use of many plant derived agents are seem to be relatively free of the undesirable toxicities of classical chemotherapeutic agents may have very well effect in the control of tumorigenesis.

Plants and their active principles Included in this Study

Piper longum

Piper longum is an important medicinal plant used in traditional medicine by many people in Asia and Pacific islands especially in Indian medicine (James, 1999). *Piper longum* is a component of medicines reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions (Singh et al, 1992). Other reported beneficial effects of *Piper longum* include analgesic and diuretic effects, relaxation of muscle tension and alleviation of anxiety (Singh and Blue Menthal, 1997). Piperine was the first amide isolated from piper species and was reported to display central nervous system depression, antipyretic and anti-inflammatory activity (Virinder et al, 1997).

Piperine

Piperine is the active phenolic component of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). Black pepper and long pepper is being used as a spice or component of indigenous system of medicines (Virinder et al, 1997).

Piperine is a potent inhibitor of mixed function oxygenase and it is a nonspecific inhibitor of p450 isoenzymes (Atal, 1985). Constituents of piper species have inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro* (Stohr et al, 2001). We have reported its antimetastatic activity and its inhibitory potential of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production. It has been reported that Piperine could inhibit the lung metastasis of B16F-10 melanoma cells by the in the inhibition of the activation of NF-κB and the proinflammatory cytokine gene expression. (Pradeep and Kuttan, 2004)

Thuja occidentalis L

Arbor vita (*Thuja occidentalis* L.) is a native European tree widely used in homeopathy and evidence-based phytotherapy. *Thuja occidentalis*, commonly known as Arbor vitae or white cedar, is indigenous to eastern North America and is grown in Europe as an ornamental tree. Today, it is mainly used in homeopathy as mother tincture or dilution (Deutscher et al 1985; Deutscher et al 2003). The fresh plant (related to the dry substance) contains essential oil, coumarins, water-soluble polysaccharides, flavanoids, and tannic agents (Harnischfeger and Stolze, 1983). A critical factor for *Thuja* use as a medicinal herb is its content of essential oil. The immunopharmacological potential of *Thuja* has been investigated in various *in vitro* and *in vivo* test models (Höld et al, 2000; Teuscher et al, 2004).

Thuja polysaccharide

The fresh plant (related to the dry substance) contains 4.9% water-soluble polysaccharides. High molecular weight glycoproteins/polysaccharides are highly relevant for the activity of the plant (Neth et al, 1995) *Thuja* polysaccharides

(TPS) inhibited human immunonodeficiency virus (HIV)-dependent cell death. The *Thuja* polysaccharides fraction caused an increase in the proliferation rates of spleen cells from mice. High molecular weight subfractions of TPS proved to be highly mitogenic on peripheral blood leukocytes. It was demonstrated that the mitogenic and cluster-forming activity of TPS causes T-cell induction particularly of the CD4-positive T-helper/inducer cells in connection with an increased production of interleukin-2 (IL-2). This indicates that not only proliferation but also a differentiation to fully functional T-helper cells takes place. Only T cells, not B cells, were stimulated by TPS. *Thuja occidentalis herba* led to an increased secretion of the cytokines IL-1, IL-6 and tumor necrosis factor-α (TNF-α) in the cell culture supernatant, with regard to TNF-α (Bodinet et al, 1999).Because of the proven immunomodulatory efficacy of the preparation, it was assumed that the positive effect of this extract exerted on infected mice is mediated first of all by its immunostimulating activity and that the direct antiviral activity is rather of secondary importance (Bodinet et al, 2002).*Thuja occidentalis* is widely used in homeopathy and evidencebased phytotherapy. Its immunopharmacological potential has been demonstrated in numerous *in vitro* and *in vivo* test models showing its immunostimulating and antiviral activities.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

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Chapter 2

Materials and methods

Materials

Plant materials

Authenticated *Piper longum* fruits and *Thuja occidentalis* were obtained from Amala Ayurveda Pharmacy, Thrissur, India. A voucher specimen of *Piper longum* (NO BSI. 64852) and *Thuja occidentalis* (CP01) is kept in the herbarium of Amala Ayurvedic Hospital, Thrissur, India. Piperine was obtained as a gift from Dr. Majeed, Sami Chemicals, Bangalore, India. A Partially purified polysaccharide fraction from the leaf and small twigs of *Thuja occidentalis* was prepared according to the protocol of Chintalwar et al (Chintalwar 1999).

Cells Used

L929 mouse lung fibroblast cells, B16F-10 metastatic mouse melanoma cells, K 562-leukemic cells and EL-4 thymoma cells were obtained from National Centre for Cell sciences, Pune, India. Dalton's Lymphoma Ascites (DLA) cells (arose as a spontaneous Carcinoma of thymus) and Ehrlisch ascites Tumor cells (EAC) were obtained from Adayar Cancer Institute, Chennai, India. Human Umbilical Vein Endothelial Cells were isolated from the umbilical cord of neonatal according to the protocol of Jaffe et al (1973).

Animals used

BALB/c mice, C57BL/6 mice, Swiss albino mice were purchased from National institute of Nutrition, Hyderabad, India. The animals were housed in well-ventilated cages in air-controlled rooms. They were fed with normal mouse chow (Sai Durga Feeds, Bangalore, India) and water ad libidum. All the animal experiments were carried out according to the rules of Animal Ethics Committee, Govt. of India.

Chemicals

Casein	Hi Media Laboratories, Mumbai, India.
Dulbecco's Modified Eagles Medium	
Fluid Thioglycollate Medium	
L-Glutamine	
Medium 199	
Minimum Essential Medium	
Rosewell Park Memorial Institute Medium	
Trypsin	
TBA (Thiobarbituric acid)	

Foetal Calf Serum : Biological Industries, Kibbutz, Israel.

Aprotinin	Sigma chemicals, St. Louis, USA
Benzamidine	
Bromophenol blue	
Collagen solution type I (from calf skin)	
Concanavalin – A	
Collagenase Type VIII (From Clostridium histolyticum)	
Collagen solution Type I (From calf skin)	

DEPC (Diethylpyrocarbonate)

DTT (Dithiotheritrol)

EGTA

Ethidium bromide

ECM-Gel

(from Engelbreth Holrn-Swarm mouse sarcoma)

Gelatin Type A (from Porcine skin)

Guanidinium thiocyanate

γ-GT substrate (r-glutamic acid γ-ρ nitroanilide)

L-γ Glutamyl p-nitroanilide

Glycyl glycine

4, 6 Glucuronic acid lactone

Hydroxyproline

Leupeptin

MTT (Methyl thiazol tetrazolium bromide)

2-mercaptoethanol

PMSF

Sodium acetate

Sodium citrate

Triton X-100

Sigma Chemicals, St. Louis, USA

Acetyl acetone	Sisco Research Laboratories, Mumbai, India
Acrylamide	
1, 4- Bis(phenyloxazol-2yl) Benzene (POPOP)	
2.5 Diphenyl oxazole (PPO)	
Folins reagent	
Glucosamine hydrochloride	
HEPES buffer	
N,N-Methylene Bisacrylamide	
Papain (Extracted from Papaya latex)	
Lipopolysacchride	: Difco Laboratories, USA
Polycarbonate filters:	: Nucleopone, Cambridge, USA.
^{3}H-Thymidine	BARC, Mumbai, India.
Sodium Chromate ($Na_2Cr^{51}O_4$) (Specific Activity > 50mci/mg)	
Diffquick stain set	: Dade behring, USA.
methyl cholanthrene	: Aldrich Chemicals, U.S.A)
Human recombinant FGF-bssic	Pepro Tech, USA.
Human recombinant VEGF	

Crystal Violet	Romali, Mumbai, India
Trypan blue	
DNase, RNase free water	Genei, Bangalore, India
DNTP mixture	
Taq DNA polymerase	

Reagent Kits

BD Mercury Transfactor Kit : BD Biosciences, USA

Cells to cDNA Kit : Ambion Inc, USA

Gelcode Blue Stain Reagent : Pierce Inc, USA

Mouse ELISA Kits of IL-1ß, IL-6, TNF-γ, GM-CSF : Endogen Inc, USA

Mouse ELISA Kits of VEGF and TIMP-1 : R&D Systems,USA

Mouse Pro-Inflammatory Cytokine Multiplex PCR Kit : Biosource Inc, USA

Mouse quantikine m-RNA kit for VEGF : do

Tissue Culture Wares

Medium filtering assembly : Millipore, USA

Polycarbonate membrane filter : Whatman, USA

(13 µm, 8 mm, PVP free)

Cellulose syringe filtering apparatus : Sartorius, Germany

Tissue culture flask (25 cm^2) — Tarson, India

Tissue Culture Petri dish (90mm). — Tarson, India

96 well flat bottom culture plate — Tarson, India

24-well tissue culture plate : Laxbro, India.

Blind well chamber (Modified boyden Chamber) : Nucleopore Cambridge USA

Instruments used

Automatic Gamma Counter : PerkinElmer, USA

CO_2 Incubator : Napco, Canada

Deep Freezer : Remi, Chennai, India

Disc electrophoresis unit : Balaji Scientific Service Chennai, India

Electronic Balance : Schimadzu, Japan

ELISA-Reader :AwarenessTechnology,Gujarat, India

Gel Documentation system : Vilber Lourmat, Torcy, France

High speed cooling centrifuge : Remi, Chennai, India

Inverted Microscope : Leica, Germany

Lyophilizer : Labconco Inc, USA

Minicycler – Thermocycler	: MJ Research, USA
Rack Beta Liquid Scintillation Counter	: Wallac, Finland
Spectrophotometer	: Elico, India
Spinwin Microcentrifuge	: Tarson, India
Submerged electrophoresis unit	: Bangalore, Genei, India
Tissue homogenizer	: York Scientific Industrues, Delhi
Transilluminator	: Vilber Lourmat, USA

Reagents

A. Phosphate buffered saline (PBS)

NaCl	-	8.00 g
KCl	-	0.20g
KH_2PO_4	-	0.20g
$Na_2 HPO_4. 2H_2O$	-	1.44g

Dissolved in distilled water and made up to 1000 ml pH was adjusted to 7.2 with 1N HCl. Sterilized by autoclaving at 15 lbs for 15 min.

B. Phosphate buffer (0.2 M)

Solution-A. Na_2HPO_4	-	0.2M (27.80g of Na_2HPO_4/litre)
Solution-B. NaH_2PO_4	-	0.2M (53.65g of NaH_2PO_4/litre)

Solutions A and B were mixed in a proportion to get the desired pH.

C. PBS – EDTA

EDTA - 20mg.

Dissolved in 100 ml PBS and sterilized by autoclaving.

D. Trypsin solution

Trypsin - 200 mg.

Dextrose - 20 mg

Dissolved in 100 ml ice-cold PBS-EDTA and sterilized by filtration through 0.2

μm membrane filter and stored at $^{-}20^{0}$C.

E. 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.

F. Scintillation fluid

PPO - 2.5 g.

POPOP - 0.25 g.

Naphthalene - 100g

The reagents were dissolved in a final volume of one litre. Kept in dark with out exposing to sun light at any stage.

G. Crystal Violet

Crystal violet - 50.0 mg.

20% methanol - 100 ml.

Solution filtered and used

H. Trypan blue

Trypan blue stain - 100 mg

Normal saline (0.85% NaCl) - 100 ml

Trypan blue was dissolved in saline by overnight stirring. Any suspended particles were removed by filtration.

I Eosine

Eosine - 500mg

Saline (0.9%) - 100ml (Final volume)

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

J. Harris haematoxylin

Haematoxylin - 5g

Ethyl alcohol - 50ml

Potassium alum - 50mg

Potassium iodide - 50mg

Distilled water - 950ml

Hematoxylin 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 hematoxylin was added to the alum solution. The mixture was cooled and potassium was added and filtered.

METHODS

Tissue Culture

Sterilization of Glass-wares

All glass wares 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 glass wares were rinsed in distilled water and dried in a hot air oven. These were then autoclaved at a pressure of 15 lbs for 15 minutes, dried and used for experiments.

Preparation of Culture Media and Sterility Checking

DMEM (9.98g/l), MEM (10.3g/l), RPMI (10.3g/l) and 199 (12.45g/l) medium were prepared in autoclaved double distilled water, pH adjusted to 7.2 using sodium bicarbonate, L-glutamine (2mM) was added as additional supply and filtered under negative pressure using a 0.22μm cellulose filter. Sterility of the medium was tested using fluid thioglycollate medium. One ml of the filtered medium was inoculated into 10 ml of sterile thioglycollate (29.96g/l) and incubated at 37°C for 7 days and checked for visible contamination. Broad spectrum antibiotics such as penicillin (100units/ml) streptomycin (100μg/ml) and foetal calf serum (10%) were added to the medium prior to use.

Maintenance of L929 cell lines in Tissue culture

Mouse lung fibroblast cell line was maintained in culture for long time to

start with the tissue culture technique. The spent medium was removed from the confluent bottles and the cells were washed thrice with PBS EDTA. Trypsin solution (0.lml) containing 0.02% EDTA was added and incubated for 3-4 minutes at 37°C and the bottles were tapped to dislodge the cells. MEM (5ml) containing 10% goat serum and antibiotics was added. Cells were dispersed into single cells 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°C. The spent medium was changed every 3rd day and was sub cultured every week.

Maintenance of B16F-10 cell lines in tissue Culture

The Metastatic B16F-10 cell line was also maintained in culture in similar manner. The spent medium from confluent bottles was removed and washed three times with PBS. Trypsin solution free of EDTA was used to dislodge the cells. 10ml DMEM containing 10% FCS and antibiotics were added and the cells were dispersed to make single cell suspension by repeated pipetting, using a 10 ml pipette. An aliquot of the cell suspension was added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37°C. The spent medium was changed every 3rd day and was sub cultured every week. Added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37°C.

Maintenance of K562 and EL-4 cells in culture

Human leukemic cell line K562 and thymoma cell (EL-4) lines non adhering cells. They were maintained in culture as suspension culture. 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 sub cultured every 3rd day.

Preparation of cells for in vitro studies

For experiments 70-80% confluent cultures were used. Monolayer cells were washed three times with PBS and the cells were harvested by mechanical dislocation using a cell scraper. Cell number was adjusted and the viability was checked by trypan blue exclusion method (Kuttan et al, 1985). Cell suspension with more than 95% viability was used for experiments.

Determination of cell viability

Trypan blue exclusion method (Kuttan et al, 1985) was used to check the cell viability. 0.1 ml of cell suspension was mixed with 0.1 ml of 1% trypan blue, while non-viable cells take up the dye and thus appeared blue in colour. The number of stained and unstained cells was counted separately.

$$\% \text{ Of Dead cells} = \frac{\text{Number of dead cells}}{\text{Number of total cells}} \times 100.$$

Isolation and maintenance of human umbilical vein endothelial cells (HUVECs)

Endothelial cells were obtained from human umbilical cord veins. A sterile technique was utilized in all manipulations of the cord. The cord was served from the placenta soon after birth, placed in sterile container filled with buffer. The umbilical vein was canulated with a blunt 14 gauge needle 2cm long, and the needle with a twine. The vein was perfused with cord buffer to wash out the blood. The other end of the cord was then tightly tied with a twine. 2ml of

0.2% sterile collagenase in the cord buffer was then infused into the umbilical vein and incubated at 37°C for 20 minutes. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord and centrifuged at 250xg for 10 minutes. The cells were resuspended in 199 medium containing 20% FCS, penicillin, streptomycin and L-glutamine and made single cell suspension. The cell suspension was transferred into 3 bottles and sufficient volume of fresh medium with VEGF (2ng/ml) and FGF (2ng/ml) was added. The cells were fed twice a week with a complete change of fresh culture medium. Subculture the cells using 0.01% EDTA and 0.1% collagenase. These cells were used for experiments from 3rd passage to 6th passage.

Maintenance of experimental animals

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

Maintenance of tumor cell lines in animals

Dalton's lymphoma Ascites (DLA) cells and Ehrlich Ascites Carcinoma (EAC) cells were propagated in the peritoneal cavity of Swiss albino mice and maintained by transplanting the cells every two weeks. Tumor cells were aspirated from the peritoneal cavity, washed with PBS and 1X10^6 cells were injected intraperitoneally to induce ascites tumor.

B16F-10 melanoma cells were propagated in C57BL/6 mice as transplantable solid tumors. 1X10^6 cells were injected subcutaneously to the hind limb of the mouse. After 10-15 days, the animal was sacrificed; tumor was

mashed and processed in PBS. $1X10^6$ viable cells were injected to another set of animals.

In vitro cytotoxicity studies

DLA or EAC cells ($1X10^6$) were incubated with various concentrations of the test compounds in a final volume of 1ml adjusted with PBS for 3h at 37°C. Control tubes contained tumor cells only. After incubation the viability of cells were determined by the trypan blue dye exclusion method (Kuttan et al, 1985).

Long term *in vitro* cytotoxicity studies in tissue culture

Cells growing in log-phase were used for this experiment. Cells were seeded in 96-well flat bottom tissue culture plate (5000cells/well) containing 200µl complete medium and incubated for 24h at 37°C with 5%CO_2 atmosphere. After incubation, various concentrations of the test compounds were added to the wells and the incubation was continued for 48h. Before 4h of the completion of incubation, 20µl of MTT (5mg/ml) was added to each well. After the incubation period the plates were centrifuged, supernatant was removed and 100µl of 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 (Cole, 1986; Campling et al, 1991). The percentage of dead cells was determined using the formula,

$$\% \text{ of dead cells} = \frac{\text{OD of drugs treated}}{\text{OD of control}} \times 100.$$

Antitumor studies

Solid tumor development

Swiss albino mice (6-8 week old) were used in this study. DLA cells were collected from the peritoneal cavity of the mice, washed in PBS and viability was checked. The cell number was adjusted to ($1X10^6$ cells/0.1ml) and was injected subcutaneously to the right hind limbs of mice. The radii of developing tumor was measured using vernier calipers at 3 days intervals for 1 month and tumor volume was calculated using the formula $V= 0.4ab^2$, where 'a' and 'b' represents the major and minor diameter, respectively (Atia and Weiss, 1966).

Ascites tumor

Swiss albino mice were induced ascites tumor by injecting $1X10^6$ cells/ animals to the peritoneal cavity. The death pattern of animals due to tumor burden was noted and the percentage of increase in lifespan was calculated using the formula (T-C)/CX100 were, 'T' and 'C' represent the number of days that treated and control animals survived, respectively (Kuttan et al, 1988).

Carcinogenesis

BALB/c mice (6-8 weeks old) were used for this study. Hair was shaved from the dorsal side of mice. All animals were administered with a single dose of 20-MC (200µg/0.1ml DMSO/mouse) subcutaneously on the dorsal side. This dosage has been shown to produce sarcoma development in mice by 8-12 weeks (Joy et al, 2000). Sarcoma developments as well as survival of the animals were noticed up to 180 days.

I. Immunological parameters

General techniques

Collection and trypsinization of SRBC

Sheep blood was freshly collected from the slaughters house in equal volumes of sterile Alsever's solution. Cells were washed three times with PBS (pH 7.2). Supernatant discarded and the pellet was suspended in Hanks balanced salt solution (HBSS). 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%.

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 (Moudgil and Singh, 1997). According to the antibody titre value, serum was diluted and used for the experiments.

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.

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.

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 thy same way for spleen and the thymocytes were suspended in RPMI-1640 medium containing 10%foetal calf serum.

Hematological parameters

Determination of Haemoglobin (Cheesbrough et al, 1991)

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,

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

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 corners square was counted (Cheesbrough et al, 1991).

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 haemocytometre, allowed to settle at the bottom of the chamber for 2 minutes and counted under a microscope using 10Xobjective. The total WBC counts was determined using the formula,

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

Where,

Dilution factor = 1/20

Depth = 1/10mm

Area counted = 4sq.mm

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

Differential count of leucocytes (Chesbrough et al, 1991)

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 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.

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 thigh 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 37% 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 color and cells were counted under microscope using oil immersion.

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 (Moudgil and Singh, 1997).

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.

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.

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 (Justo et al, 2003).

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-5µg/ml and 2.5µg/ml; Con-A -10µg/ml and 5µg/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) were measured in a liquid scintillation counter.

^{51}Cr-release assay

^{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 were resuspended in few drops of FCS. 100µCi of $Na_2{}^{51}CrO_4$ was added and incubated at 37^0C for Ih 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.

Determination of Natural Killer cell- mediated cytotoxicity

Labeled 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 96well round bottom 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).

Calculations

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

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 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.

Determination of antibody-dependent complement-mediated cytotoxicity

Principle

When tumor cells are incubated with specific antibodies in presence of complement, the classical pathway will be activated leading to the lysis of target cells

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)

II. Antimetastatic studies

***In vivo* antimetastatic studies**

Determination of the Metastatic potential of B16F-10 melanoma cells

C57BL/6 mice were used to study Metastatic ability of B16F-10 cells. Pulmonary colony forming ability of B16F-10 cells was carried out as described by Fidler (Fidler, 1978). B16F-10 melanoma cells ($1X10^6$ cells/0.05ml PBS) were injected in each animal via lateral tail vein. Animals were sacrificed after 21 days of tumor induction. The lungs were dissected and blood was collected by heart puncture. The lungs were used for morphological examination using a dissection

microscope and the blackish Metastatic colonies were counted. Then the lung tissue was subjected to the estimation of collagen hydroxy proline (Bergman and Loxley, 1970), hexosamines (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) and histopathological analysis. Serum sialic acid levels were determined according to the procedure of Bhavanandan et al (Bhavanandan et al., 1981). The serum r-GT (Szasz, 1976) levels were also assayed. The rate of survival was also observed.

Biochemical parameters

Estimation of protein by Lowry method.

Principle

The 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.

Sodium potassium tartarate - 1ml(2%)

$CuSO_4$ - 1ml (1%)

Na_2CO_3 - 98ml (2% in 0.IN NaOH)

Solution B

Folinis phenol reagent - IN, 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 vortex mixed reaction mixture, incubated at room temperature for 30minutes. Optical density read at 660nm.

Estimation of Hydroxyproline (Bergman and Loxley, 1970).

Principle

Hydroxyproline present in sample were oxidized by chloramine T. The colored product is more stable in the presence of high concentration of isopropanol.

Reagents

1. Oxidant solution

Sodium acetate	- 5.7 g.
Trisodium citrate	- 3. 75g.
Citric acid	- 0.55g.
Isopropanol	- 38.5 ml.
Double distilled water	- 61.5 ml.

2. Ehrlich's reagent.

p- dimethylaminobenzaldehyde	- 4.4g
Perchloric acid	- 10.2g (6%)
Isopropanol	- 25 ml (final volume)

3. Chloramine T.

Chloramine T	- 1.75g /25ml (oxidant solution prepared on the day of use).

Procedure

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

Estimation of uronic acid

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.

Extraction of uronic acid from the tissue was carried out according to the method of 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 pH 5.5 containing 0.005M cysteine and 0.005M disodium salt of EDTA at 65°C for 24h. An aliquot of the sample containing approximately 5-15mg uronic acid was estimated by the method of Bitter and Muir (Bitter and Muir, 1962).

Procedure

Aliquot (5ml) of sulphuric acid reagent prepared with 0.025M sodium tetra borate in con.H_2SO_4 was taken in tubes and cooled at 4°C for sometime. Iml

of sample or standard glucoronolactone solution containing 5-20mg was layered on the acid. Tubes were closed with glass stoppers and mix well .Tubes were kept in a boiling water bath for 10 minutes 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 color thus developed was read at 530nm. Uronic acid content of the tissue was expressed as µg/100mg net weight.

Estimation of Hexosamine (Elson and Morgan, 1933).

Preparation of 2% acetyl acetone in 0.5M Na_2CO_3

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

Preparation of Ehrlich's reagent

Dissolve 1.33g of p-dimethyl aminobenzaldehyde (PDAB) in 100ml of 1:1 ethanol and con. HCl.

Lyophilized tissue samples (5mg) were hydrolyzed with 2N HCl (5ml) at 100°C 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 15 minutes. After cooling under tap water, 5ml 95% ethanol and 1ml of Ehrlich's reagent (1.33% p-dimethylaminobenzaldehyde in 1:1 ethanol: conc.HCl mixture) were added and mixed thoroughly. The purple red color developed was read after 30 minutes at 530nm. Water containing blank and

standard glucosamine solutions of various concentrations was also treated similarly to get a standard curve. Hexosamine contents of tissues were expressed as μg/100mg lyophilized tissue.

Estimation of serum sialic acid (Bhavanandan et al., 1981).

Principle

Hydrolyzed serum liberated sialic acid forms a colored compound with thioharbituric acid.

Reagents

H_2SO_4 - 0.2N.

Periodic acid - 25μM in 62.5mM H_2SO_4.

Sodium arsenite - 0.2% in 0.5M HCl.

Thiobarbituric acid - 0.6 % (PH 9.0).

Dimethyl sulphoxide (DMSO).

Procedure

Serum (200μl) was hydrolyzed using 0.2N H_2SO_4 at 80°C for 1h. The hydrolysate was oxidized with 50μl of periodic acid (25μM) and incubated at 37°C for 30 minutes. This oxidation was terminated by adding 50μl of sodium arsenite. Then 100μl of 0.6%. TBA was added and heated in boiling water bath for 7.5 minutes. After heating, equal volume of DMSO was added to intensify the color and read at 549nm and 632nm.

Estimation of γ-glutamyl transpeptidase (GGT)

Principle (Szasz, 1976)

γ-glutamyl transpeptidase catalyses the transfer of γ-glutamyl moiety of γ-

glutamyl donor to a variety of acceptors.

Reagents

L-γ-glutamyl-p- nitroanilide	-2.5mM
Glycyl glycine	-20mM
Tris- HCl	-0.05M.
NaCl	-75 mM.

Procedure

The standared assay mixture contained (1ml) of 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 spectrophotometer.

Histopathological analysis

The lung tissues were fixed in 10% formaldehyde. The tissues were dehydrated for in alcohol series, cleaned in xylene and embedded in paraffin. About 4µm thick sections were taken on a glass slide and stained with haematoxylin and eosin and visualized under the microscope for histological changes (Culling, 1976).

Determination of the survival rate of Metastatic tumor bearing animals.

Animals were injected with B16F-10 melanoma cells ($1X10^6$) intravenously. The mortality rate of the animals was noted and the percentage increase in life span (% ILS) was calculated from the formula, % ILS= T-C X100. Where 'T' represent the number of survival days of the control animals.

In vitro antimetastatic studies

Collagen matrix invasion assay

The invasion assay was carried out in Boyden chambers (Blind well chambers) as described by Albini et al (Albini et al., 1987). The lower compartment of the chamber was filled with a mixture of serum free DMEM and conditioned medium from B16F-10 cells in 10:1 ratio. Type IV collagen-coated (25µg/membrane) polycarbonate filter membrane was placed in between the upper and lower compartments of the chamber. B16F-10 melanoma cells ($1X10^5$cells/150µl DMEM) were then seeded in to the upper chamber and incubated at 37°C in 5% CO_2 atm for 10h. After incubation the medium from the upper chamber was removed and the cells on the upper side of the filter were removed by a cotton swab. The membrane filter was separated and placed on membrane filter was separated and placed on a glass slide in an upside down position. It was fixed in methanol for 7-10 minutes and stained for 2 minutes with crystal violet. Cells that penetrated the polycarbonate filters were counted under a microscope. Results were calculated as % inhibition of invasion using the formula.

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

Tumor cell motility assay

B16F-10 melanoma cells ($1X10^5$/150µl) were seeded into the upper compartment of blind well chamber containing polycarbonate filters without collagen coating. The lower chamber was filled with DMEM without FCS.

Chambers were incubated for 24h at 37°C in 5% CO_2 atmosphere and the number of cells migrated to the lower chamber was counted using a haemocytometer. Percentage of inhibition of motility was calculated by using the above formula.

Tumor cell adhesion assay (Inokuchi et al, 1991)

Principle

Transformed cells have higher adhesive attachment rates to a variety of homotypic or heterotypic attachment (Nicolson, 1978).

Procedure

Adhesion assay was done according to the procedures of Inokuchi et al (Inokchi et al., 1991). B16F-10 cells were ($5X10^3$ cells/well) added to collagen Type- 1 (25µl/ well) coated flat bottom wells of 96 well titre plate and incubated for 5h at 37°C in 5% CO_2 atmosphere. After incubation, medium was removed and the wells were washed with PBS. Adhering cells were fixed with 5% formaldehyde for 10 minutes and stained with crystal violet for 2 minutes each. The cells were counted under an inverted microscope.

Gelatin zymography

Principle

Proteases of tumor cell supernatant were initially resolved on SDS-polyacrylamide gels, which were incorporated with gelatin. Following incubation of the gel in the activation buffer, protease separated on the gel will break down the gelatin and appears as transparent zones against dark back ground of stain.

Reagents

1. 0.25 Sucrose- 0.01M Tris HCl buffer, PH 7.4

Sucrose - 85.87g

Tris-HCl - 1.21g.

Distilled water - 1000 ml (final volume).

2. 0.1M Tris- Hcl; pH 8.8 (10 mM)

Tris-HCl - 15.7g

Distilled water -1000ml (final volume)

pH adjusted with NaOH.

3. 0.1M Tris-HCl; pH 6.8 (10mM)

Tris HCl -15.7g

Distilled water -1000 ml. (final volume)

pH adjusted with NaOH.

4. Trypsin solution

Trypsin 75µg/ ml in 0.1Tris-HCI, with

10 mM $CaCl_2$, P H 8.

5. Activation buffer

Tris –HCl - 15.7g

$CaCl_2.2H_2O$ - 1.47g

Distilled water -1000 ml (final volume)

Preparation of gels

Resolving gel

11% Polyacrylamide gels with 0.1% SDS and 0.8% gelatin

29.2% acrylamide + 0.5% bisacrylamide - 11 ml

0.1M Tris-HCI (pH 6.8) -1.2

20% SDS	-0.15 ml
20% Ammonium per sulphate	-0.10ml
Gelatin (16mg/2ml distilled water, heated to dissolve)	-2 ml
TEMED	-0.045 ml

Mix and pour at room temperature.

5% Stacking gel

29.2% acrylamide + 0.5% bisaerylamide	- 1.67ml
0.1m Tris HCl, PH 6.8	- 1.75ml
20% SDS	-0.10ml
20% Ammonium per sulphate	-0.10ml
Distilled water	-6. 36ml
TEMED	-0.02ml

Sample buffer

Tris base	-3g
Glycine	-14.2g

Make up to ltwith Distilled water.

2% Triton x-100

Triton x- 100	-2ml
0.1m Tris-HCl, pH 7.8	-100ml (final volume).

10mM EDTA Solution

EDTA	-372. 24mg
0.1M Tris Hcl (pH 7.8)	-1000ml.

Procedure

SDS-PAGE was performed according to the procedure of Billings et al (Billings et al, 1991). The medium from subconfluent culture bottles were removed, cells were washed with PBS cod incubated in serum free medium (DMEM) at 37°C for 24h. After incubation, medium was collected, centrifuged and supernatant was used for zymographic analysis. After determining the protein concentration, supernatant (equivalent 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), without reducing agent, loaded on 0.1% SDS, 11% polyacrylamide gels containing 0.8% gelatin and carryout electrophoresis at 10°C for 3h with a constant current of 2MA per tube until tracking dye reach the periphery of the gels.

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

III. Antiangiogenic studies

Antiangiogenic studies *in vivo*

The antiangiogenic activities of the the test materials were studied in C57BL/6 mice. Angiogenesis was incduced by injecting $1x10^6$ B16F-10 melanoma cells intradermaly on the shaven ventral skin of the mice. The angiogenesis induced animals were sacrificed on 9^{th} day after tumor inoculation. The ventral skin was cut removed, washed in PBS and the number of tumor directed capillaries per cm^2 around the tumor was counted using dissection microscope (Kishi et al, 2000).

Blood was collected from angiogenesis induced animals at two time points viz, day 1 and day 9 from the caudal vein in a sterile manner. Serum was separated and used for cytokine profiling. Cytokines such as IL-1β, IL-2, IL-6, TNF-α, GM-CSF, VEGF and an endogenous inhibitor of TIMP-1 were assayed using respective ELISA kits.

Antiangiogenic studies *in vitro*

Rat aortic ring assay

Rat aortic ring assay was used as the *in vitro* angiogenesis study model (Nicosia, 1998). Dorsal aorta from a freshly sacrificed Sprague Dawley rat was cut removed in a sterile manner, rinsed in ice cold PBS to remove blood and any membranous tissue. It was then cut into ~1mm thick transverse sections using surgical blades. Each segment was placed in a collagen precoated 96 well tissue culture plate. The rings were incubated for 24h at 37°C in medium, afterwards

exchanged for conditioned medium from B16F-10 melanoma cells. The rings were further incubated for six days and then analyzed by phase contrast microscopy for any microvessel out growth from the aorta.

***In vitro* studies using HUVECs**

Endothelial cell motility assay.

Endothelial cells (HUVECs) were grown to confluence in 96 well tissue culture plates coated with gelatin. A clear area was then scraped in the monolayer with a 20µl tip and fresh medium along with or without the test compounds were added (Gua et al, 2002). The culture were further incubated for 24h and then fixed using methanol and stained with crystal violet. Migration of cells into the wounded area was evaluated with an inverted microscope and photographed.

Tube formation assay (Gupta et al, 2002)

ECM gel (25µl) was added to 96 well plate and incubated at 37°C for 30 minutes. Endothelial cells were added to the solidified gel and further incubated for 48h in 5% CO_2 atmosphere in 199 medium supplemented with 20ng/ml VEGF and 20ng/ml FGF. Supernatant was then removed cells were fixed and stained using Diff-Quick stain set. Tube formation was examined using an inverted microscope.

IV. *In Vitro* Apoptotic Studies

Morphological Analysis

To detect the morphological changes, B16F-10 melanoma cells were incubated in the presence and absence of test products at 37^0C in the presence of 5% CO_2 for 48hours. The cells were washed thrice with PBS, and stained with

haematoxylin and eosin. Apoptosis was characterized by the morphological changes such as chromatin condensation, nuclear condensation, cellular membrane blebbing and formation of apoptotic bodies.

Extraction of DNA for DNA-ladder Analysis

Reagents

Cytoplasm Extraction Buffer

Tris-HCl pH 7.5	-	10mM
NaCl	-	150 mM
$MgCl_2$	-	5 mM
Triton X-100	-	0.5 %

DNA Lysis Buffer

Tris-HCl pH 7.5	-	10 mM
NaCl	-	400 mM
EDTA	-	1 mM
$MgCl_2$	-	5 mM
Triton X-100	-	1 %

Tris-EDTA Buffer

Tris-HCl, pH 8.0	-	10 mM
EDTA	-	1 mM

To detect the morphological changes, B16F-10 melanoma cells were incubated in the presence and absence of natural products at 37^0C in the presence

of 5% CO_2 for 48hours. The cells were washed thrice with PBS, incubated with 1 ml of cytoplasm extraction buffer on ice for 20 min, and pelleted by centrifugation. The pellet was resuspended in DNA lysis buffer for 20 min on ice and then centrifuged for 5 min (1000g, 4^0C). The supernatant obtained was incubated over night with RNase (2µg/ml) at room temperature and then with proteinase K (50µg/ml) for 2hours at 37^0C. DNA was extracted using phenol-chloroform (1:1) and precipitated with ice-cold 100% ethanol. The DNA precipitate was centrifuged at 10000g for 15 min and the pellet was air dried and dissolved in 50µl of Tris-EDTA buffer. The extracted DNA was resolved on 1.5% agarose gels.

V. Gene Expression Studies

cDNA Synthesis

Cells to cDNA^{TM} II kit from Ambion Inc, U.S.A., were used for producing cDNA directly from mammalian cells in culture without isolating RNA. Gene expression was carried out by RT-PCR method. It is one of the main methods for measuring mRNA levels from a small number of cells. The cDNA thus produced is used in the polymerase chain reaction (PCR).

Principle

The crude cell lysate is subjected to RT-PCR without purifying the RNA by using the cells to cDNA II kit. Cells from tissue culture were washed in PBS and then heated in cell lysis buffer. This treatment has two important effects. Cell

lysis buffer lyse the cells and releases the RNA into the cell lysis II buffer, and by heating the endogenous RNases get inactivated and thus protects the RNA from degradation. Next the crude cell lysate is treated with DNase I to degrade genomic DNA and the mixture is heated a second time to inactivate the DNase-I. Finally the reverse transcription and PCR were carried out using two steps RT-PCR strategy.

Procedure

$1X10^4$cells/well B16F-10 melanoma cells were seeded in the 96-well flat bottom titre plate using DMEM, supplemented with 10% FCS and antibiotics (penicillin 100units/ml and streptomycin 100µg/ml) and incubated for 12h at 37°C in 5% CO2 atmosphere. After incubation medium was aspirated and the cells were washed with ice cold PBS. 100µl Ice cold cell lysis buffer was added to the wells and the plate was immediately transferred to a water bath, at 75°C for 15 minutes. The lysed cells were then transferred to 200 µl nuclease free micro centrifuge tubes. 2µl DNase-1 per 100µl cell lysis buffer were added and incubated for 15 min at 37°C. DNase was inactivated by heating at 75°C for 5 min.

Preparation of master mix-I

Cell lysate containing total RNA	10 µl
dNTP Mix	4 µl
Ologo $(dT)_{18}$	2 µl
Nuclease free water	16 µl

The reagents were assembled in a nuclease free micro centrifuge, 10µl cell

lysate was added. The reagents were then mixed, centrifuged briefly and placed on crushed ice. The remaining Reverse transcription reagents (master mix 2) were also added to the same microcentrifuge tubes as follows.

Master mix-II

10X RT Buffer	2 µl
M-MLV Reverse Transcriptase	1 µl
RNase Inhibitor	1 µl

The reagents were mixed by vortexing and centrifuged briefly. The reaction mixture was incubated at 42°C for 60 minutes followed by further incubation at 90°C and then cooled to 4°C for 5 minutes. Then cDNA can be stored in -70 °C.

Determination of the gene expression level of k-ras, prolyl hydroxylase, lysyl oxidase, nm-23, MMP and TIMP

C57BL/6 mice were induced metastasis by using B16F-10 melanoma cells. The animals were sacrificed on 21st day. The lungs were excised and RNA was isolated using a modified method of single step guanidium thiocynate phenol-chloroform by Chomczynski et al (Chomczynski and Sacchi, 1987). cDNA was prepared from the RNA. PCR was performed using specific primers of k-ras, prolyl hydroxylase, lysyl oxidase (LOX), Nm-23, MMP-1, MMP-2, TIMP 1 and TIMP 2. PCR products were analyzed by agarose gel electrophoresis and visualized using the ethidium bromide dye.

Sequences of primers used in this study are given below.

Mouse Gene	Primer sequence	Expected product size
kras	5'-TGTGGATGAGTACGACC-3' 5'-ACGGAATCCCGTAACTC-3'	338bp
Prolyl hydroxylase	5'-CGGGATCCTAGACCGGCTAACAAGTA-3' 5'-GGAATTCCAAGCAGTCCTCAGCTGT-3'	317bp
Lysyl oxidase	5'-CTACATCCAGGCTTCCACG-3' 5'-TCTCCTCTGTGTGTTGGCAT-3'	283bp
Nm-23	5'-CTCAGCCTTAATTTTTTCCCCC-3' 5'-TTAACTTCCGACACTGGGTGT-3'	310bp
MMP-2	5'-GAGTTGGCAGTGCAATACCT-3' 5'-GCCGTCCTTCTCAAAGTTGT-3'	354bp
MMP-9	5'-AGTTTGGTGTCGCGGAGCAC-3' 5'-TACATGAGCGCTTCCGGCAC-3'	327
TIMP-1	5'-CTGGCATCCTCTTGTTGCTA-3' 5'-AGGGATCTCCAGGTGCACAA-3'	414bp
TIMP-2	5'-AGACGTAGTGATCAGGGCCA-3' 5'-GTACCACGCGCAAGAACCAT-3'	525bp

Detection of Proinflammatory cytokine gene expression by RT-PCR.

PCR was performed with Message Screen^{TM} Mouse Inflammatory Cytokine Set Multiplex PCR kit from Biosource International, USA. This kit is designed to detect the expression of Mouse GAPDH, IL-6, IL-1ß, GM-CSF, TNF-α and IL-12p40 genes. The PCR primers have similar melting temperature (Tm's) and no obvious 3'end overlap, which would enhance the amplification of multiple targets. The target PCR products generated from positive control cDNA, is included in this kit. This kit provides a quick and simple method for analyzing these genes expression and normalizes the expression of these genes against GAPDH.

Materials provided in the kit

List of primers and the expected length of the amplified products

Target	Expected product size
Mouse GAPDH	557 bp
Mouse IL-6	484 bp
Mouse IL-1ß	430 bp
Mouse GM-CSF	375 bp
Mouse TNF-α	290 bp
Mouse IL-12p40	239 bp

Procedure

All the reagents were thawed before starting experiments, mixed thoroughly and centrifuged briefly. All the reagents were assembled in nuclease free micro centrifuge tubes as follows.

2 X PCR Buffer	25 µl
10 X MPCR Primer pairs	5 µl
Taq DNA polymerase	5 µl
Nuclease free water	9 µl
cDNA sample or positive control	5µl

Reaction mixtures were vortexed and centrifuged briefly before and after adding the cDNA sample. PCR thermal cycling was performed according to the protocol in the following condition.

Steps	Conditions	Temperature	Duration
i	Initial Denaturation	95^0C	1 min
ii	Denaturation	94^0C	1 min
iii	Annealing	60^0C	4 min
iv	Denaturation	94^0C	1 min
v	Annealing	60^0C	2.5 min
vi	Extension	72^0C	1 min
vii	Go to step iv and repeat 40 cycles		
viii	Final extension	72^0C	10 min
ix	Store at	4^0C	

Detection of Bcl–2, Caspase-3 and p53 gene expression

PCR was performed with primers obtained from Maxim Biotech, Inc, USA for the cDNA detection of Bcl–2, Caspase-3 and p53. The cDNA

synthesized by the above method was directly used for PCR amplification.

Procedure

All the reagents were thawed before starting the experiments, mixed and centrifuged briefly. All the reagents were assembled in nuclease free micro centrifuge tubes according to the protocol of primer kit as follows.

Master mixture preparation

250µl each of pre-mixed primers were added to each tube of optimized PCR buffer. This master mixture was aliquoted and used for preparation of reaction mixture as follows for further PCR amplification.

Master Mixture	40µl
Taq DNA polymerase	0.2µl
cDNA sample	10µl

Reaction mixture was vortex and centrifuged briefly before and after adding the cDNA sample. PCR thermal cycling was performed according to the protocol of Maxim Biotech, Inc at the following conditions.

Steps	Conditions	Temperature	Duration
i	Initial Denaturation	96^0C	1 min
ii	Denaturation	94^0C	1 min
iii	Annealing	58^0C	1 min
iv	Extension	72^0C	1 min
v	Go to step ii and repeat 35 cycles		
vi	Final extension	72^0C	10 min
vii	Store at	4^0C	

Detection of PCR products

10µl of each PCR product was analyzed by 2% agarose gel electrophoresis. It was prepared as follows.

i. Sufficient electrophoresis buffer (1X TEB buffer 0.08M Tris-phosphate and 0.002M EDTA) used to fill the electrophoresis tank.

ii. Edges of a clean, dry, plastic gel tray were sealed with sealing tape and the comb was placed.

iii. Required percentage of agarose gel was prepared.

iv. Ethidium bromide was added to a final concentration of 5µg/ml to the slurry, then poured to the sealed gel tray and allowed to cool for 30-45minutes and the gel tray placed in the electrophoresis tank after removing the sealing tape.

v. 10µl of PCR products mixed with 2µl of 10X gel loading buffer and loaded into the wells.

vi. The samples were resolved at 100v until the dye has migrated up to the ¾th length of the gel.

vii. The gels were examined using a gel-documentation system.

VI. Transcription factor profiling

Transcription Factor profiling was done with BD Mercury Transfactor kit obtained from BD Biosciences. This kit provides rapid, high throughput detection of specific transcription factor activities in cell extracts. Using an enzyme-linked immunosorbent assay (ELISA)-based format, the Transfactor Kits detect DNA

binding by specific transcription factors (Shen et al, 2002).

Principle

Each Transfactor kit is provided in a 96-well format with oligonucleotides containing the consensus binding sequences for each transcription factor coated on the wells. When cell extracts containing the transcription factors are incubated in the wells, the DNA binds to their consensus sequences. A specific primary antibody was then used to detect bound transcription factors. A horse radish peroxidase conjugated secondary antibody was used to detect the bound primary antibody. The enzymatic product was measured with standard micro titer plate reader.

Transfactor ELISA procedure

Cytosolic and nuclear extract procedure

The cytosolic or nuclear extract from cell line culture was prepared according to the following procedure (Dyer and Herzog, 1995; Lee et al, 1998), Which is designed for extraction from 1×10^7cells. After cells have been treated with desired conditions, extracts are prepared for transfactor analysis.

All steps were performed at 4°C unless otherwise specified. Reagents were kept at 4°C during the procedure, and should not be used until fully defrosted. Tubes and reagents were kept on ice when not centrifuging. All reagents were centrifuged at 4°C in a pre-cooled rotor.

Materials required

10 X Pre Lysis - Buffer

100mM HEPES (pH 7.9)

15mM $MgCl_2$

100mM KCl

Pre -extraction buffer

20mM HEPES (pH 7.9)

1.5mM $MgCl_2$

0.42M NaCl

0.2mM EDTA

25% (v/v) glycerol

Protease inhibitor cocktail

Aprotinin (1mg/ml)	–	5µl
Leupeptin (1mg/ml)	–	5µl
PMSF (100 mM in Isopropanol)	–	5µl
DTT (100 mM in D.W.)	–	10µl
Benzamidine (1mg/ml)	–	5µl
Triton × 100 (10% in D.W)	–	10µl
Tris pH 7.4 (1M)	–	20µl

NaCl (5M)	–	50µl
EDTA (0.5M)	–	5µl
EGTA (10mg/ml)	–	5µl

Make up this cocktail into 1 ml and store at–22^{0}C.

1 X Lysis buffer

10 X Pre-Lysis buffer	–	150µl
0.1 M DTT	–	15µl
Protease inhibitor cocktail	–	15µl
DD H_2O	–	1.32µl

Cell lysis

1. Cells were collected after incubation using a cell scraper, and transferred to a clean centrifuge tube, centrifuged for 5min at 450xg. Supernatant was decanted.
2. The cell pellet were rinsed with ice cold PBS and centrifuged for 5min at 450xg and supernatant were decanted.
3. Cell pellet volume was estimated using a micropipette and this volume was used in subsequent step of this procedure.
4. Five times volume of lysis buffer to the cell pellet volume was added to the cell pellets.
5. The cell pellets were resuspended gently with out the formation of foam and incubated for 15min on ice.
6. The cell suspension was centrifuged for 5min at 420×g and the supernatants were

discarded and the cell pellet resuspended in a volume of lysis Buffer equal to twice the cell pellet volume.

Cell disruption

1. Cell suspension in the lysis buffer was filled in a syringe with narrow gauge needle (No. 27). The cell suspension ejected with a rapid stroke. This was repeated for 10 times.
2. The disrupted cell suspension was centrifuged at 10000-11000×g for 20 min.
3. Transfer the supernatant was transferred to a fresh tube and this fraction is known as the cytosolic fraction.

Nuclear extraction

1. Nuclear extraction buffer by mixing the following reagents as follows,

   Pre- extraction Buffer – 147µl

   0.1 M DTT – 1.5µl

   Protease inhibitor cock-tail – 1.5µl

2. The crude nuclear pellet obtained in Step 5 (above) suspended in a volume of nuclear extraction buffer equal to two-third (2/3) of the cell pellet volume
3. The nuclei were disrupted by using a fresh syringe.
4. Nuclear suspension were allowed to shake gently for 30 min at 4°C and centrifuged at 20000-21000×g for 5min.
5. The supernatant were transferred to a clean, chilled test tube and this fraction is the nuclear extract.
6. The protein concentration of the nuclear extract was measured by BradFord

method.

7. The supernatant was aliquoted in to small vials and stored at -70^{0}C.

Determination of protein concentration of the nuclear extract by BradFord Method

This is a rapid, simple and sensitive method for the estimation of proteins in a sample extract. The color development is virtually complete in 2 min and the color is stable for 1h. The procedure is based on the interaction of a dye, Coomassie Brilliant Blue with proteins. The unbound dye has an absorbance maximum at 465 nm. However on interaction with proteins the dye turns blue and its absorbance at 595 nm the amount of protein in a sample solution can be quantitatively estimated.

Materials and reagents

1. BradFord Reagent.

Dissolve 100 mg Coomassie Brilliant Blue G250 in 50 ml of ethanol, add 100ml of 85% phosphoric acid make the volume to 1L with water.

2. 0.1 M phosphate buffer (pH 7.5)

Procedure

0.1 ml of sample solution was made up to the volume of 1 ml with 0.1 M phosphate buffer (pH 7.5). 5 ml of Brad Ford reagent was added to the sample and mixed thoroughly. Absorbance was recorded at 595 ηm against the blank and the protein concentrations were determined from the standard curve.

Trans factor ELISA Procedure

Assay wells were incubated with 150µl of 1X Transfactor buffer for 15min. at room temperature. Nuclear extracts (6µl) were made up to 50µl with 1X Transfactor buffer and added to the assay wells and incubated 60min at room temperature. Wells were washed with 1X Transfactor buffer and incubated with 100µl primary antibody at room temperature for 60min, then washed with 1X Transfactor buffer and incubated with 100µl of secondary antibody at room temperature for 30 min. Wells are washed with 1X Transfactor buffer and incubated with 100µl of TMB substrate to each well and incubated at room temperature for 10min. After seeing the blue color development the reaction were stopped by adding 100ml of sodium azide stop solution. Absorbance was measured at 655ηm.

VII. Statistical Data Analysis

Statistical significance of the data was calculated using Students's 't' test to determine the significance between group (x) and group (y), the value 't' was found from the equation,

$$t = \frac{X - Y}{S\sqrt{1/nx + 1/ny}}$$

Where, X - Mean of samples (x)

Y - Mean of samples (y)

nx - Sample size (x)

ny – Sample size (y)

S was found from the equation

$$S = \sqrt{\frac{(nx-1)\,sx^2 + (ny-1)\,sy^2}{(nx+ny) - 2}}$$

Where, sx - Standard deviation of x

sy - Standard deviation of y

By knowing the degree of freedom (nx+ny)-2, Statistical significance (Pvalue) was deduced from the't' distribution table.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

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Chapter 3

Antitumor and anticarcinogenic activity of Piper longum, Piperine, Thuja occidentalis and Thuja polysaccharide fraction

Introduction

A tumor that is not capable of indefinite growth and does not invade the healthy surrounding tissue extensively is benign. A tumor that continues to grow and becomes progressively invasive is malignant. Carcinomas are tumors arising from endodermal or ectodermal tissue. Sarcomas are arising from mesodermal connective tissue.

A variety of bioactive compounds and their derivatives has been shown to inhibit carcinogenesis in a number of experimental systems involving initiation, promotion and progression (Huang et al, 1994). Methylcholanthrene is a highly carcinogenic poly-cyclic aromatic hydrocarbon. The most common isomer is 3–methylcholanthrene (3-MCH) Subcutaneous injection of mice with (3-MCH) resulted in sarcomas at the site of injection. Plants contain abundant quantities of these substances and have consistently been shown to be associated with a lower risk of cancers at almost every site (Steinmetz and Potter, 1991). Efforts, therefore, are being made to identify naturally occurring antitumor and anticarcinogens which would prevent or slow down the cancer induction and its subsequent development (Chuang et al, 2000). This chapter focuses attention on the antitumor and anticarcinogenic activity of *Piper longum*, Piperine, *Thuja occidentalis* and Thuja Polysaccharide fraction using *in vitro* models.

Materials and methods

Animals

Male Swiss albino mice (6-8 weeks) weighing 25-30 gm were used for this study.

Cells

L929, B16F-10, CHO, Vero, EL-4, K562cells, Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells were used for this study.

Drug administration

In antitumor study, mice were treated with 10 doses of *Piper longum* extract (10mg/dose/animal), Piperine (1.14mg/dose/animal) *Thuja occidentalis* extract (5mg/dose/animal) and Thuja polysaccharide fraction (TPS) (1mg/dose/animal) intraperitoneally for 10 consecutive days.

Determination of the *in vitro* cytotoxic activity of *P.longum*, Piperine, *T.occidentalis* and TPS to DLA and EAC cell.

Dalton's lymphoma ascites (DLA) cells and Ehrlich ascites carcinoma (EAC) cells ($1X10^6$) were incubated with various concentrations (2-500µg/ml) of *P.longum*, Piperine, *T.occidentalis* and TPS in a final volume of 1ml for 3h at 37^0C. After incubation the viability of cells were determined by the trypan blue dye exclusion method (Talwar, 1974).

Determination of the cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS to different cell lines using MTT assay.

Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS was determined using L929, B16F-10, CHO, Vero, EL-4, K 562, DLA and EAC cells in culture as described in chapter 2. Cells were seeded in 96-well flat bottom titre plates (5000 cells/well) and allowed to adhere for 24h at 37°C with 5% CO_2

atmosphere. Different concentrations of *P.longum*, Piperine, *T.occidentalis* and TPS (25-100 μg/ml) were added and incubated further for 48h. Before 4h of the completion of incubation 20μl of MTT (5mg/ml) was added (Cole, 1986; Campling et al, 1991). Percentage of cell viability was determined using an ELISA plate reader set to record absorbance at 570nm.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on solid tumor development

Solid tumor was induced by injecting DLA cells ($1x10^6$cells/animal) subcutaneously to the right hind limbs of three groups (6mice/group) of Swiss albino mice. Group I animals were kept as untreated control. Group II-V animals were treated with alcoholic extract of *P. longum*, Piperine, *T.occidentalis* and TPS respectively for 10 consecutive days. The radii of developing tumor were measured using vernier calipers at 3 days intervals for one month and tumor volume was calculated using the formula $V=0.4ab^2$ where 'a' and 'b' represents the major and minor diameter respectively (Atia et al, 1966). This was compared with untreated control.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the survival of ascites tumor bearing animals.

Three groups (6mice/group) of Swiss albino mice were induced ascites tumor by injecting $1X10^6$cells/animals to the peritoneal cavity. Group I was inoculated with EAC cells alone and kept as untreated control. Group II-V received *P.longum*, Piperine *T.occidentalis* and TPS respectively for 10

consecutive days. The death pattern of animals due to tumor burden was noted and the percentage of increase in lifespan was calculated using the formula (T-C)/CX100 were 'T' and 'C' represent the number of days that treated and control animals survived respectively.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on 3-Methyl cholanthrene induced sarcoma in mice.

Hair was shaved from the dorsal side of Swiss albino. All animals were administered with a single dose of methyl cholanthrene (200µg/0.1ml DMSO/mice) subcutaneously on the dorsal side. Thereafter the animals were randomly divided into five groups (15mice/group).Group I was treated with a single dose of 3-methyl cholanthrene alone, which served as control. Group II-V was treated with *P.longum*, Piperine, *T.occidentalis* and TPS respectively for 6 days in a week for 20 weeks. Sarcoma development as well as survival of the animals was noticed up to 180 days.

Results

Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS towards DLA cells

The Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS towards DLA cells were estimated using the trypan blue exclusion method (Table I). *P.longum* and Piperine showed 50% and 100% Cytotoxicity towards DLA cells at a concentration of 250µg/ml. At 50µg/ml concentration of *T.occidentalis* and TPS cause 70% and 83% Cytotoxicity respectively towards the DLA cells.

Table I

Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS to Dalton's lymphoma ascites (DLA) cells (*in vitro*)

Concentration (μg/ml)	Percentage of cell death			
	P.longum	Piperine	*T.occidentalis*	TPS
500	100	100	ND	ND
250	50	96	100	ND
100	12	30	100	100
50	0	25	70	83
10	ND	6	28	37
5	ND	0	7	19
2	ND	ND	0	7

DLA cells (10^6) were incubated with different concentrations (100-500μg/ml) of *P.longum*, Piperine, *T.occidentalis* and TPS. % of dead cells was determined by trypan blue exclusion method.

Table II

Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS to Ehrlich ascites carcinoma (EAC) cells.

Concentration (μg/ml)	Percentage of cell death			
	P.longum	Piperine	*T.occidentalis*	TPS
500	100	100	ND	ND
250	100	85	100	ND
100	48	15	100	100
50	4	3	90	63
10	0	1	32	33
5	ND	0	4	11
2	ND	ND	0	0

EAC cells (10^6) were incubated with different concentrations of (100-500μg/ml) of *P.longum*, Piperine, *T.occidentalis* and TPS. % of dead cells was determined by trypan blue exclusion method.

P.longum and Piperine produced 100% cytotoxicity towards EAC cells at a concentration of 250μg/ml. 100μg/ml concentration of *T.occidentalis* and TPS gives 100% cytotoxicity towards EAC cells (Table II).

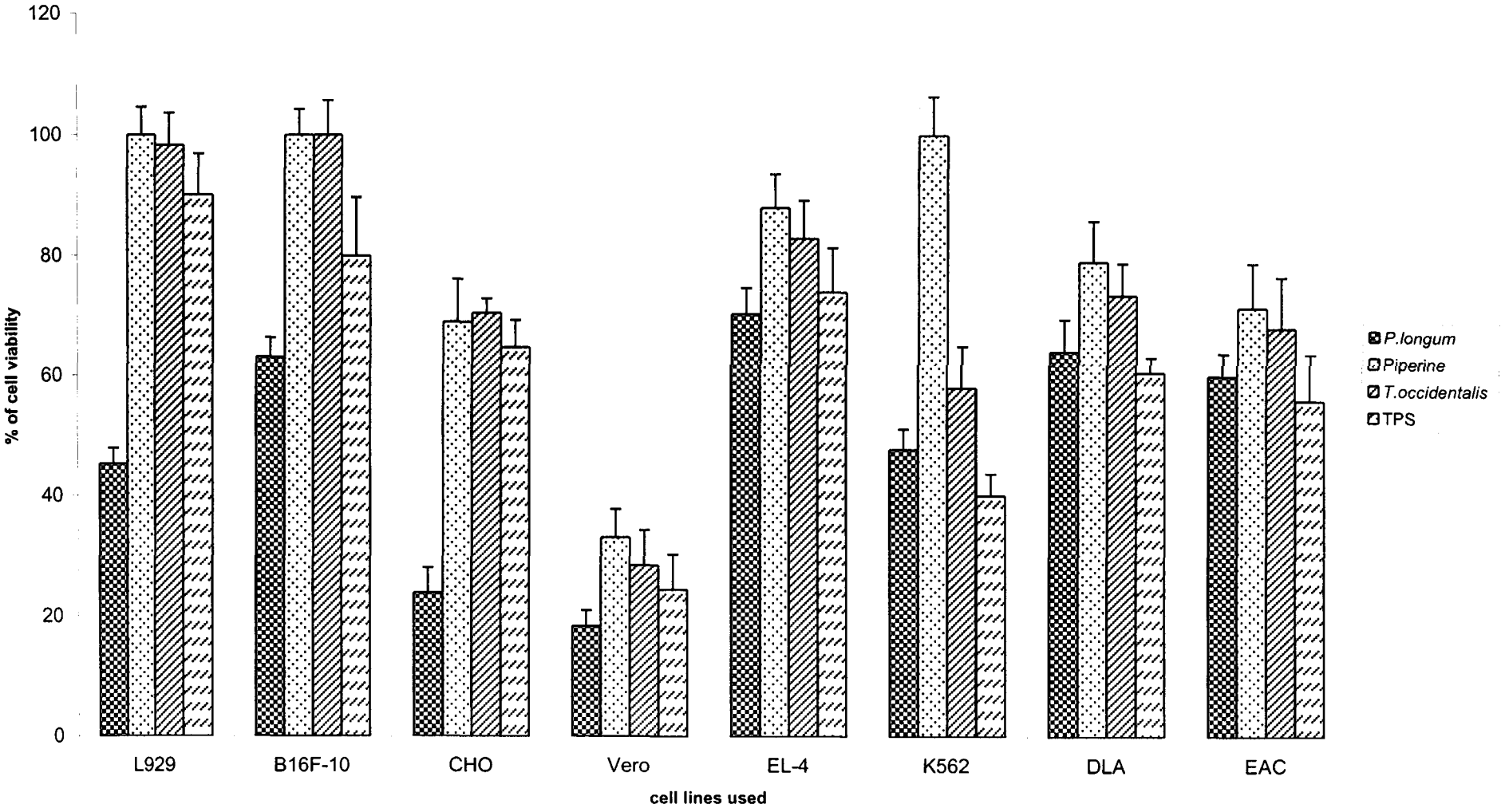
Cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS towards different cells in culture

P.longum, Piperine, *T.occidentalis* and TPS was found to be cytotoxic towards L929, B16F-10, CHO, Vero, EL-4, K-562, DLA and EAC cells in culture (Fig. I). *P.longum* at a concentration of 100μg/ml was found to be 45.3%, 63.1%, 70.4%, 47.8%, 64.1% and 60% toxic towards L929, B16F-10, EL-4, K-562, DLA and EAC cells. It produced only 23.9% and 18.4% toxicity towards CHO and Vero cells at the same concentration.

Treatment with Piperine at a concentration of 100μg/ml produced maximum toxicity of 100% towards L929, B16F-10 and K562 cells. It produced 88% toxicity towards EL-4 cells followed by DLA (79.1%), EAC (71.4%), CHO (69%), and Vero cells (33.2%).

T.occidentalis at a concentration of 100μg/ml of produced maximum toxicity (100%) towards B16F-10 cells followed by L929 (98.3%), EL-4 (83%), DLA (73.5%) CHO (70.5%), EAC (68%), K562 (58%) and followed by Vero cells (28.6%). In case of TPS the maximum toxicity was produced towards B16F-10 (90%), L929 (80%) followed by EL-4 (74%), CHO (64.7%), DLA (60.6%) EAC (55.9%), K562 (40.1%) and Vero cells (24.5%). at a concentration of 100μg/ml. all compounds were less toxic towards normal Vero cells.

Figure I Effect of *P.longum* , Piperine, *T.occidentalis* and TPS on different cell lines



Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on solid tumor development

Administration of *P.longum*, Piperine, *T.occidentalis* and TPS significantly reduced the solid tumor formation induced by DLA cells (Fig II). Tumor volume of control animal was 1.7cc^3 on 22nd day while that of *P.longum*, Piperine, *T.occidentalis* and TPS treated animals was only 0.59 cc^3, 0.29 cc^3, 0.21 cc^3 and 0.87 cc^3 respectively on the same day.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the survival of ascites tumor bearing animals.

Life span of ascites tumor bearing mice treated with *P.longum*, Piperine, *T.occidentalis* and TPS was found to be significantly increased (Table III). Control animals survived only 14.4 days after the tumor induction while the *P.longum*, *T.occidentalis* and TPS treated animals survived 20.6, 21.5 and 22.5 days with an increase in life span of 43%, 49.3% and 56.2% respectively. Piperine treatment was found to be more effective showing 65.2% increase in the life span of the tumor bearing animals.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on 3-Methyl cholanthrene induced sarcoma in mice.

The effect of 3-Methyl cholanthrene induced sarcoma development in mice in the presence and absence of *P.longum*, Piperine, *T.occidentalis* and TPS. All the compounds tested here significantly delayed or reduced the sarcoma development in mice induced by 3-Methyl Cholanthrene. In the case of controls

Figure II Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on solid tumor reduction

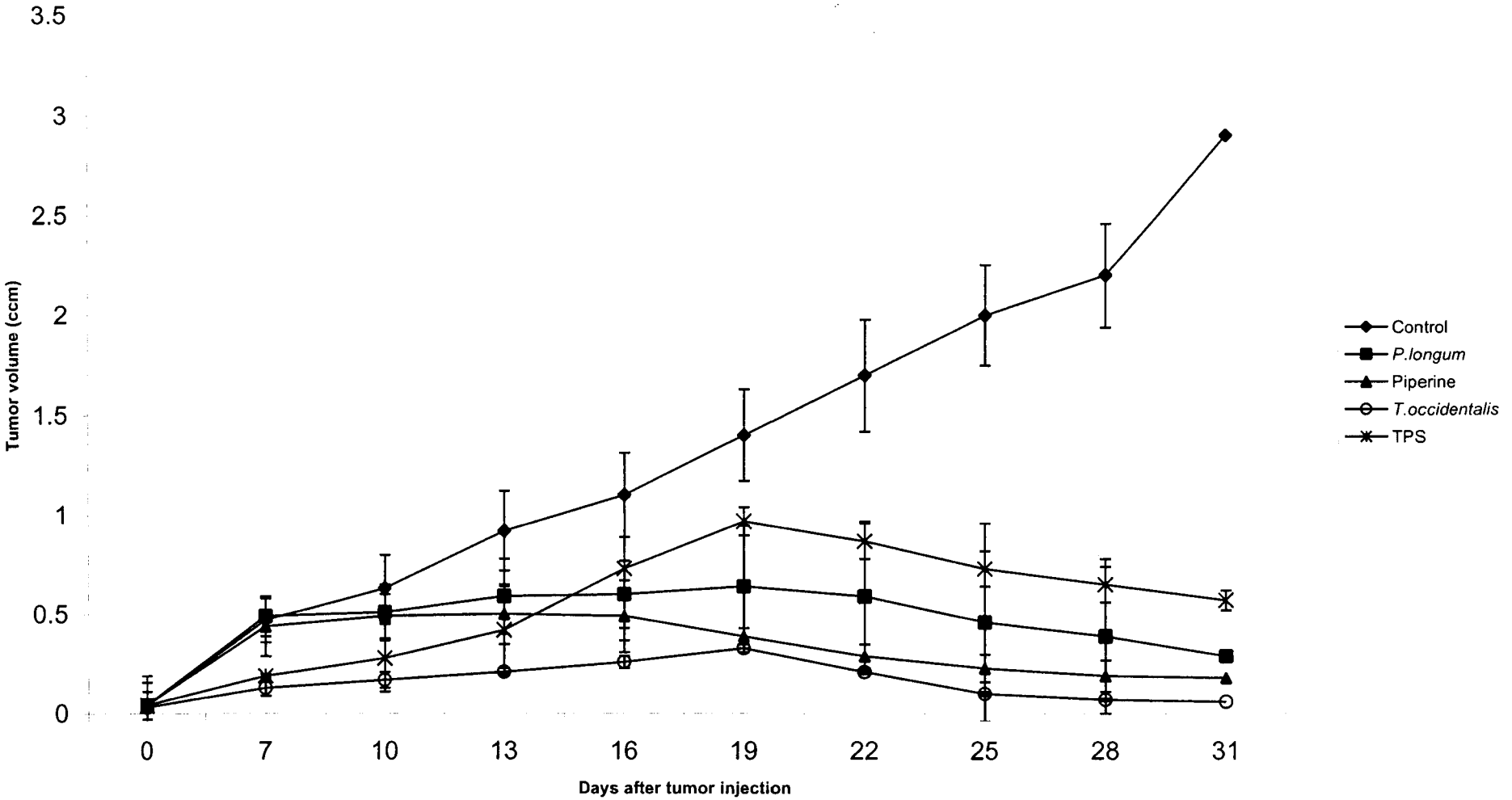


Table IV

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS in the development of sarcoma induced by 3- Methyl cholanthrene

Days	Number of animals developed sarcoma				
	Control	*P.longum*	Piperine	*T.occidentalis*	TPS
60	2	0	0	0	0
80	4	0	0	1	1
100	11	1	0	2	2
120	14	3	2	3	2
140	14	5	4	3	4
160	15	6	4	4	5
180	-	7	4	5	6

Animals were administered with a single dose of methyl cholanthrene (200µg/0.1ml DMSO/mice) subcutaneously on the dorsal side. Thereafter the animals were treated with *P.longum*, Piperine, *T.occidentalis* and TPS respectively for 6 days in week for 20 weeks. Sarcoma development was noticed up to 180 days. Values are the mean ± SD statistically significant from untreated control. *P<0.001

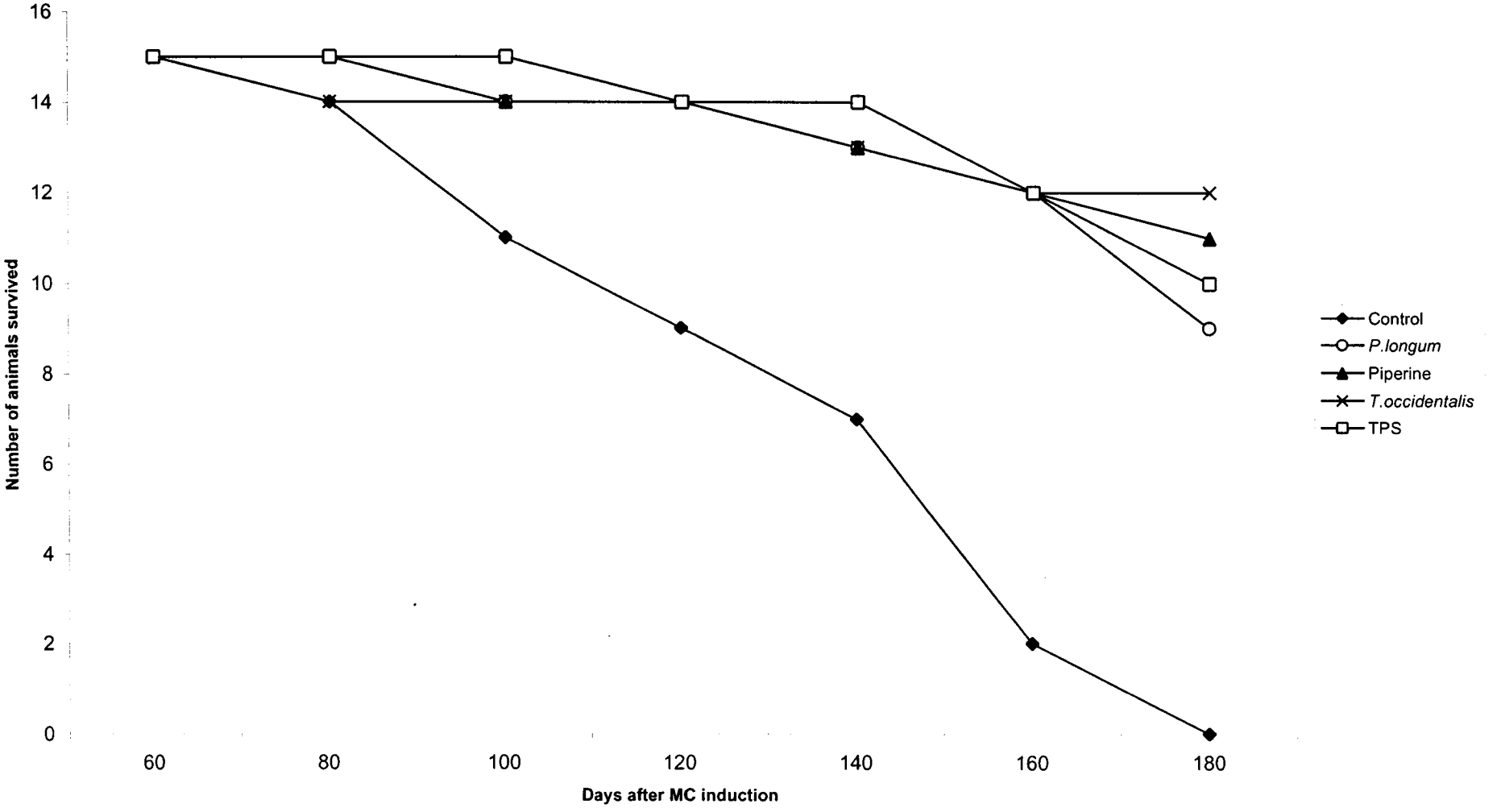
Table III

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the life span of ascites tumor bearing animals.

Treatement	Mean survival days	Percentage of increase life in span (%ILS)
Control (tumor alone)	14.4±0.8	–
Tumor+*P.longum*	20.6±1.7	43.0
Tumor+Piperine	23.8±2.4	65.2
Tumor+*T.occidentalis*	21.5±1.2*	49.3
Tumor+TPS	22.5±2.0	56.2

Animals were treated with 5 doses of *P.longum*, Piperine, *T.occidentalis* and TPS after injecting Ehrlich ascites carcinoma (EAC) cells (10^6cells/animal). Survival pattern was observed for 30 days. Values are the mean ± SD statistically significant from untreated control. *P<0.001

Fig. III Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the survival of sarcoma bearing animals



14/15 animals developed tumors at 120 days while the tumor development was significantly reduced with the simultaneous treatment with *P.longum* (3/15), Piperine (2/15), *T.occidentalis* (3/15) and TPS (2/15) (Table IV). These results also reflected at final evaluation at 180 days. All the 15 animals developed tumors in the case of controls while the tumor development was significantly reduced in the compounds treated animals (*P.longum* 7/15, Piperine 4/15, *T.occidentalis* 5/15 and TPS 6/15.

There was also a significant increase in the number of animals survived by drug treatment (Fig. III). In the case of control none of the animals survived at 180 days while the survival rate was significantly increased in the groups simultaneously treated with *P.longum* (9/15), Piperine (11/15), *T.occidentalis* (12/15) and TPS (10/15).

Discussion

The main objective of this study was to focus on the antitumor and anticarcinogenic activity of alcoholic extract of *P.longum* and its component Piperine. We also studied these properties of *T.occidentalis* and its polysaccharide fraction (TPS). The dosage of *P.longum*, Piperine, *T.occidentalis* and TPS was selected on the basis of cytotoxicity. The results of MTT assay using *P.longum*, Piperine, *T.occidentalis* and TPS shows a cell specific activity towards different cell line. These compounds were found to be toxic towards transformed cells less toxic to normal Vero cells.

Administration of *P.longum*, Piperine, *T.occidentalis* and TPS could significantly inhibit the growth of solid tumor induced by DLA cells and these

compounds could also increase the lifespan of mice bearing ascites tumor induced by Ehrlich ascites carcinoma cells. The results indicate that administration of *P.longum*, Piperine, *T.occidentalis* and TPS also could significantly delay the sarcoma development in mice model by 3-Methyl cholanthrene and increased their survival rate. Hence the results indicated that the *P.longum*, Piperine, *T.occidentalis* and TPS could act as a non toxic anticarcinogenic agent which process antitumor property also.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

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Chapter 4

Effect of Piper longum, Piperine, Thuja occidentalis and Thuja polysaccharide fraction on the immune system of experimental animals

Introduction

One of the major drawbacks of the current cancer therapeutic practices such as chemotherapy and radiation therapy is suppression of immune system (Devasagayam et al., 2002). The immunomodulating property of IL-2, IL-4, IL-7 etc. promoted their use in the treatment of cancer patients. But their unique and diverse side effects such as cardiovascular toxicity, pulmonary toxicity, haematological toxicity etc. made limitations in their use (Ognibene et al., 1998; Rosenberg et al., 1994). There are two effector pathways that account for T cell mediated cytotoxicity namely granule exocytosis pathway and Fas pathway. There is considerable evidence to support that these two pathways mediate tumor cell killing *in vivo* by T cells and it has been suggested that these pathways account for cytotoxicity mediated by CTLs against tumor cells (Bente et al, 1996) NK cells, comprising approximately 15% of all circulating lymphocytes can cause early production of cytokines and chemokines and lyse tumor cells without prior sensitization (Watzl and Long, 2000). The linking of antibody to target cells with the Fc receptors of a number of cell types, particularly natural killer (NK), can direct the antibody dependent cell mediated cytotoxic (ADCC) activities. Low NK cell activity has been associated with poor prognosis in advanced cancer patients (Anatoly et al, 1993). Therefore a number of immunotherapeutic approaches aiming to enhance NK cell activity have been investigated.

We have reported the immunomodulatory activity of some plants such as *Viscum album* (Kuttan et al., 1992), *Tinospora cordifolia* (Mathew et al., 1999), *Withania somnifera* (Davis et al., 2000) etc.

In this chapter the effect of *P.longum* extract, Piperine, *T. occidentalis* extract and Thuja TPS on the immune system as well as host defense response, as demonstrated by enhanced NK cell, ADCC, ACC and CTL activity and proliferation of spleen, thymus and bone marrow cells in the presence and absence of mitogens are investigated.

Materials and methods

Animals

BALB/c mice (4-6 weeks) and C57BL/6 mice (4-6 weeks) weighing 20-25g were used for this study.

Cells

L929 cells, K562 human leukemic cells, Ehrlich ascites carcinoma (EAC) cells and EL-4 thymoma cells and Sheep red blood cells (SRBC) were used for this study.

Drug administration

In all the immunological parameters the BALB/c mice were treated with 5 doses of alcoholic extract of *Piper longum* (10mg/dose/animal), Piperine (1.14mg/dose/animal) *Thuja occidentalis* (5mg/dose/animal) and TPS (1mg/dose/animal) intraperitoneally for 5 consecutive days.

Determination of the effect of *P.longum*, Piperine, *T. occidentalis* and TPS on haematological parameters

BALB/c mice were grouped in to IV (6 mice /group). Group I, II, III and IV were treated with 5 doses of *P.longum* extract, Piperine, *T. occidentalis* extract

and TPS respectively. Blood was collected from the caudal vein and parameters such as total WBC count (Haemocytometer), differential count (Leishman's stain), haemoglobin content (cyanmethemoglobin method) (Drabkin and Austin, 1932) as well as body weight were recorded prior to the drug treatment and every third day for one month.

Determination of the effect of *P.longum*, Piperine, *T. occidentalis* and TPS on the bone marrow cellularity and α-esterase positive cells

Bone marrow cellularity was determined by the method of Sredni et al (Sredni et al., 1992). The animals were divided in to five groups (6mice/group). Group l was untreated control; group II, III, IV and V were treated with 5 doses of *P.longum*, Piperine, *T.occidentalis* and TPS intraperitoneally for 5 consecutive days respectively. The animals were sacrificed 24 h after the last dose of drug treatment. Bone marrow cells from femur was collected and made into single cell suspension and the cell number was determined using haemocytometre.

The number of α-esterase positive cells were determined by the azodye coupling method (Bancroft and Cook, 1984). A smear of bone marrow cells from the above preparation was made on clean glass slides, air-dried, stained with α-naphthyl acetate and pararosaniline hydrochloride, counter stained with heamatoxyline. The number of α-esterase positive cells were expressed out of 4000 cells.

Determination of the effect of *P.longum*, Piperine, *T. occidentalis* and Thuja TPS on circulating antibody titre

In this study five groups of BALB/c mice (6 mice/group 20-25g body

weight) were used. Group I kept as untreated control, group II and III animals were pretreated with alcoholic extract of *P.longum* and Piperine. Group IV and V were treated with *T.occidentalis* and TPS fraction. All the animals were immunized with SRBC ($2.5X10^8$cells/animal, ip) after the last dose of drug. Blood was collected from the tail vain, serum separated and heat inactivated at 56^0C. Antibody titre was conducted by hemagglutination according to the method of Singh et al (Singh et al., 1984) using SRBC as the antigen in 96 well round bottom titre plates.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on plaque forming cells in spleen

Five groups of BALB/c mice (7 mice /group; 4 weeks old) were used in this study. Group 1 was kept as untreated control. Group II, III, IV and V were treated with 5 doses of alcoholic extract of *P.longum*, Piperine, Thuja and TPS respectively. All the animals were immunized with SRBC ($2.5x10^8$ cells /animal, ip) after the last dose of drug. The animals were sacrificed on different days; spleen was processed to single cell suspension and used for the determination of the antibody producing cells by Jern's Plaque assay (Jerne and Nordin, 1963).

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on CTL production *in vivo*

Alloimmunization was carried out by injecting the spleen ($2X10^7$) cells from C57BL/6 mice, subcutaneously to normal, *P.longum*, Piperine, Thuja and TPS treated BALB/c mice. Winn's neutralization test was carried out according to the method of Kobayashi et al (Kobayashi et al, 1992). Alloimmune spleen cells

from BALB/c mice (effector cells) were mixed with complete medium containing $5x10^5$ EL-4 cells (target cells). The cells were incubated for 1hour at 37°C in 5% CO_2 atmosphere and 0.2 ml of this mixture was injected intraperitoneally to 8 groups of BALB/c mice. Group I animals received EL-4 cells alone. Group II animals received EL-4 cells incubated with normal spleen cell suspension, group III, IV, V and VI animals received EL-4 cells incubated with normal spleen cells and continued with 10 doses of *P.longum*, Piperine, *T.occidentalis* and TPS respectively. Group VII-X animals received EL-4 cells incubated with *P.longum*, Piperine, *T.occidentalis* and TPS treated spleen cells and group XI-XIV received EL-4 cells incubated with *P.longum*, Piperine, *T.occidentalis* and TPS treated spleen cells and continued the administration of these compounds for 10 days. The animals were observed daily for 80 days after tumor inoculation. Increase in mean survival days of the treated group compared with the control animals were considered as the indication of CTL activity.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the proliferation of spleen cells, Bone marrow cells and thymocytes *in vitro*.

Spleen, thymus and bone marrow from BALB/c mice were collected and processed aseptically to single cell suspension, washed and suspended in RPMI containing 10% FCS and antibiotics to detect its proliferation in the presence and absence of mitogens (Justo et al, 2003). Cells ($5X10^4$cells/well) were cultured in 96-well 'U' bottomed titre plate with various mitogens (Con A 10µg/ml, PHA 2.5µg/ml, LPS 10µg/ml and PWM 10µg/ml), in the presence and absence of *P.longum* extract (20µg/ml), Piperine (10µg/ml), *T. occidentalis* extract

(5μg/dose/animal) and TPS (1μg/dose/animal). Final volume was adjusted to 250μl with RPMI and incubated at 37°C for 48h in the presence of 5% CO_2. Cultures were set up in triplicate and the proliferation was assayed by adding 1μCi of thymidine (^3H) to each well and further incubated for 16-18 h at 37°C. After the incubation, cells were lysed using 5N NaOH and transferred to 5 ml scintillation fluid and radioactivity was measured using a rack beta liquid scintillation counter.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on cell mediated immune response in Ehrlich ascites tumor bearing animals.

BALB/c mice (4-6 weeks old) grouped into (12 no/group) three groups. Group I was kept as untreated tumor bearing control animals. Group II and III animals were treated with *P.longum* extract, Piperine, *T. occidentalis* and TPS for 5 consecutive days and along with last dose of drug treatment, Ehrlich ascites carcinoma (EAC) cells ($1X10^6$cells/animal) were injected intraperitoneally. After tumor inoculation the animals were sacrificed at different time points, spleen and blood was collected and processed. Spleen cells were used as effector cells to determine the NK-cell (Tsavoris et al, 2002) and ADCC activity by 4 h ^{51}Cr-release assay (chapter 2). Serum was used as anti-EAC antibody for ACC by trypan blue exclusion method (Kuttan et al, 1985)

Results

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on haematological parameters

Administration of the alcoholic extract of *P.longum*, Piperine, *T. occidentalis* and TPS was found to increase the total WBC count in BALB/c mice (Fig I). The maximum increase in the animals treated with *P.longum* (142.8%) and Piperine (140.6%) were observed on 15^{th} day after drug administration. But incase of Thuja extract (134.7%) and TPS (132.3%) was observed on 12^{th} day after drug administration. There was no appreciable change in the differential count, body weight and Hb content after the administration of *P.longum* and *T. occidentalis* (data not shown).

Effect of the effect of *P.longum*, Piperine, *T.occidentalis* and Thuja TPS on the bone marrow cellularity and α-esterase positive cells

The effect of *P.longum*, Piperine *T. occidentalis* and TPS on the bone marrow cellularity and α-esterase positive cells is given in Table I. Administration of alcoholic extract of *P.longum* ($29.5x10^6$cells/femur), Piperine ($24.0x10^6$cells/femur) *T.occidentalis* ($29.7x10^6$cells/femur) and TPS ($26.1x10^6$cells/femur) showed a significant enhancement in the bone marrow cellularity compared to the normal control (14.3 cells$x10^6$ cells/femur) animals.

Moreover the number of α-esterase positive cells was also found to be increased significantly in *P.longum* (1398 cells/4000 bone marrow cells, Piperine

Fig 1. Effect of *P.longum* , Piperine, *T.occidentalis* and TPS on total WBC count

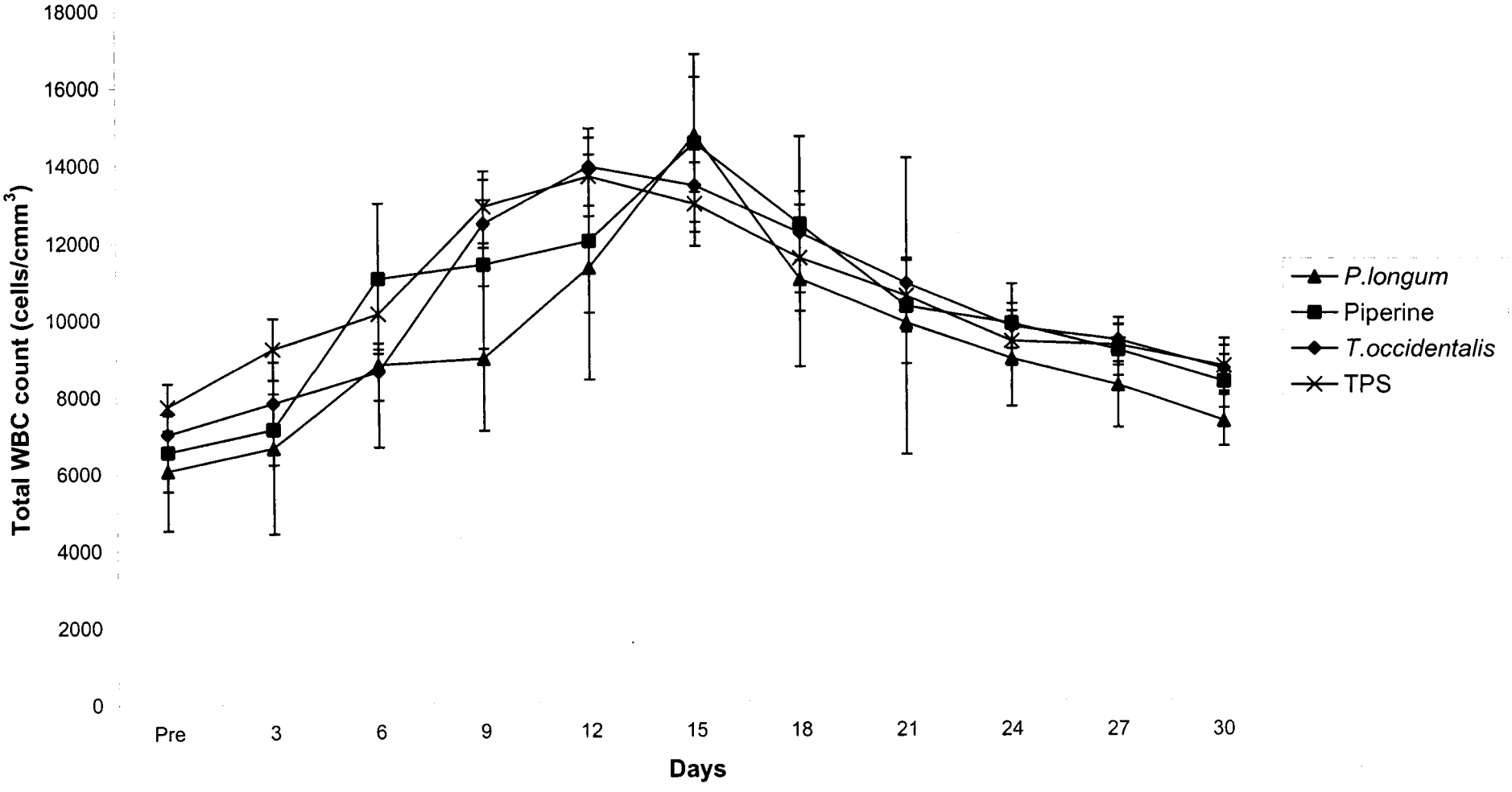


Table I

Effect of *P.longum*, Piperine *T.occidentalis* and TPS on bone marrow cellularity and α-esterase activity.

Treatment	Bonemarrow cellularity (Cells/femur)X10^6	Number of α-esterase positive cells /4000 cells
Normal	14.3±0.2	779.5±57.2
P. longum	29.5±0.8	1398.3±112.9
Piperine	24.0±0.4	1236.6±108.5
T. occidentalis	29.7±0.8	1426.6±135.8
TPS	26.1±1.9	1134.0±148.1

Treated animals received 5 doses of *P.longum* extract (10mg/dose/animal), Piperine (1.14mg/dose/animal) *T.occidentalis* extract (5mg/dose/animal) and TPS (1mg/dose/animal). Bone marrow cells were collected from femur. Values are the mean ± SD statistically significant from untreated control. *P<0.001

(1236.6 cells/4000 bone marrow cells), *T. occidentalis*(1426.6 cells/4000 bone marrow cells) and TPS (1134cells/4000 bone marrow cells) treated animals compared to the normal animals (779.5 cells/4000 bone marrow cells).

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on circulating antibody titre

The enhancement of total antibody production by the administration of *P.longum* extract, Piperine *T. occidentalis* and TPS is shown in Fig II. The maximum antibody titre value of 256 for alcoholic extract of *P.longum* and 512 for Piperine treated animals was observed on 18^{th} day after immunization. Thuja and TPS showed the maximum antibody titre value of 128 and 256 respectively on 15^{th} day after immunization and it persisted for few days.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on plaque forming cells (PFC) in spleen

The effect of *P.longum* extract, Piperine *T. occidentalis* and TPS on the number of plaque forming cells is shown in fig III. The maximum increase in the plaque forming cells in alcoholic extract of *P.longum* (306.25PFC/10^6spleen cells) and Piperine (358.1PFC/10^6spleen cells) treated groups were observed on 6^{th} day after the immunization. While in case of *T. occidentalis* (284 PFC/10^6spleen cells) and TPS (257PFC/10^6spleen cells) it was observed on 5^{th} day after the immunization.

Figure II. Effect of *P.longum* , Piperine, *T.occidentalis* and TPS on antibody titre

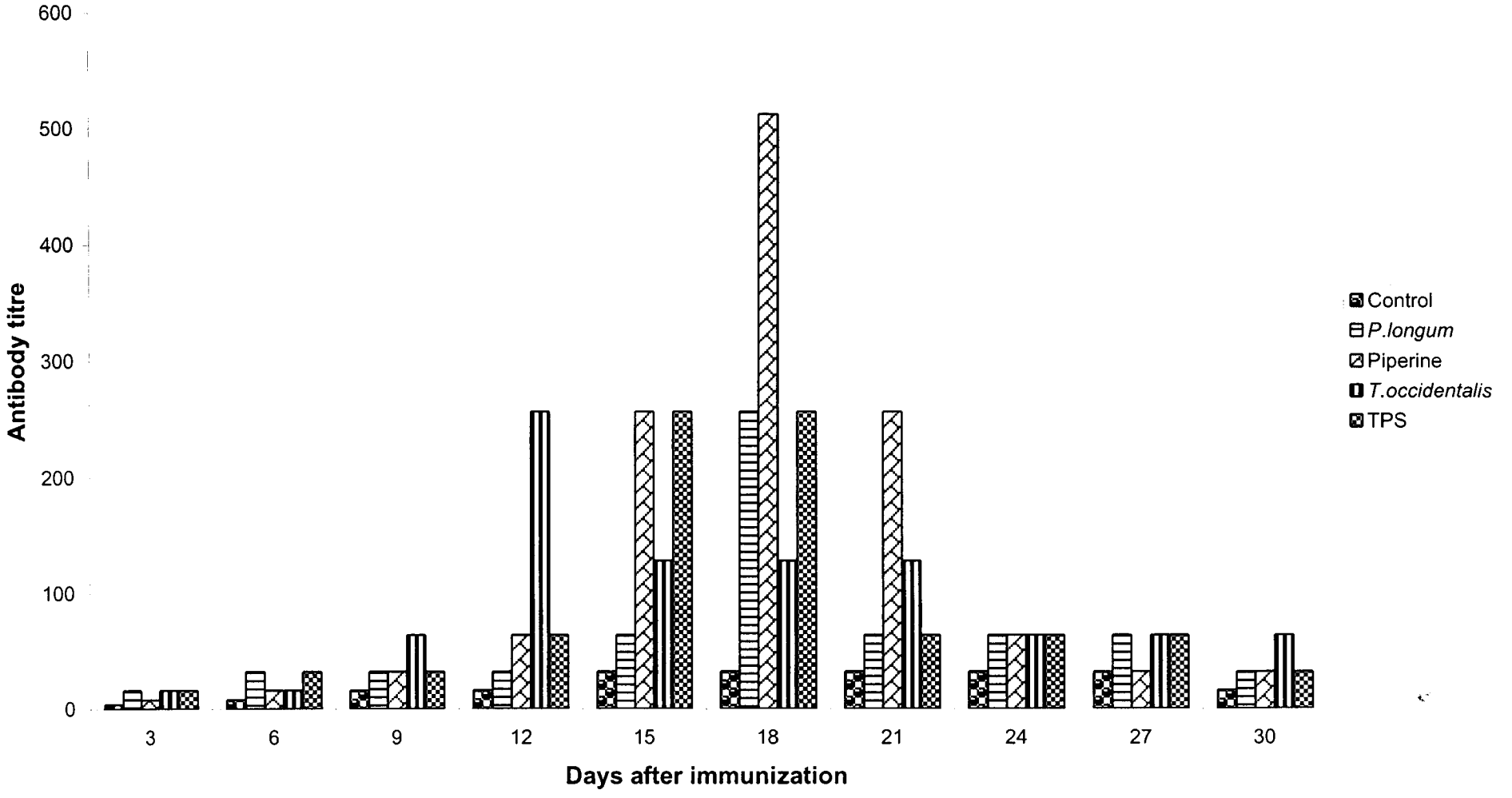
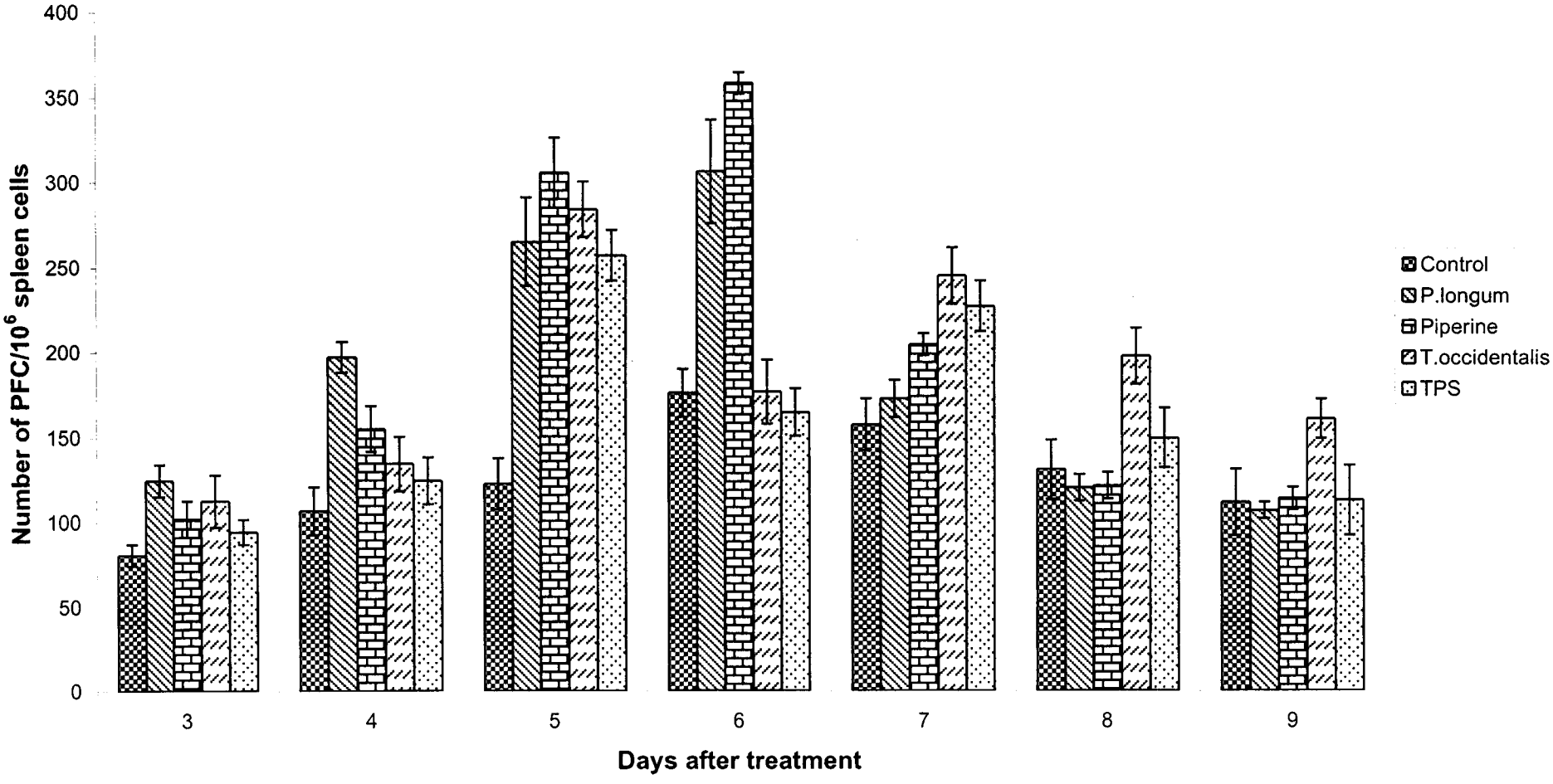


Figure III. Effect of *P.longum* , Piperine, *T.occidentalis* and TPS on plaque forming cells



Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on CTL production *in vivo*

Effect of *T.occidentalis* and TPS on the generation of CTL *in vivo* is given in the Table II. The survival rate of animals in the untreated tumor bearing (EL-4 alone) was 26.8±4.4 days. When the animals were injected with normal spleen cells, the survival rate was 36.8±4.2 days. When EL-4 cells were incubated with *P.longum*, Piperine *T.occidentalis* and TPS the survival rate was 38.3±4.02, 41.7±4.16, 43.5±4.06, and 40.3±5.1 days respectively. When the administration of these compounds were continued in the above groups the survival rate was significantly increased up to 48.6±5.7, 52.9±3.5, 54.4±5.8 and 50.2±3.9 days respectively

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the proliferation of spleen, thymus and bone marrow cells *in vitro*

The effect of *P.longum* extract, Piperine Thuja and TPS on the proliferation of spleen, thymus and bone marrow cells is shown in Table III to V. Maximum increase in the spleen proliferation was seen when the cells were treated with PHA along with *P.longum* (CPM 6904±568.2), Piperine (CPM 5765±440.4), Thuja (CPM 6649±460) and TPS (CPM 6342.4±482.1) followed by Con A, LPS and PWM stimulation when compared to the untreated control (CPM 1327±39.5) (Table III).

A synergistic enhancement on bone marrow cell proliferation was obtained when PHA stimulated cells were treated with *P.longum* (CPM 3547.6± 149.6), Piperine (CPM 3686± 230.4), Thuja (CPM 3484±156.4) and TPS (CPM

Table II

Effect of *P.longum*, Piperine *T.occidentalis* and TPS on CTL generation (*in* vivo)

Treatment	Survival rate of animals (days)				
		P.longum	Piperine	Thuja	TPS
EL-4 alone	26.8± 4.4	-	-	-	-
EL-4+ normal spleen	36.8± 4.2	-	-	-	-
EL-4+normal spleen+drug	-	40.4±3.8	44.6±3.4	46.9±3.7	43.1±3.7
EL-4+ treated spleen	-	38.3±4.0	41.7±4.1	43.5±4.0	40.3±5.1
EL-4+ treated spleen+drug	-	48.6±5.7	52.9±3.5	54.4±5.8	50.2±3.9

Alloimmunization was carried out by injecting the spleen (2X 10^7) cells from C57BL/6 mice,to normal, *P.longum*, Piperine, Thuja and TPS treated BALB/c mice. Winn's neutralization test was carried out according to the method. The cells were injected intraperitoneally to treated and untreated BALB/c mice. The animals were observed daily for 80 days after tumor inoculation

Table III

Effect of *P.longum*, Piperine *T.occidentalis* and TPS on spleen cell proliferation

Treatment	Counts per minutes (CPM)				
	No Mitogen	PHA (5µg/ml)	Con A (10µg/ml)	PWM (10µg/ml)	LPS (10µg/ml)
Normal Spleen	1327 ± 39.5	4561.3 ± 551.4	4639.6 ± 206.4	3352 ± 228.7	4307.2 ± 363.0
Normal Spleen + *Piper longum*	4310.3 ± 391.9*	6904± 568.2*	5248.3 ± 224.0*	4553.6 ± 185.5*	4828.6± 312.4*
Normal Spleen + Piperine	3864.6± 342.2*	5765 ± 440.4*	6347.6 ± 435.5*	5735.3± 335.5*	5424.6 ± 234.9*
Normal Spleen + *Thuja occidentalis*	4208.3 ± 476.9*	6649± 460*	6266 ± 475.9*	4916.6 ± 139.8*	5439.3 ± 321.1*
Normal Spleen + TPS	4006.7 ± 307.9*	6342.4 ± 482.1*	5976.3± 496.3*	4754.6 ± 510.4*	5132.1 ± 462.3*

* $P < 0.001$ compared to normal. Spleen cells were cultured in the presence and absence of *P. longum* (10µg/ml) and Piperine (10µg/ml), *T.occidentalis* (5µg/ml) and TPS (1µg/ml) with different mitogens and the rate of proliferation was checked. All the stimulations were done in triplicate.

Table IV

Effect of *P.longum*, Piperine *T.occidentalis* and TPS on bone marrow proliferation

Treatment	Counts per minutes (CPM)				
	No mitogen	PHA (5µg/ml)	Con A (10µg/ml)	PWM (10µg/ml)	LPS (10µg/ml)
Normal	1176.6± 116.0	3077± 143.0	1668.3± 109.2	1990.6± 227.9	3070± 210.2
Normal BM+ *Piper longum*	1882.3± 159.2*	3547.6± 149.4*	2569± 219*	2137.3± 107.5*	3517.3± 168.8*
Normal BM + Piperine	1735± 140.84*	3686± 230.4*	2067± 130.4*	2393.3± 160.6*	3724± 144.8*
Normal BM + *T. occidentalis*	1546± 95.6*	3484± 156.4*	2013.3± 186*	1899.6± 174.7*	3324± 221.1*
Normal BM + TPS	1223± 106.6*	3065± 210.5*	1978.4± 175.4*	1743.9± 205.3*	3086± 352.4*

*P< 0.001 compared to normal

Bone marrow (BM) cells were cultured in the presence and absence of *P.longum* (10µg/ml) and Piperine (10µg/ml), *T.occidentalis* (5µg/ml) and TPS (1µg/ml) with different mitogens and the rate of proliferation was checked. All the stimulations were done in triplicate

Table V

Effect of *P.longum*, Piperine *T.occidentalis* and TPS on thymocyte proliferation

Treatment	Counts per minutes (CPM)		
	No mitogen	Con A (10µg/ml)	PWM (10µg/ml)
Normal	1577.6 ± 127.8	4073.3 ± 143.7	3639.3± 188.7
Normal thymocytes+ *Piper longum*	4043.3± 969.1*	4418± 335.5*	3992.6± 288.4*
Normal thymocytes+ Piperine	4174.8 ± 408.6*	4723.1± 508.6*	4228.9± 523.1*
Normal thymocytes+ *T. occidentalis*	3528.6± 230.6 *	4823.3± 161.6*	5055.6± 167.4*
Normal thymocytes+ TPS	3428± 331.4*	4693.1± 452.4*	4943.6± 509.5*

*P < 0.001 compared to normal

Thymocytes were cultured in the presence and absence of *P. longum* (10µg/ml) and Piperine (10µg/ml), *T.occidentalis* (5µg/ml) and TPS (1µg/ml) with different mitogens and the rate of proliferation was checked.

3065±210.5). LPS also could significantly stimulated the proliferation of bone marrow cells followed by Con A and PWM induced stimulation. In control animals it was only 1176.6±116 CPM (Table IV).

Thymocyte proliferation was significantly enhanced by *P.longum*, Piperine, *T. occidentalis* and TPS along with the T-cell mitogens such as Con A (CPM 4418±335.5, CPM 4723.1±508.6, CPM 4823.3±161.6 and CPM 4693.1±452.4respectively) and PHA compared to control (CPM 1577.6 ± 127.8) (Table V)

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on natural killer (NK) cell activity

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the activation of NK cells in normal and tumor bearing mice is given in Fig.IV and V. NK cell activity of *P. longum,* Piperine, *T.occidentalis* and TPS was enhanced to maximum level on 5^{th} day (42.1%, 40.3%, 53.3%, and 52.1% cell lysis respectively) after last dose of drug administration in normal mice. In normal animals the maximum lysis observed was only 6%. The NK cell activity in untreated tumor bearing animals was (20% cell lysis) observed only on 11^{th} day.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on antibody dependent cellular cytotoxicity (ADCC)

P. longum, Piperine, *T.occidentalis* and TPS significantly enhanced the ADCC in normal as well as tumor bearing animals (Fig.VI, VII). The maximum rate of ADCC was observed on 9^{th} day after *P.longum* and Piperine (55.9%, 46.2%cell lysis respectively) administration in normal animals. A maximum cell

Figure IV. Effect of *P.longum* and Piperine on natural killer cell activity

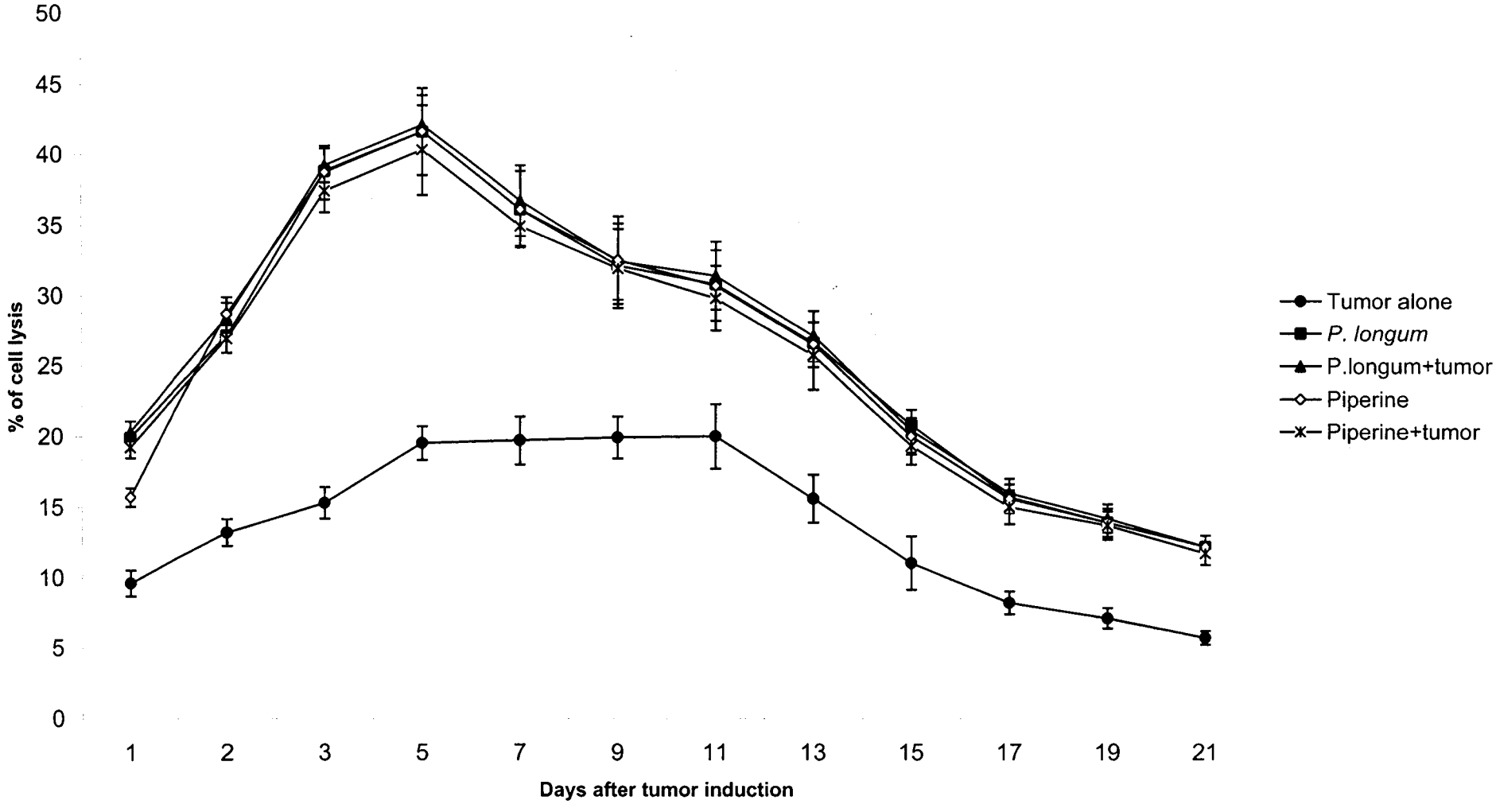


Fig. V Effect of *T.occidentalis* and TPS on Natural killer (NK) cell activity

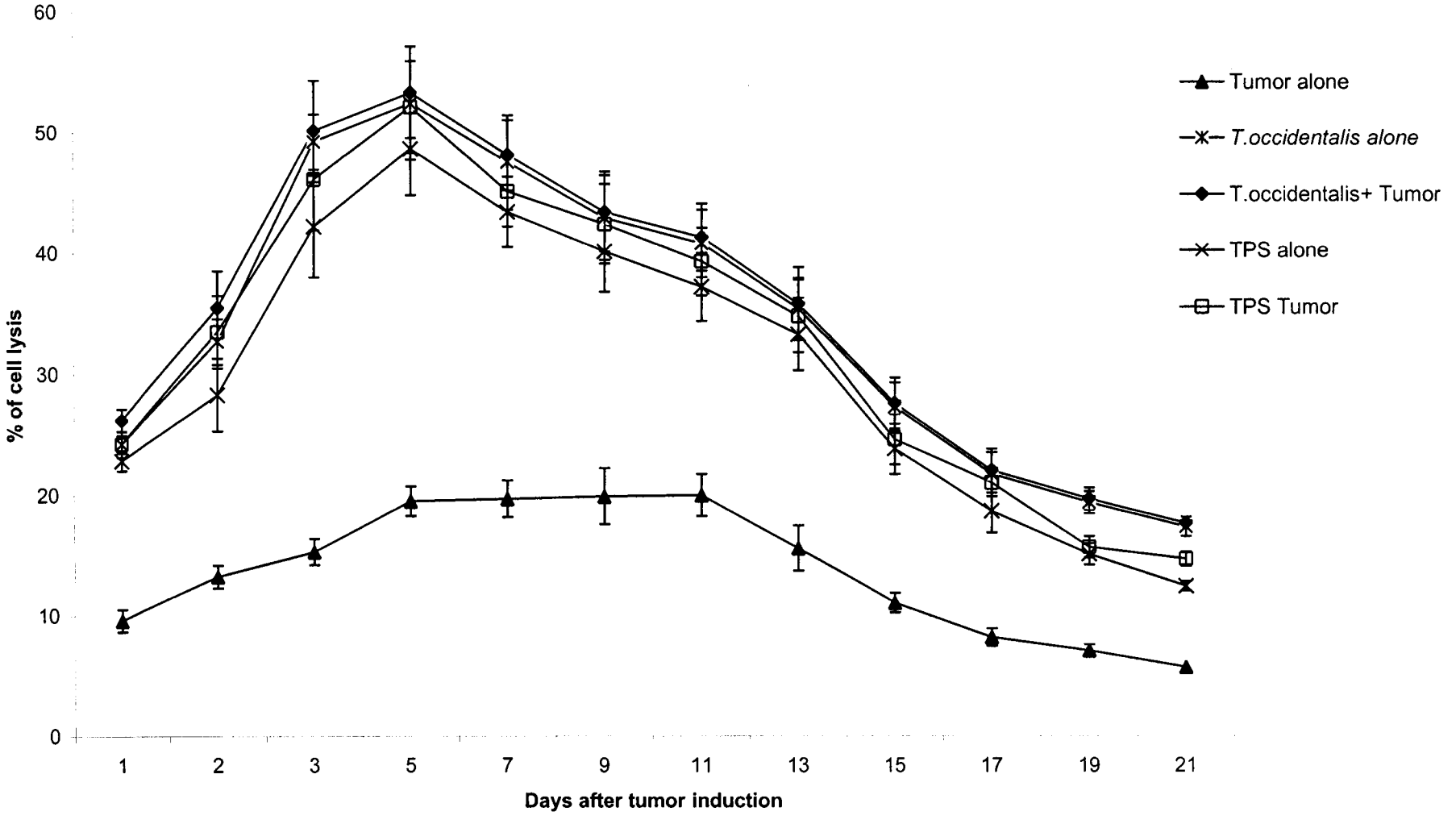


Figure VI. Effect of *P.longum* and Piperine io antibody dependent cellular cytotoxicity (ADCC)

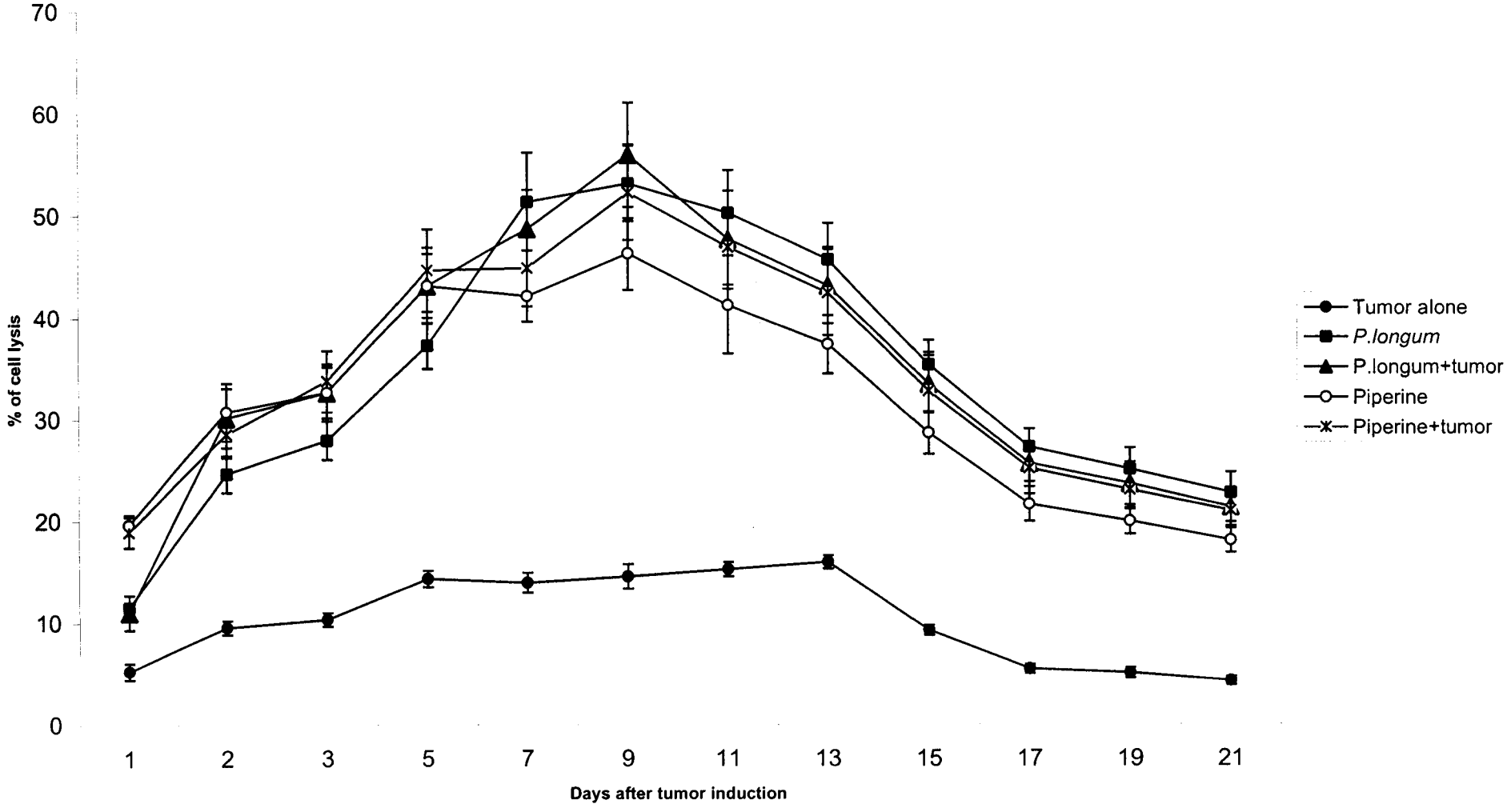
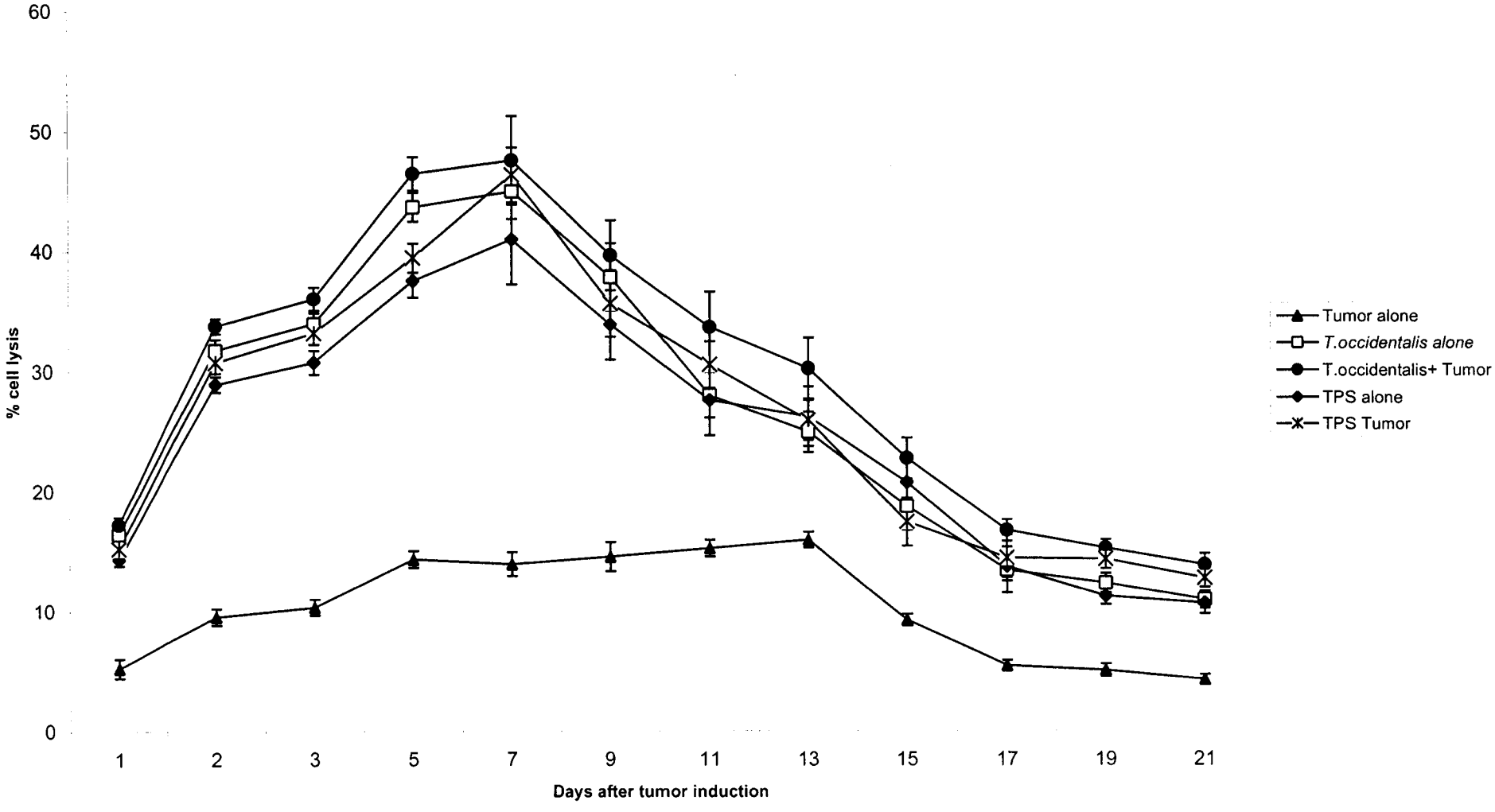


Fig. VII Effect of *T.occidentalis* and TPS on antibody dependent cellular cytotoxicity (ADCC)



lysis of 47.5% and 46.3% was observed on 7th day after tumor inoculation in *T.occidentalis* and TPS whereas in control animals, there was only 15.9% cell lysis on 13th day.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on antibody-dependent complement mediated cytotoxicity (ACC).

The effect of *P.longum*, Piperine *T.occidentalis* and TPS on ACC is shown in Fig.VIII and IX. Maximum cell lysis was observed on 15th day. *P.longum* (25.6%), Piperine (24.6%) *T.occidentalis* (30%) and TPS (27.1%) treated animals showed a maximum cytotoxicity on 17th day after tumor inoculation. While in untreated control animal, the maximum cytotoxicity was only 14.3% on 17th day.

Discussion

The main objective of this study was to focus on the immunomodulatory activity of *P.longum*, Piperine *T.occidentalis* and TPS. Immunoregulation is a complex balance between regulatory and effector cells and any imbalance in immunogical mechanism can lead to pathogenesis (Steven et al., 1988). Immunosuppression is one of the main obstacles in the conventional cancer treatment such as chemo and radiotherapy.

The dosage of *P.longum*, Piperine *T.occidentalis* and TPS was selected on the basis of cytotoxicity. 10mg/dose/animal for *P.longum* extract, 1.14mg/dose/animal for Piperine (5mg/dose/animal) for *T.occidentalis* and (1mg/dose/animal) for TPS is the lowest concentration with maximum activity. Administration of *P.longum*, Piperine *T.occidentalis* and TPS showed increased number of total WBC count. This indicates they can stimulate the haemopoietic

Fig. VIII Effect of *P.longum* and Piperine on Antibody dependent complement mediated cytotoxicity (ACC)

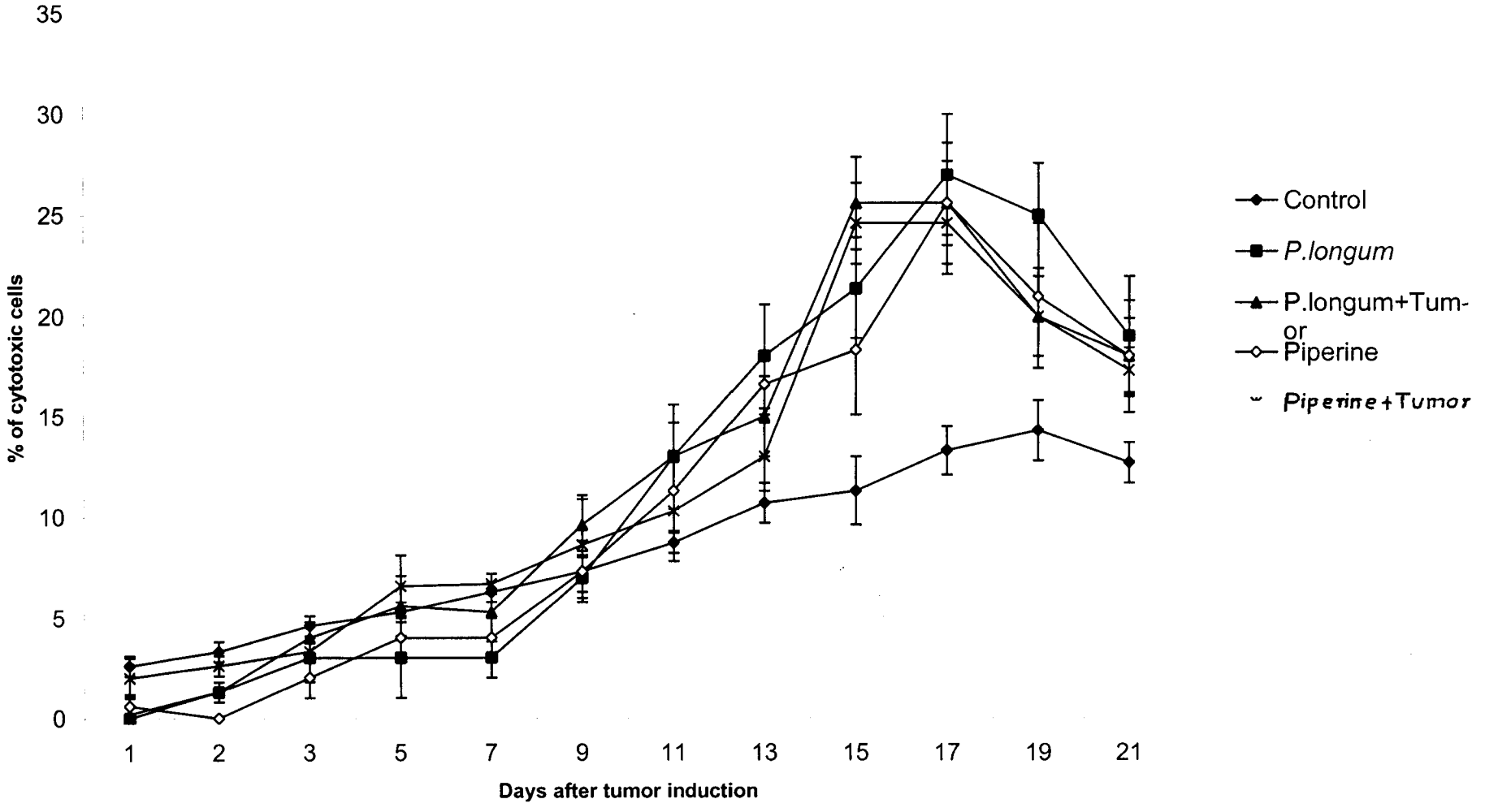
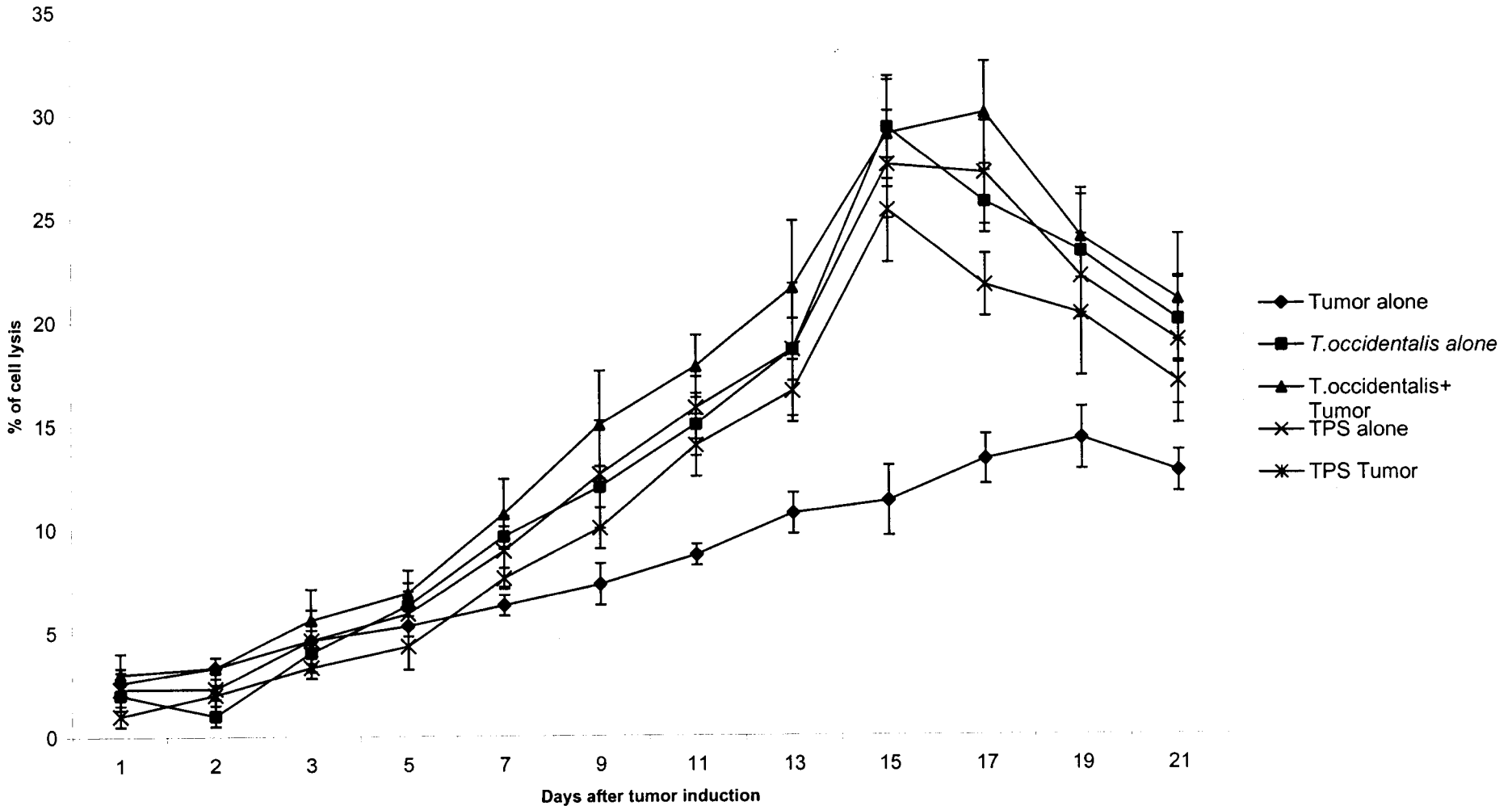


Fig. IX Effect of *T.occidentalis* and TPS on antibody dependent complement mediated cytotoxicity (ACC)



system. The differential count shows the drug did not alter the ratio of different WBC types.

Bone marrow serves as the major source of all blood cells including lymphocytes. Administration of *P.longum*, Piperine *T.occidentalis* and TPS showed an increase in bone marrow cellularity and α-esterase positive cells indicating its effect on stem cell proliferation and maturation.

P.longum, Piperine *T.occidentalis* and TPS was found to increase the circulating antibody titre and antibody forming cells indicating its stimulatory effect on the humoral arm of immune system.

P.longum, Piperine *T.occidentalis* and TPS was found to activate specific immune cells Treatment with these compounds was found to enhance the proliferation of spleenocytes, thymocytes and bone marrow cells directly and in the presence of different mitogens such as PHA, Con A, PWM and LPS. T-cells and B-cells contribute to the major effector mechanisms in cell-mediated immunity (Greaves et al, 1974).

Administration of *P.longum*, Piperine *T.occidentalis* and TPS enhanced the activity of non-specific immune cells such as NK cells. They are part of the host's innate or front line defense mechanism, as they require no adaptation of the host or immunization in order to react against certain tumor cells and virus infected cells (Shayana et al, 2004). Administration of these compounds enhanced the NK cell activity compared to tumor bearing control animals.

The presence of humoral immunity is not consistently correlated with

increased tumor resistance in the host (Benjamin et al 1982), but the antibodies can mediate cell destruction either via binding with complement or by acting as an opsonin to facilitate phagocytosis by macrophages or other phagocytic cell bearing Fc receptors and ADCC. ADCC is the co-operative interaction of humoral and cell mediated immune effector. The expression of NK cell activity and ADCC activity at an early stage of the tumor may lead to the exitration of tumor mass by enhancing the immunological status. *P.longum*, Piperine *T.occidentalis* and TPS was found to enhance ADCC and ACC activity in normal and tumor bearing animals and the maximum activity occurred much early compared to control animals.

Immunomodulatory activity of *P.longum*, Piperine *T.occidentalis* and TPS may be due to the combined action of humoral and cell mediated immune responses. One of the major objectives of the immunotherapy is to modulate immune responses for selected objectives. It include augmentation of cell mediated immunity and cytotoxic antibody. Modulation of immune responses may be achieved through the use of immunomodulators, also called immunopotentiators. Use of plant products as immunomodulators is still an interesting area in the research field.

The present study indicates that the *P.longum*, Piperine *T.occidentalis* and TPS could stimulate the stem cell proliferation and differentiation. It can also activate both cell mediated and humoral immune responses in mice. These results suggest that *P.longum*, Piperine *T.occidentalis* and TPS are a potent immunomodulator and could be used as adjuvant during cancer therapy.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

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Chapter 5

Antimetastatic activity of Piper longum, Piperine, Thuja occidentalis and Thuja polysaccharide fraction in in.vivo as well as in.vitro models.

Introduction

The process of cancer metastasis is multifaceted and complex since it depend upon the interaction of invasive tumor cells and their products. If any agent can interrupt any of these steps in the metastatic cascade, the probability for its clinical trials will be promising. Cytotoxic T-lymphocyte and NK cell activity play an important role in the immunological surveillance in neoplasia and metastasis (Brittenden, 1990). Therefore a number of immunotherapeutic approaches aiming to enhance NK cell activity have been investigated.

The proinflammatory cytokines such as IL-1ß, IL-6, TNF-∝, GM-CSF act as autocrine growth factors for tumor cells. These cytokines could be prometastatic or proangiogenic and their deregulated expression directly correlates with the metastatic potential of several human carcinomas (Frederick et al, 2003). The MMPS and TIMP, have a significant role in facilitating tumor invasion and metastasis (Curran et al, 2004), not only through their direct role in degrading extracellular matrix but also by interaction with other biological systems implicated in tumor invasion including cell adhesion molecules, cytoskeletal proteins and growth factors (Conor et al, 2002). Expression level of MMP-2 and MMP-9 are associated with tumor metastasis for various human cancers (Polette et al, 1996). Metastatic tumor express high levels of type IV collagenase activity than the non-metastatic tumors (John and Lynn, 1995).

Since immunity is suppressed in cancer and during cytoreductive therapy of cancer, use of indigenous drug in cancer therapy was found to be highly

indicative. The Piperine shows a potent antimetastastic activity using B16F-10 melanoma cells (Pradeep and Kuttan, 2002). In this chapter the antimetastatic activity of *P.longum*, *T. occidentalis* extract and Thuja TPS were studied using *in vitro* as well as *in vivo* models.

Materials and methods

Animals

Male C57BL/6 mice (6-8 weeks; 20-25g) were used for this study.

Cells

B16F10 melanoma cells, a highly metastatic cell line, K-562 leukemia cells and EL-4 thymoma cells were used for this study.

IN VIVO ANTIMETASTATIC STUDIES

Drug administration

The animals were intraperitoneally treated with of alcoholic extract of *Piper longum* (10mg/dose/animal), Piperine (1.14mg/dose/animal), *Thuja occidentalis* (5mg/dose/animal) and TPS (1mg/dose/animal) simultaneously with metastatic tumor cells from the same day of tumor inoculation. The drugs were continued for 10 consecutive days.

Metastasis induction

The animals were induced metastasis by injecting B16F-10 melanoma cells (10^6cells/animal) via the lateral tail vein (Fidler, 1978).

Determination of the effect of *P.longum*, *T.occidentalis* and TPS on the lung tumor nodule formation

The animals were induced metastasis as described above and were

separated into four groups (14mice/group). The Ist, IInd, and IIIrd group were treated with *P.longum, T. occidentalis* and TPS respectively. Group IV was kept as untreated control. The animals (8 animals from each group) were sacrificed after 21 days of tumor inoculation, lungs were excised and the lung tumor nodules were counted. The lungs were used to estimate the levels of lung collagen hydroxyproline, lung uronic acid, and lung hexosamine (Bergman and Loxley, 1970; Elson and Morgan, 1933; Bitter and Muir, 1962), and were also used for histopathological analysis. Blood was collected by heart puncture, serum separated and used to estimate serum sialic acid (Bhavanandan et al., 1981) and gamma-glutamyl transpeptidase (GGT) level (Szasz, 1976) as described in chapter 2.

Determination of the effect of *P.longum*, *T.occidentalis* and TPS on the survival rate of metastatic tumor bearing animals

Six animals from each group of the previous experiment were observed for their survival rate and the percentage of increase in life span (ILS) was calculated as described in chapter 2.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on cell mediated immune response in metastatic tumor bearing animals.

C57BL/6 mice (4-6 weeks old, male) were divided into nine groups with 12 animals in each group. Group I was kept as untreated control and group II- V animals were intraperitoneally treated with five doses of *P.longum*, Piperine, *T.occidentalis* and TPS. Group I, and VI-IX animals were treated as above and the animals were induced metastasis using B16F-10 melanoma cells. At various time intervals, animals

were sacrificed, spleen and blood was collected and processed. Spleen cells were used as effector cells for assaying the NK-cell activity (Gupta, 1993) and ADCC by 4 h ^{51}Cr-release assay (Tadaki et al, 1999). Serum was used as anti-EAC antibody for ACC by trypan blue exclusion method (Kuttan et al, 1985) as explained in chapter 2.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on cytokine production in metastatic tumor bearing animals.

Five groups of C57BL/6 mice were induced metastasis. Group I was kept as untreated metastatic tumor bearing controls. Group II-V animals were treated intraperitoneally with *P.longum*, Piperine, *T.occidentalis* and TPS respectively. Three animals from each group were sacrificed at two different time points (7^{th} and 21^{st} days after tumor inoculation). Blood was collected, serum separated and cytokines such as IL-1β, IL-6, TNF-α, GM-CSF, IL-2, and TIMP were assayed using respective ELISA kits.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the expression level of k-ras, nm-23, prolyl hydroxylase, lysyl oxidase, MMP 2 and 9 and TIMP 1 and 2

Five groups of C57BL/6 mice were induced metastasis using B16F-10 melanoma cells. Group I was served as untreated control. Group II- IV were treated with *P.longum*, Piperine, *T.occidentalis* and TPS respectively for 10 consecutive days. The animals were sacrificed on 21^{st} day. The lungs were excised and RNA was isolated using guanidium thiocynate by the method of Chomczynski et al. PCR was performed using specific primers of k-ras, nm-23, prolyl hydroxylase, lysyl oxidase (LOX), MMP-9, MMP-2, TIMP 1 and TIMP 2.

PCR products were analyzed by agarose gel electrophoresis and visualized using the ethidium bromide dye.

In vitro antimetastatic studies

Drug exposure

Based on the cytotoxicity analysis B16F-10 melanoma cells were treated in the presence or absence of non-toxic concentrations of *Piper longum* (2, 5 and10μg/ml), *Thuja occidentalis* (1, 2 and 5μg/ml) and TPS (0.01, 0.05 and 1μg/ml) for *in vitro* analysis.

Determination of the effect of *P.longum*, *T.occidentalis* and TPS on the inhibition of B16f-10 melanoma cell growth.

B16F-10 melanoma cells (5000cells/well) were plated in a 96 well flat bottom titre plate and incubated at 37°C in 5% CO_2 atmosphere. After 24 hours different concentrations (1-200μg/ml) of the *P.longum*, *T.occidentalis* and TPS were added and the incubation was continued for 48 h under the same conditions. Cell viability was determined by the MTT assay as described in chapter-2.

Tumor cell adhesion assay

Tumor cell adhesion assay was carried out by the method of Inokuchi et al (Inokuchi, 1990) as described in Chapter2. Briefly, B16F-10 melanoma cells were seeded on to type-I collagen coated wells of flat-bottomed titre plates, in the presence and absence of different concentrations of *P.longum, T. occidentalis* and TPS 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.

Collagen matrix invasion assay

The invasion assay was carried out in Boyden chambers as described by Albini et al (Albini et al, 1987). Briefly the lower compartment of the chamber was filled with DMEM and a type IV collagen coated polycarbonate filter membrane was placed in between the two chambers. B16F-10 cells ($1X10^5$/0.15ml) were then seeded on to the upper chamber in the presence and absence of different non toxic concentrations of *P.longum, T. occidentalis* and TPS. The chambers were then incubated at 37°C for 10h in a 5% CO_2 atmosphere. After incubation period the medium and cells were removed from the upper chamber and the membrane was fixed as explained in chapter 2. The triplicate results were expressed as percentage inhibition of invasion.

Tumor cell motility assay

Tumor cell motility assay was performed in the same manner as the invasion assay except that polycarbonate filters were collagen free. *P.longum, T. occidentalis* and TPS were added along with B16F-10 melanoma cells to the upper compartment of the Boyden chamber. After incubation at 37°C for 24h, the number of cells migrating to the lower chamber was determined using a haemocytometer. The results are expressed as percentage inhibition of motility.

Gelatin zymography

SDS- PAGE was performed according to the modified procedure of kau et al (kau et al, 2003). B16F-10 melanoma cell culture supernatant of treated [*Piper*

longum (2, 5 and10µg/ml), *Thuja. occidentalis* (1, 2 and 5µg/ml) and TPS (0.01, 0.05 and 1µg/ml)] and untreated cells were subjected to zymographic analysis with or without trypsin activation. 5µl of trypsin solution (75µg/ml) in 0.1M Tris-HCl, 10mM $CaCl_2$ buffer (pH 8.0) and incubated for 1h at room temperature. Samples were mixed with an equal volume of 2X sample buffer and loaded on to 11% polyacrylamide gels containing 0.8% gelatin. Electrophoresis was carried out until the bromophenol blue reached the periphery. Gels were then washed with 2% Triton x-100 in 0.1M Tris-HCl, 10mM $CaCl_2$ at 37°C for 18h followed by staining with Gel code for 2h and visualize the clear area against the light background.

Determination of the effect of *P.longum*, *T.occidentalis* and TPS on the gene expression of IL-1ß, IL-6, GM-CSF, TNF-∝ and IL-12p40

B16F-10 cells ($2X10^4$cells/well) were grown in 96-well titre plate and the cells were incubated in the presence and absence of *P.longum* (10µg/mL), *T.occidentalis* (5µg/ mL) and TPS (1µg/ mL) for 4h at 37°C in 5% CO_2 in serum free medium. Total RNA was extracted from B16F-10 cells and cDNA was synthesized, using moloney murine leukemia virus reverse transcriptase. PCR was performed with Biosource message screen™ Mouse inflammatory cytokine multiplex PCR kit. This kit has been designed to detect the expression of mouse GAPDH, IL-1ß, IL-6, GM-CSF, TNF-∝ and IL-12p40 genes. The PCR primers have similar Tm's and no obvious 3' end overlap to enhance amplification of multiple targets. The target PCR products also generated from a positive control cDNA which was included in this kit. PCR products were analysed by agarose gel

electrophoresis and visualized by ethidium bromide.

Results

IN VIVO ANTIMETASTATIC STUDIES

Effect of *P.longum*, *T.occidentalis* and TPS on lung tumor nodule formation

There was a significant reduction in lung tumor nodule formation when the animals were treated with alcoholic extract of *T occidentalis* followed by TPS and *P.longum* (Table I).

The untreated control animals developed a massive number of tumor nodules on their lungs and were assigned an arbitrary number of 250 (Hill, 1994). A better inhibition of tumor nodule formation was observed when the *T occidentalis* extract was administered (74.4%) and TPS (71.8%). When the extract of *P.longum* was administered there was a moderate inhibition (57.6%) of tumor nodule formation.

Effect of *P.longum*, *T.occidentalis* and TPS on the survival rate of tumor bearing animals

The effect of alcoholic extract of *P.longum*, *T.occidentalis* and TPS on the survival rate of metastatic tumor bearing mice is shown in Table 2. Control metastatic tumor bearing mice survived only up to 32 days (Table I). When the animals were treated with, *T.occidentalis,* TPS, and *P.longum*, the lifespan of the animals was increased to 120.39%, 109.6%, and 94.53% respectively.

Table I

Effect of *P.longum*, *T.occidentalis* and TPS on lung tumor nodule formation and survival rate of metastatic tumor bearing animals

Treatment	No. of nodules per lung	% inhibition of tumor nodule	% of increase in life span (%ILS)
Control	250	-	-
P. longum	106.0 ± 9.8	57.6	109.6
T. occidentalis	64.0 ± 10.5	74.4	120.39
TPS	71.3 ± 12.7	71.5	109.6

B16F-10 melanoma cells (10^6) were injected via the lateral tail vein. *P. longum* (10mg/ml), *T.occidentalis* (5mg/ml) and TPS (1mg/ml) was administered intraperitoneally for 10 consecutive days. Values are the mean ± SD. Statistically significant from untreated controls.

*$P < 0.005$

Effect of *P.longum*, *T.occidentalis* and TPS on the lung collagen hydroxyproline content, lung uronic acid and the lung hexosamine content

The effect of *P.longum*, *T.occidentalis* and TPS on the lung collagen hydroxyproline content is shown in Table II. Tumor bearing animals showed a drastically increased level of lung collagen hydroxyproline (21.13 μg/mg protein) compared to normal animals (0.98 μg/mg protein). This elevated level was significantly reduced when the animals were treated with the extract of Thuja (4.75 μg/mg protein) followed by TPS (6.73 μg/mg protein) and *P.longum* (12.35 μg/mg protein).

The effect of *P.longum*, *T.occidentalis* and TPS on lung uronic acid content is shown in Table II. Uronic acid content of normal animals is 35μg/100mg tissue. The tumor bearing control animals showed elevated levels of uronic acid in their lung tissue (349.5μg/100mg tissue), which was significantly reduced, in the treated animals. Treatment of Thuja extract simultaneously with the tumor cells lowered the uronic acid level to 105.5μg/100mg tissue. *P.longum* and TPS treatment also showed significant reduction in uronic acid level (155.6μg/100mg tissues, 117.4μg/100mg tissues respectively). These reduced levels of uronic acid indicate the decreased lung fibrosis in drug-treated animals.

The effect of *P.longum*, *T.occidentalis* and TPS on the lung hexosamine content is shown in Table II. The hexosamine content of the normal animals is 0.53mg/100mg lyophilised tissue. There was an increased level of lung hexosamine content in the control tumor bearing animals (4.03mg/100mg lyophilised tissue) and a reduced level in the treated animals. Administration of

Table II

Effect of *P.longum*, *T.occidentalis* and TPS on the lung collagen hydroxyproline, lung uronic acid, lung hexosamine levels of tumor bearing animals.

Treatment	Lung collagen Hydroxyproline (µg/mg protein)	Lung uronic acid (µg/100 mg tissue)	Lung hexosamine (mg/100 mg lyophylised tissue)
Normal	0.98 ± 0.07	35.0± 1.9	0.53± 0.03
Control	21.13± 2.03	349.5± 29.7	4.03± 0.41
P. longum	12.35± 1.6*	155.6± 10.1*	2.05± 0.30*
T. occidentalis	4.75± 0.7*	105.5± 14.9*	1.22± 0.26*
TPS	9.66± 1.9*	130.6± 16.5*	1.86± 0.32*

Experimental design is given in Table-2. Animals were sacrificed on the 21st day and their lungs were excised. Values are the mean ± SD. Statistically significant from untreated control.

*$P < 0.001$

Thuja extract showed a better inhibition of lung hexosamine content (1.22mg/100mg lyophilised tissue) followed by the administration of TPS and *P.longum* (1.81mg/100mg lyophilised tissue, 2.05mg/100mg lyophilised tissue respectively). The reduced level of lung hexosamine also indicates a decreased tumor burden.

Effect of *P.longum*, *T.occidentalis* and TPS on the serum sialic acid and GGT levels

The effect *P.longum*, *T.occidentalis* and TPS on the serum sialic acid level of metastatic tumor bearing animals is given in Table III. The serum sialic acid level was drastically elevated in the control group (102.9µg/ml serum) compared to normal animals (23.2µg/ml). *T.occidentalis* as well as TPS treatment significantly lowered the sialic acid levels (35.98µg/ml and 42.36µg/ml serum respectively). *P.longum* extract treatment also showed a reduction (54.6µg/ml) in serum sialic acid.

The effect of *P.longum*, *T.occidentalis* and TPS on serum GGT levels is shown in Table 4. The higher content of GGT in the control animals (105.9nmol p-nitroaniline/ml serum) was significantly reduced in treated animals. *T.occidentalis* and TPS treatment significantly reduced the GGT level to 40.67nmol p-nitroaniline/ml and 44.7nmol/p-nitroaniline/ml respectively. A reduction in the level of GGT (51.3nmole p-nitroaniline/ml) was also shown in the animals treated with *P.longum*. Normal level of GGT is 25.6nmole p-nitroaniline/ml.

Table III

Effect of *P.longum*, *T.occidentalis* and TPS on the serum sialic acid and serum GGT level of metastatic tumor bearing animals

Treatment	Serum sialic acid (μg/ml serum)	Serum GGT (nmol p-nitroaniline/ml serum)
Normal	23.2± 1.3	25.7±.1
Control	102.9± 16.3	105.9±2.3
P. longum	41.0± 8.8*	37.8± 11.9*
T. occidentalis	35.9± 4.9*	40.6± 6.8*
TPS	42.3± 3.8*	44.7± 3.9*

Experimental design is given in Table-2. The animals were sacrificed on the 21st day, blood collected by heart puncture, and serum separated. Values are the mean ± SD. Statistically significant from untreated control.

*P < 0.001

Effect of *P.longum*, *T.occidentalis* and TPS on Lung Architecture.

Histopathological analysis of lung tissue showed infiltration of neoplastic cells around main bronchioles and along the pleura in the control tumor bearing animals (Fig I). The tumor nodules were composed of polygonal tumor cells with a prominent nucleus. Intracellular melanin deposition and a clear area of necrosis were also present. Lung tissue of *P.longum*, *T.occidentalis* and TPS treated animals showed a reduction in tumor mass and there were reduced number of tumor cells in alveoli and pleura.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the cell mediated immune responses of metastatic tumor bearing animals.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on natural killer (NK) cell activity

As shown in Fig II, administration of *P.longum*, Piperine *T.occidentalis* and TPS enhanced the NK cell activity significantly. A maximum NK cell mediated cell lysis was observed after 7 days (35.5% cell lysis, 42.6% cell lysis, 39.5% cell lysis) of tumor induction in *P.longum*, *T.occidentalis* and TPS treated group respectively. But Piperine showed a maximum NK cell activity (39.6% cell lysis) on 5^{th} day. The metastatic tumor bearing control animals showed maximum NK cell activity 21.5% cell lysis and it was observed only after 13 days of tumor induction.

Figure I

Effect of *P.longum, T.occidentalis* and TPS on the histopathology of the lungs of metastatic animals.

a) Control

b) Normal

c) Treated with *P. longum*

d) Treated with *T.occidentalis*

e) Treated with TPS

127.B

Fig. I Effect of *P.longum*, *T.occidentalis* and TPS on the histopathology of the lungs of Metastatic animals

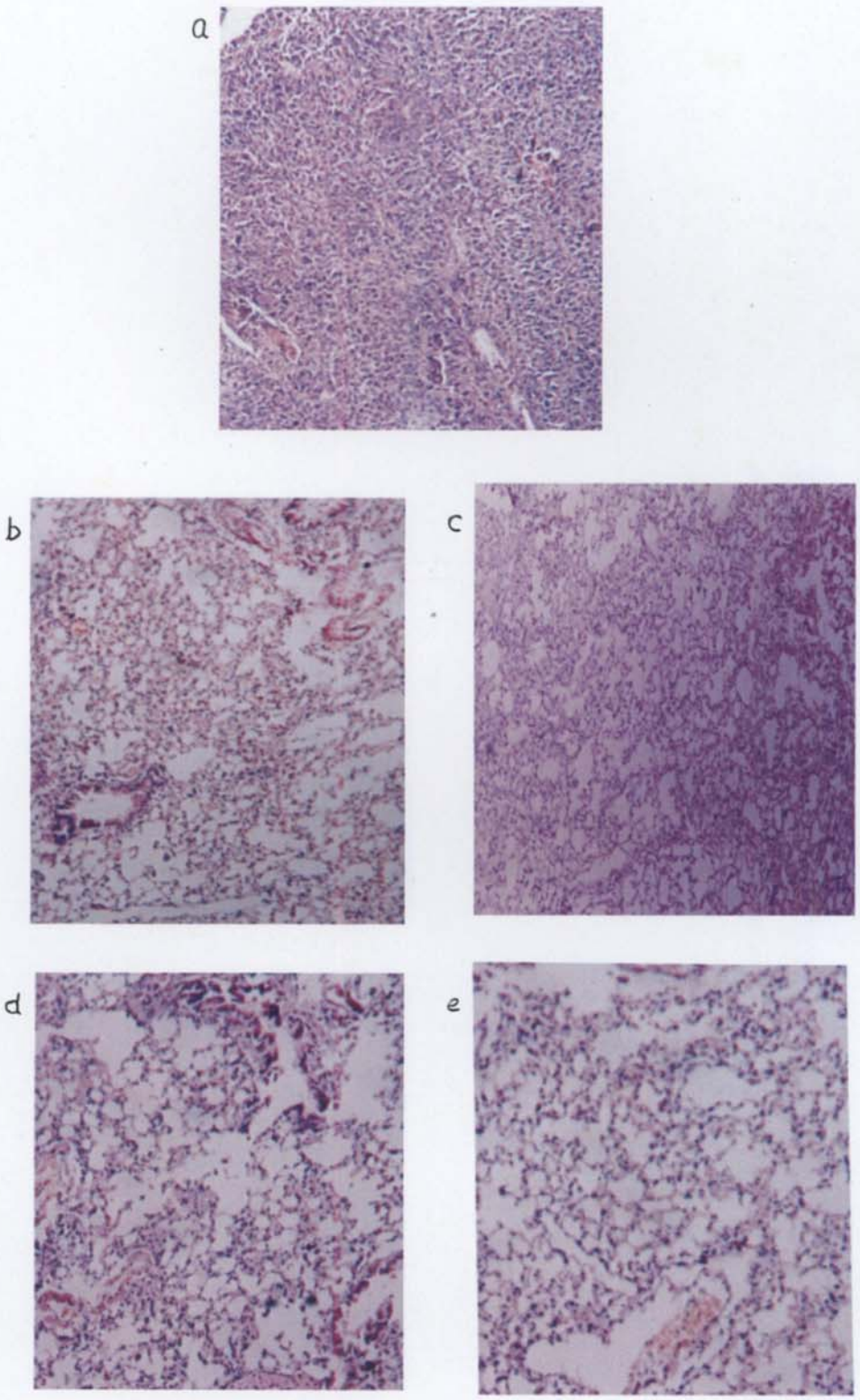
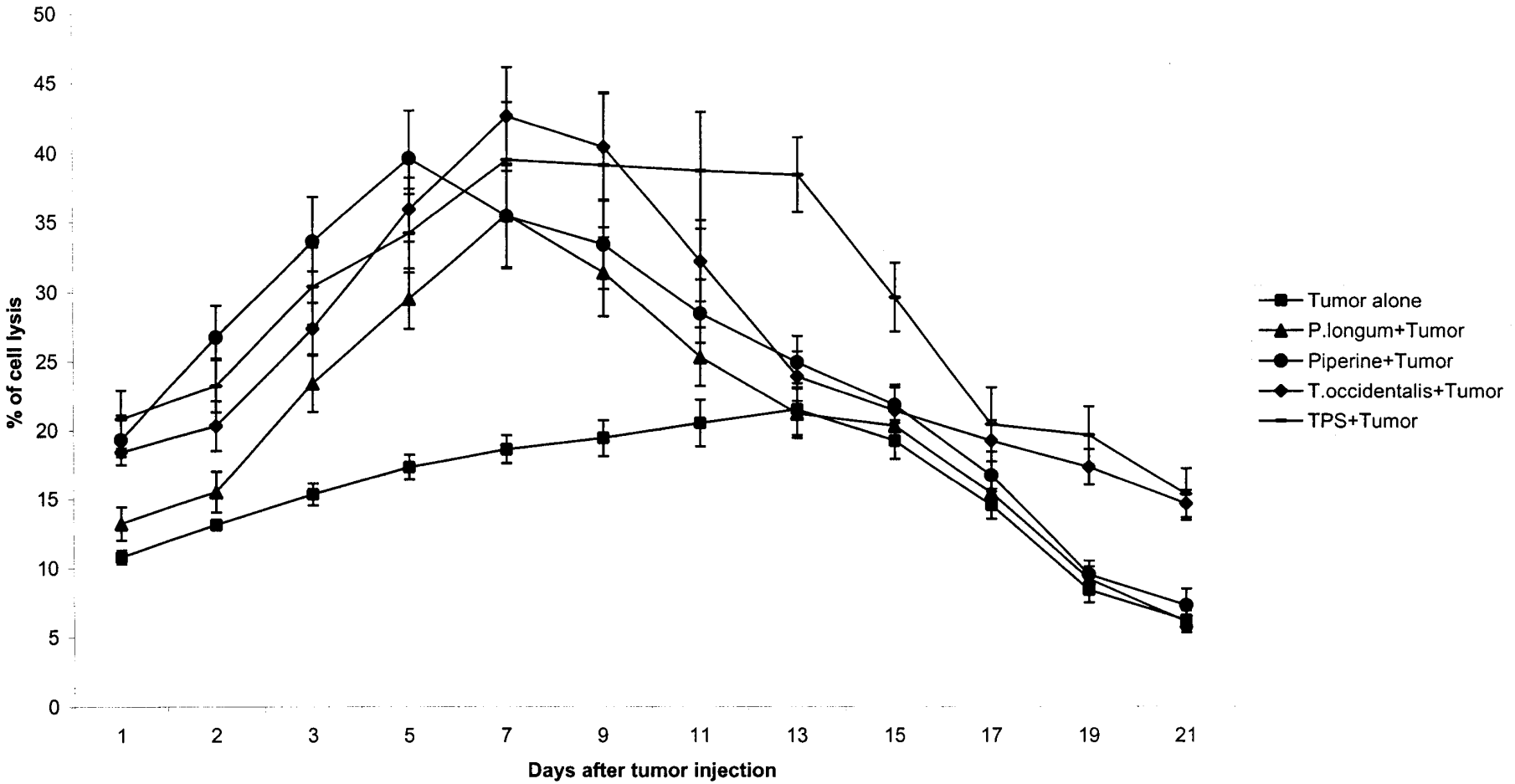


Fig. II Effect of *P. longum* , Piperine, *T. occidentalis* and TPS on Natural killer cell activity in metastatic tumor bearing animals



Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on antibody-dependent cellular mediated cytotoxicity (ADCC)

The ADCC activity of *P.longum*, Piperine *T.occidentalis* and TPS treated animals was increased to 39%, 45.2%, 43.4% and 41.7% cell lysis on 9th day respectively (Fig III), while it was only 17.4% cell lysis in control animals on the same day. The maximum ADCC activity in control animals (20.3% cell lysis) was observed only on 13th day after tumor induction.

Effects of *P.longum*, Piperine, *T.occidentalis* and TPS on antibody-dependent complement mediated cytotoxicity (ACC).

The ACC activity was elevated in the *P.longum*, Piperine *T.occidentalis* and TPS treated metastatic tumor bearing animals (Fig IV). In *P.longum*, Piperine treated animals, the maximum ACC was observed on 17th day (23.4% and 26.7% cell lysis) while in the case of *T.occidentalis* and TPS (21.7% and 20.4% cell lysis) it was observed on 15th day. Untreated tumor bearing control animals a maximum cell lysis of 14% was observed only on 17th day.

Effect of *P.longum*, *T.occidentalis* and TPS on pro-inflammatory cytokines in metastasis-induced animals

Administration of *P.longum*, *T.occidentalis* and TPS showed varying pattern of regulation of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of metastasis-induced animals during the period of study. In control animals, 7 days after tumor cell inoculation, the level of IL-1β in the serum was drastically elevated (44.8±4.9pg/ml) compared to normal level (16±3.5pg/ml). On 21st day after tumor inoculation, *P.longum*, Thuja and TPS

Fig.III Effect of *P.longum* , Piperine, *T. occidentalis* and TPS on Antibody dependent cellular cytotoxicity in metastatic tumor bearing animals

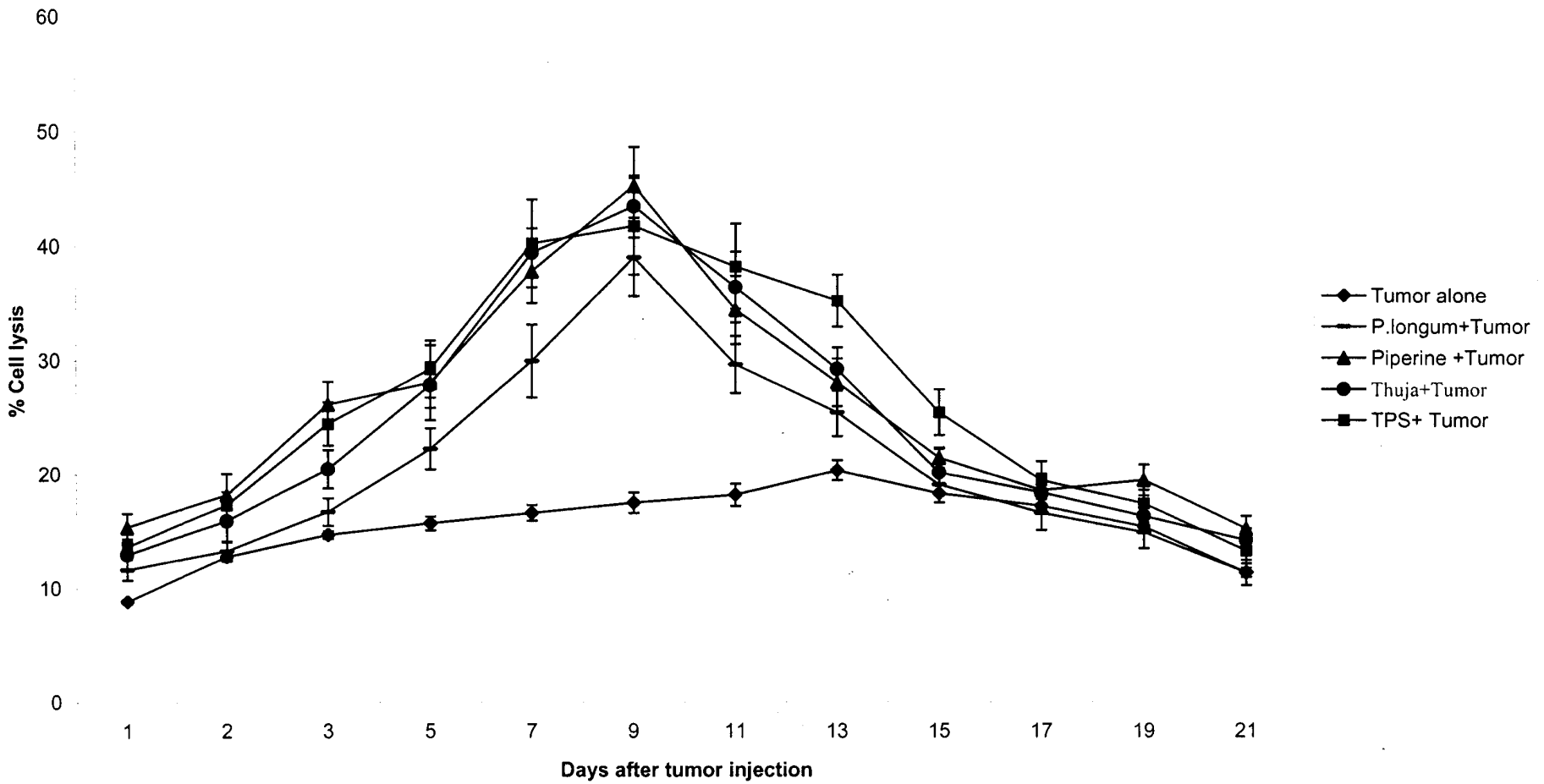


Fig. IV Effect of *P.longum* , Piperine, *T.occidentalis* and TPS on Antibody dependent complement mediated cytotoxicity in metastatic tumor bearing animals

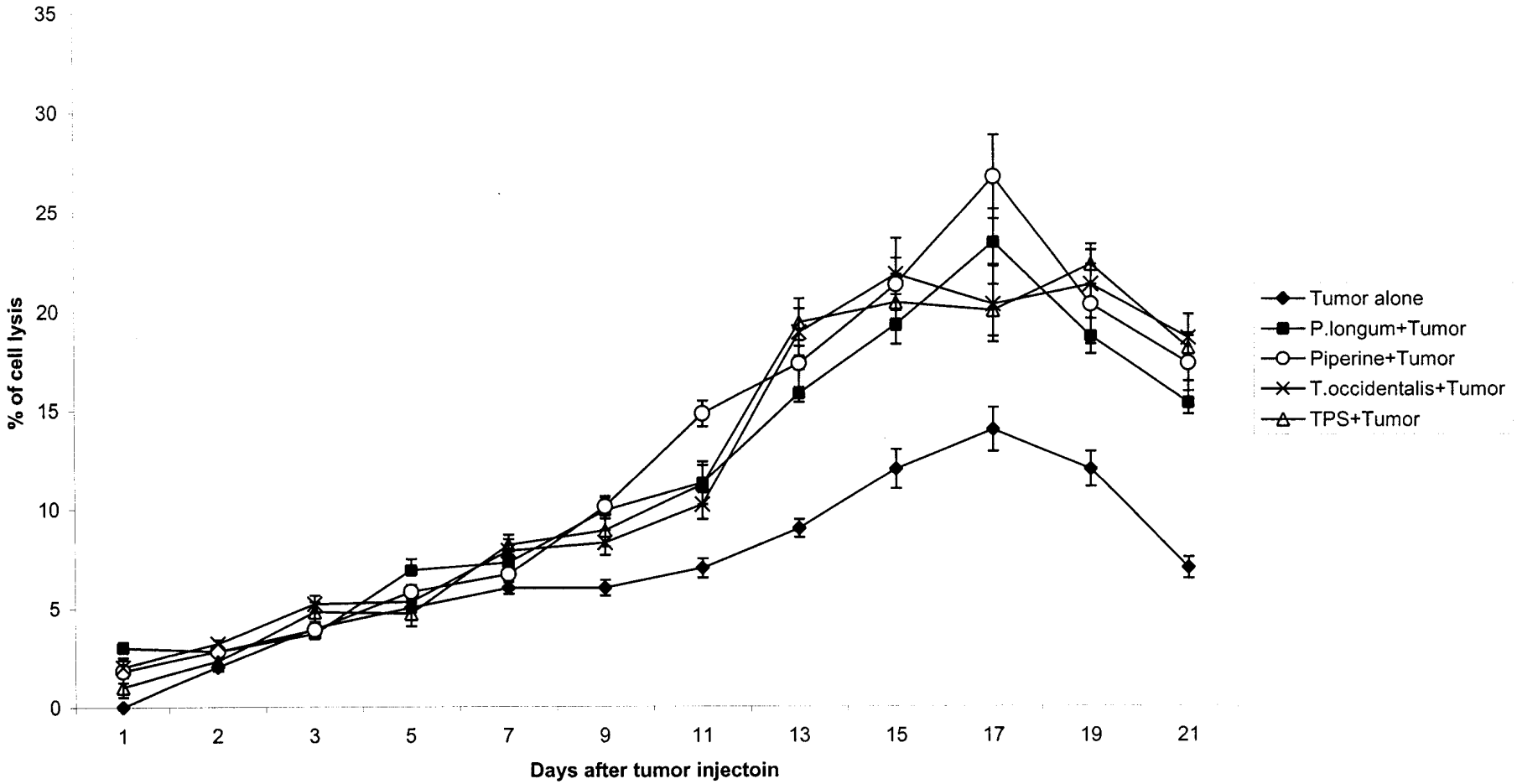


Table IV

Effect of *P.longum*, *T.occidentalis* and TPS on pro-inflammatory cytokine profile of metastasis-induced animals

	Normal	Control		*Piper longum*		*T.occidentalis*		TPS	
Cytokines (pg/ml)	Day 0	Day 7	Day 21	Day 7	Day 21	Day7	Day 21	Day7	Days21
IL-1β	16 ± 3.5	44.8± 4.9	59.4 ±5.7	40.8±3.4	36.1±2.8	38.6±2.5	20.3±2.7	39.4±3.1	23.9±2.6
IL-6	35 ± 6.5	353.4±39.1	593 ±47.3	89.8 ±9.3	310.8 ± 28.4	73.9±6.5	255.3±29.7	92.9±7.3	273.4±29.8
TNF-α	20 ± 1.8	241.8±20.7	317.2 ±26.7	146.7± 14.3	63.11 ±6.2	134.4±12.4	31.1±2.3	149.4±17.1	42.4±3.6
GM-CSF	18 ± 0.94	38.38 ±2.7	20.8 ± 1.5	33.4 ±2.7	19.4± 2.0	30.7±2.9	16.6±0.5	34.1±4.7	19.3±1.5
IL-2	23 ± 2.7	22.4± 2.0	20.9 ± 2.4	30.1 ±4.2	26.4±1.9	38.0±2.7	36.1±3.	29.8±2.4	28.5±2.8
TIMP	652 ± 46.8	514 ± 69.8	701 ± 93.4	570 ±49.8	617±38.7	656±41.3	701±93.4	593.1±21.7	623.4±82.1

All the values are mean ± SD. Blood was collected from the metastasis induced animals (see Table I) at the indicated time points after tumor challenge. Cytokine level was estimated by ELISA method.

administration effectively reduced the IL-1ß levels (36.1±2.8pg/ml, 20.3±2.7pg/ml, 23.9±2.6pg/ml respectively) to normal level, compared to the highly elevated level in the control animals (59.4±5.7pg/ml) (Table IV).

TNF-α level was also drastically elevated in the serum of control animals (317.2±26.7pg/ml) after 21st day of tumor induction compared to the level of TNF-α (20±1.8pg/ml) in normal animals. Administration of *P.longum*, *T. occidentalis* and TPS could effectively down regulate the elevated level of TNF-α to 63.11±6.2pg/ml, 31.1±2.3pg/ml and 42.4±3.6pg/ml by 21st of day of tumor induction (Table IV).

Similarly administration of *P.longum*, *T. occidentalis* and TPS could lower the elevated level of IL-6 (310.8±28.4pg/ml, 255.3±29.7pg/ml and 273.4±29.8pg/ml respectively), compared to the control animals (593±47.3pg/ml) after 21st day of tumor induction. The normal level of IL-6 is 35.3±6.5 pg/ml (Table IV).

Effect of *P.longum*, *T.occidentalis* and TPS on the colony stimulating factor levels in B16F-10 cell injected animals

The level of growth factor GM-CSF was determined using ELISA kit. In control animals, the level of GM-CSF (38.38±2.7pg/ml) in the serum was elevated after 7 days of tumor induction, compared to the normal levels of GM-CSF (18±0.94pg/ml). Administration of *P.longum* (19.4±2.0pg/ml), *T.occidentalis* (16.6±0.54pg/ml) and TPS (19.3±1.5pg/ml) could effectively lower the elevated level of GM-CSF to a normal range (18±0.94pg/ml) on 21 day after tumor induction (Table IV).

Effect of *P.longum*, *T.occidentalis* and TPS on the IL-2 and TIMP-1 profile of metastasis induced animals

The lowered level of IL-2 in the control animals (20.9±2.4pg/ml) were elevated by the *P.longum* (26.4±1.9pg/ml), Thuja (36.1±3.1pg/ml) and TPS (28.5±2.8pg/ml) administration after 21 days of metastasis induction (Table 4).

The tissue inhibitor of metalloprotease level in the serum of normal mice was 652±46.8 pg/ml, which was reduced by the induction of tumor cell to 514±19pg/ml in the untreated control animals 7 days after tumor induction. But *P.longum* (570±49.8pg/ml), *T. occidentalis* (656±41.3pg/ml) and TPS (593.1±21.7pg/ml) treatment could elevate the levels of this MMP inhibitor on the same day. Blood sample on 21st day also showed a similar TIMP-1 profile in metastasis induced animals. In control, it was only 468±13.6pg/ml and *P.longum* (617±38.7pg/ml), *T.occidentalis* (701±93.4pg/ml) and TPS (623.4±82.1pg/ml) administration could maintain the initial elevation (Table IV).

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the expression of nm-23, prolyl hydroxylase, lysyl oxidase, MMP 2, MMP 9, TIMP 1 and TIMP 2 genes

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the gene expression of k-ras, nm-23, prolyl hydroxylase, lysyl oxidase (LOX), MMP 2, MMP 9 TIMP 1 and TIMP 2 is given in fig V and VI. Treatment with Piperine and *T occidentalis* and could effectively enhance the expression of nm-23, TIMP 1 and TIMP 2 genes. Administration of *P.longum* and TPS could not enhance the expression of these genes significantly. While the expression of k-ras, prolyl

Figure V

Effect of *P.longum,* Piperine, *T.occidentalis* and TPS on the gene expression of k-ras, nm-23, prolyl hydroxylase and lysyl oxidase in metastasis bearing mice.

a) Lane 1, Marker

b) Lane 2, Untreated control

c) Lane 3, *P.longum*

d) Lane 4, Piperine

e) Lane 5, *T.occidentalis*

f) Lane 6, TPS

g) Lane 7, GAPDH

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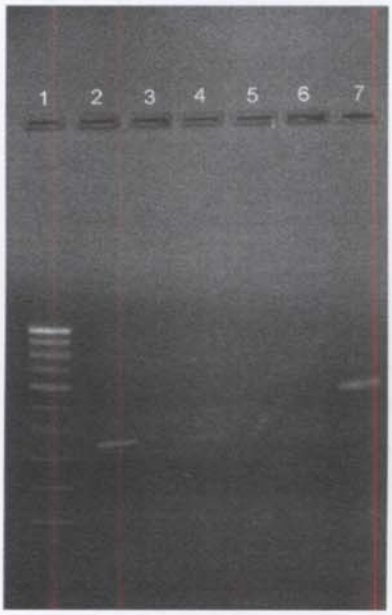
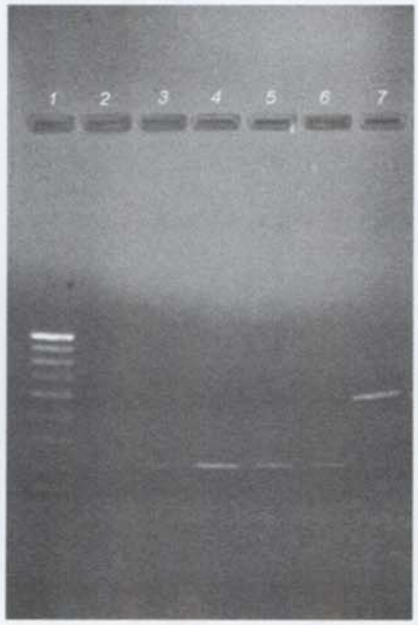
Fig. V Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the k-ras, nm-23, prolyl hydroxylase and lysyl oxidase gene expression

k-ras



nm-23



Prolyl hydroxylase

Lysyl oxidase

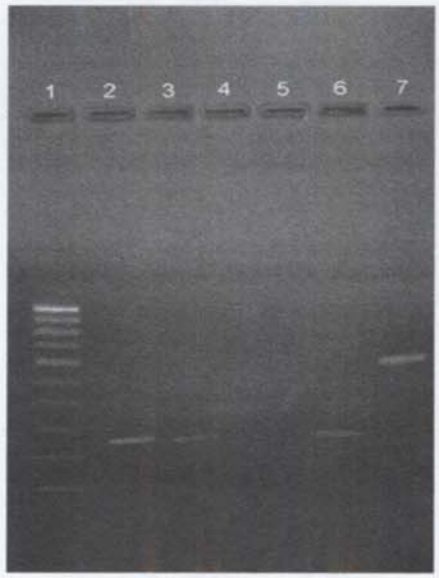


Figure VI

Effect of *P.longum,* Piperine, *T.occidentalis* and TPS on the gene expression of MMP 2, MMP 9, TIMP 1 and TIMP 2 in metastasis bearing mice.

h) Lane 1, Marker

i) Lane 2, Untreated control

j) Lane 3, *P.longum*

k) Lane 4, Piperine

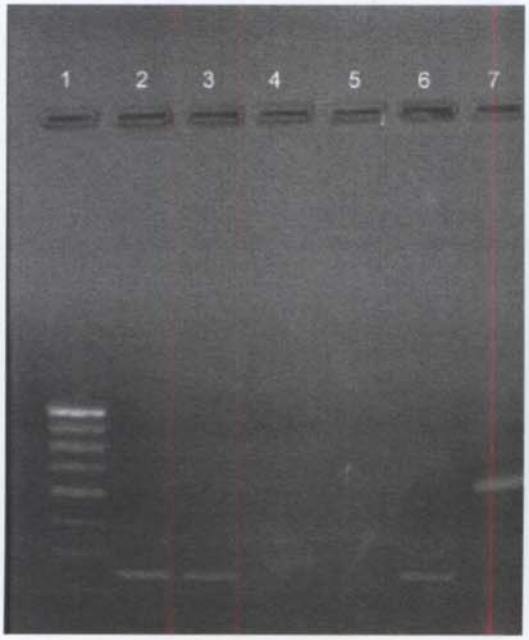
l) Lane 5, *T.occidentalis*

m) Lane 6, TPS

n) Lane 7, GAPDH

Fig. VI Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the MMP and TIMP expression

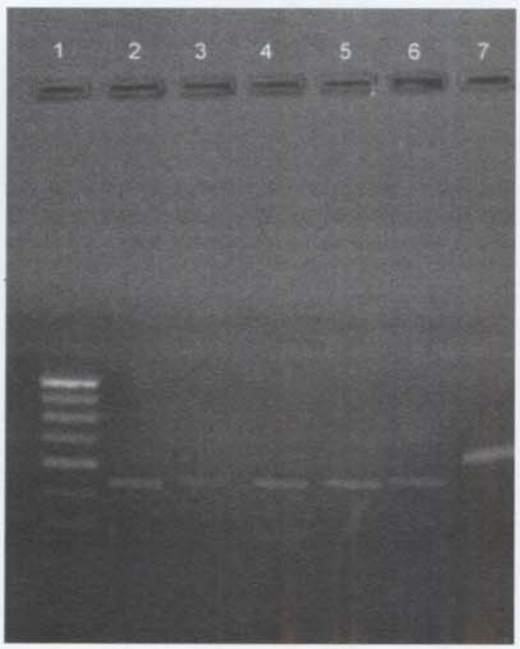
MMP-2



MMP-9

TIMP-2

TIMP-9



hydroxylase, lysyl oxidase, MMP 1, and MMP 9 genes were considerably reduced by the treatment with *P.longum*, *T. occidentalis* and TPS.

In vitro antimetastatic studies

Effect of *P.longum*, *T.occidentalis* and TPS on the Inhibition of Tumor Cell Growth

Alcoholic extracts of *P.longum, T.occidentalis* and TPS were found to be 100% cytotoxic to B16F-10 melanoma cells at a concentration of 200 µg/ml and *P.longum* was non-toxic at a concentration of 25µg/ml. *T.occidentalis* and TPS was found to be non toxic at 5µg/ml (Table V).

Effect of *P.longum*, *T.occidentalis* and TPS on the adhesion of B16F10 melanoma cells to the collagen matrix

Effect of *P.longum*, *T.occidentalis* and TPS on the adhesion of B16F10 melanoma cells to the collagen is given in Table-VI. *P.longum*, *T.occidentalis* and TPS inhibited the tumor cell adhesion in a dose dependent manner. *T.occidentalis* at concentrations of 0.5µg/ml 1µg/ml and 5µg/ml inhibited the adhesion of B16F-10 melanoma cells by 7.61%, 21.86% and 67.61% respectively. TPS (1µg/ml) and *P.longum* (10µg/ml) at higher concentrations inhibited the adhesion by 58.76% and 51.27% at high respectively.

Effect of *P.longum*, *T.occidentalis* and TPS on the Collagen matrix invasion of B16F-10 melanoma cells

The effect of *P.longum, T.occidentalis* and TPS on the invasion of B16F-10 melanoma cells is shown in Fig. VII. Metastatic B16F-10 melanoma cells show high invasive property through the collagen matrix. In untreated control,

Table V

Cytotoxicity of *P.longum*, *T.occidentalis* and TPS on B16F-10 cells in culture

Concentration µg/ml	% of cytotoxicity *P.longum*	*T.occidentalis*	TPS
200	100	100	100
100	63.2	100	54.3
50	23.7	72.4	22.1
25	7.2	46.4	15.8
10	0.0	12.4	11.4
5	0.0	4.3	5.0
2	0.0	2.1	1.0
1	0.0	0.0	0.0

B16F-10 melanoma cells 5000 cells/well were plated in a 96 well flat bottom titre plate with different concentrations of *P.longum*, *T.occidentalis* and TPS and cell viability was determined by MTT assay.

Table VI

Effect of *P.longum*, *T.occidentalis* and TPS on the Adhesion of B16F-10 melanoma cells to the collagen matrix

Treatment		% inhibition of adhesion
P.longum	10µg/ml	51.2±3.2
	5µg/ml	27.3±1.8
	1µg/ml	4.5± 2.1
T.occidentalis	5µg/ml	67.6± 5.3
	2µg/ml	21.8±1.3
	1µg/ml	7.6± 0.4
TPS	1µg/ml	58.7± 3.0
	0.5µg/ml	19.6± 1.7
	0.25µg/ml	6.7± 0.9

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 *P.longum*, *T.occidentalis* and TPS for 4h at 37^0C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.

Table VII

Effect of *P.longum*, *T.occidentalis* and TPS on the Invasion of B16F-10 melanoma cells through the collagen matrix

Treatment		% inhibition of Invasion
P.longum	10µg/ml	69.3± 1.32
	5µg/ml	55.4± 0.82
	1µg/ml	40.5± 1.21
T.occidentalis	5µg/ml	86.4± 2.34
	2µg/ml	68.5±1.83
	1µg/ml	53.4.0±2.14
TPS	1µg/ml	79.73± 2.04
	0.5µg/ml	57.34± 1.9
	0.25µg/ml	48.43± 0.89

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

Effect of *P.longum, T.occidentalis* and TPS on the invasion of B16F-10 melanoma cells

a) Untreated control

b) *P.longum* treated (2μg/ml)

c) *P.longum* treated (5μg/ml)

d) *P.longum* treated (10μg/ml)

e) *T.occidentalis* treated (1μg/ml)

f) *T.occidentalis* treated (2μg/ml)

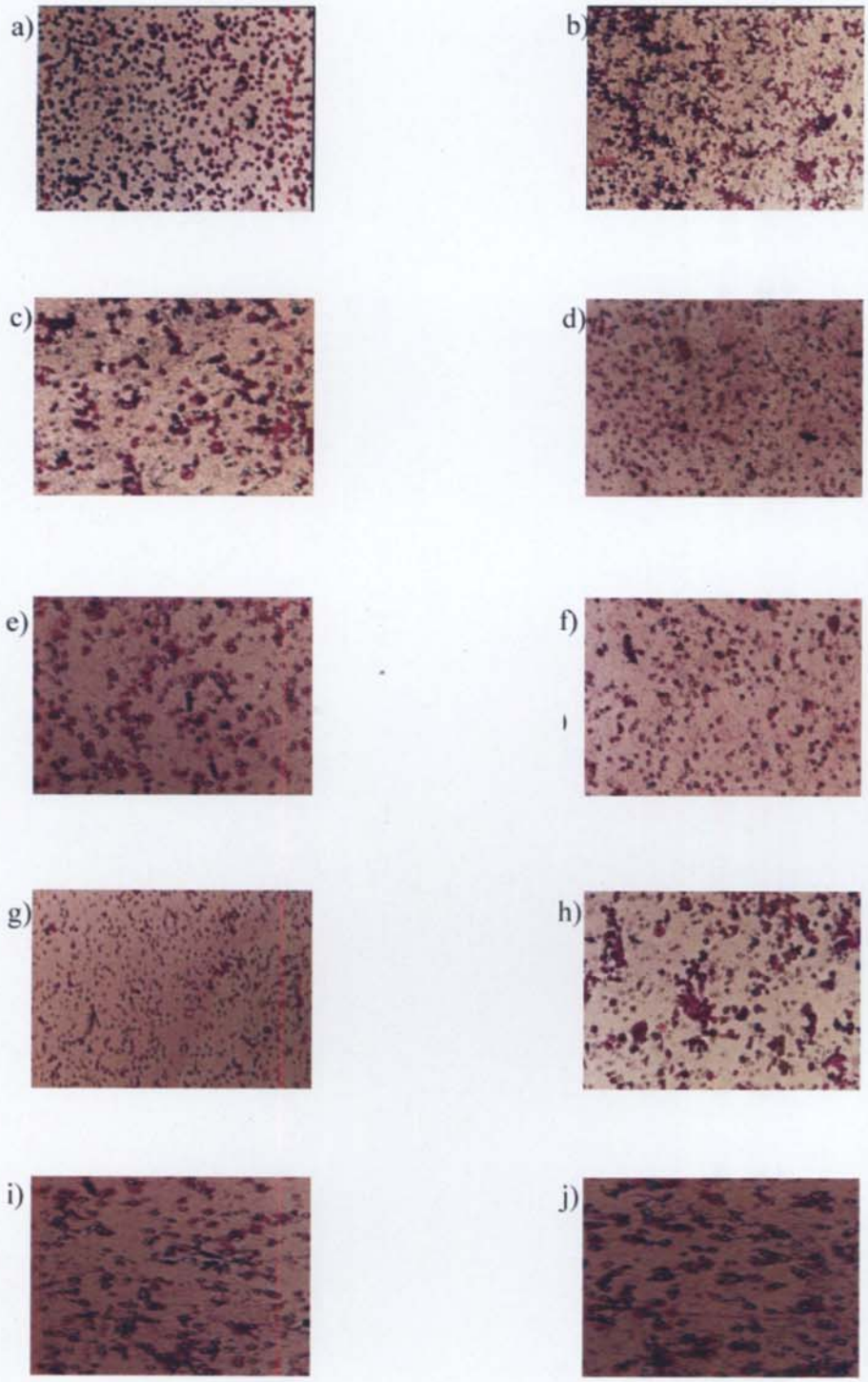
g) *T.occidentalis* treated (5μg/ml)

h) TPS treated (0.01μg/ml)

i) TPS treated (0.05μg/ml)

j) TPS treated (1μg/ml)

Fig. XII Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the collagen matrix invasion of B16F-10 melanoma cells



large number of cells were found in the lower surface of the polycarbonate membrane but *P.longum* (10µg/ml) *T.occidentalis* (5µg/ml) and TPS (1µg/ml) treatment reduced the number of the invaded cells to 86.43%, 79.73% and 69.3% respectively (Table VII).

Effect of *P.longum*, *T.occidentalis* and TPS on the migration of B16F10 melanoma cells

Inhibition of tumor cell motility by *P.longum*, *T.occidentalis* and TPS is given in Table-VIII. *P.longum* (10µg/ml), *T.occidentalis* (5µg/ml) and TPS (1µg/ml) could inhibit the motility of B16F-10 melanoma cells by 28.1%, 36.8% and 31.05% respectively.

Effect of *P.longum*, *T.occidentalis* and TPS on the matrix metalloproteinase production.

Effect of *P.longum, T.occidentalis* and TPS on the matrix metalloproteinase production is shown in Fig VIII. Conditioned medium from the untreated B16F-10 melanoma cells after the trypsin activation give the functional degradative activity identical to MMP-9 (92kDa) and MMP-2 (72 kDa). Gels loaded with Conditioned medium from the untreated B16F-10 melanoma cells without trypsin activation, did not show any clear area, indicating the inactive form of the enzyme collagenase. Trypsin activated Conditioned medium from the untreated B16F-10 melanoma cells loaded gels, after incubation with 10 mm EDTA did not show clear area which indicates, the enzyme responsible for the degradation is metalloproteinase. Condition medium from the *P.longum, T.occidentalis* and TPS treated B16F-10 cells did not give any clear degraded

Table VIII

Effect of *P.longum*, *T.occidentalis* and TPS on the migration of B16F-10 melanoma cells through the polycarbonate membrane

Treatment		% inhibition of motility
P.longum	10µg/ml	28.1±1.1
	5µg/ml	12.4± 0.8
	1µg/ml	4.2± 0.1
T.occidentalis	5µg/ml	36.8± 5.8
	2µg/ml	16.8± 1.5
	1µg/ml	6.6± 0.4
TPS	1µg/ml	31.0± 2.8
	0.5µg/ml	14.4± 1.3
	0.25µg/ml	5.1± 0.7

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

Figure VIII

Effect of *P.longum, T.occidentalis* and TPS on the production of matrix metalloproteinases

Lane1, Conditioned medium from untreated control, after the trypsin activation.

Lane2, Conditioned medium from untreated control, without trypsin activation.

Lane3, Gel incubated in the presence of EDTA.

Lane4, Conditioned medium from the cells treated with *P.longum* (2μg/ml)

Lane5, Conditioned medium from the cells treated with *P.longum* (5μg/ml).

Lane6, Conditioned medium from the cells treated with *P.longum* (10μg/ml).

Lane7, Conditioned medium from the cells treated with *T.occidentalis* (1μg/ml).

Lane8, Conditioned medium from the cells treated with *T.occidentalis* (2μg/ml).

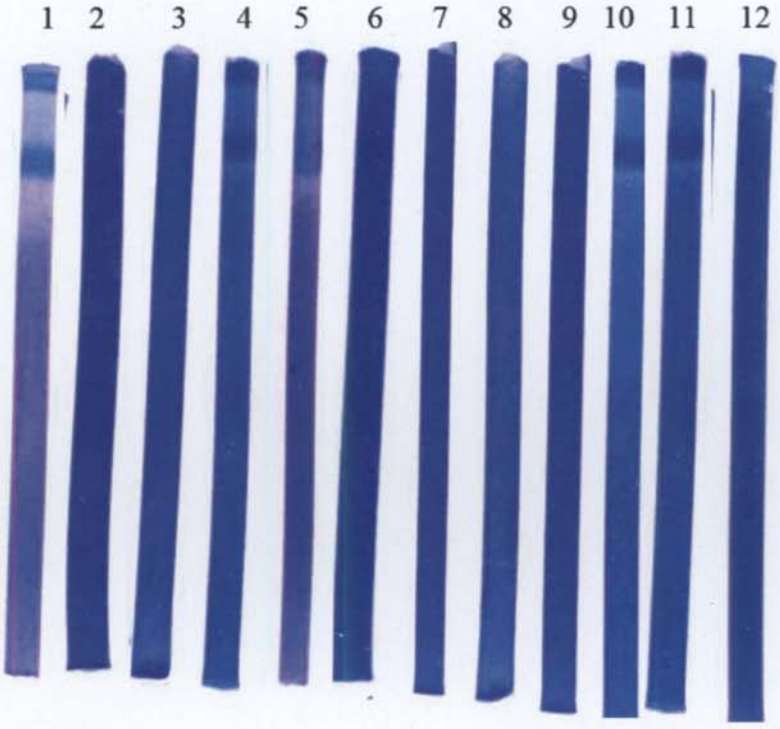
Lane9, Conditioned medium from the cells treated with *T.occidentalis* (5μg/ml).

Lane 10, Conditioned medium from the cells treated with TPS (0.01μg/ml).

Lane11, Conditioned medium from the cells treated with TPS (0.05μg/ml).

Lane12, Conditioned medium from the cells treated with TPS (1μg/ml).

Fig.IV Effect of *P.longum*, *T.occidentalis* and TPS on the production of matrix metalloproteinases



areas indicating that *P.longum, T.occidentalis* and TPS treatment could inhibit the production of matrix metalloproteinase. The gene expression analysis of MMP 2 and MMP 9 is shown in fig 8a and 8b. Treatment with *P.longum, T.occidentalis* and TPS could effectively reduce the elevated expression significantly.

Effect of *P.longum*, *T.occidentalis* and TPS on the IL-1ß, IL-6, GM-CSF, TNF-∝and IL-12p40 gene expression

Highly elevated expression of proinflammatory cytokines such as IL-1ß, IL-6, GM-CSF, TNF-∝and IL-12p40 have been observed in the metastatic B16F-10 melanoma cells. *T.occidentalis* and TPS treatment inhibited the elevated expression of proinflammatory cytokines such as IL-1ß, IL-6, GM-CSF, TNF-∝ and IL-12p40 (Fig- IX). But *P.longum,* treatment could reduce only the level of IL-6, TNF-∝and IL-12p40

Discussion

In the present study we analysed the antimetastatic activity of *P.longum*, *T.occidentalis* and TPS. The extracts of *P.longum*, *T.occidentalis* and TPS have been found to have an effective inhibition of tumor nodule formation. Their administration was found to inhibit the metastatic spread of B16F-10 melanoma cells in the mice. Maximum inhibition of metastasis was observed by the administration of *T.occidentalis* followed by TPS and *P.longum* when compared to the untreated control. Tumor nodules are the metastatic colonies of B16F10 melanoma cells and it promotes the lung fibrosis and collagen deposition in the lung. This inhibition of tumor nodules correlated with an increase in the lifespan of the metastatic tumor bearing animals.

Figure IX

Effect of *P.longum, T.occidentalis* and TPS on the proinflammatory cytokine gene expression

Lane1, Marker

Lane 2, Positive Control

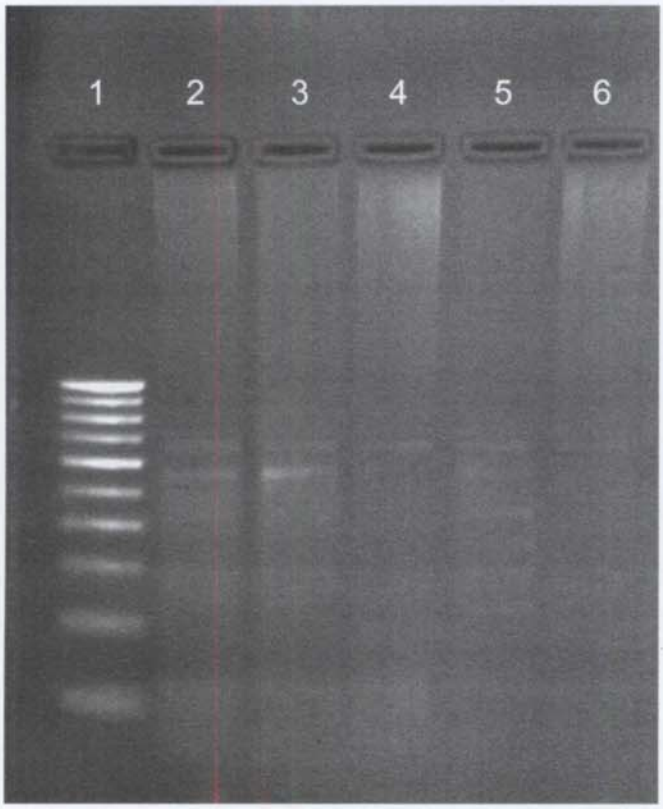
Lane 3, Untreated control

Lane4, *P.longum* treated (10μg/ml)

Lane6, *T.occidentalis* treated (5μg/ml)

Lane7, TPS treated (1μg/ml)

Fig. IX Effect of *P. longum*, *T.occidentalis* and TPS on the proinflammatory cytokine gene expression



The state of lung fibrosis was evaluated by estimating the lung collagen hydroxyproline content, because during lung fibrosis collagen is deposited massively in the alveolus of lungs. A part of collagen is hydroxyproline (Voet and Voet, 1995), which results the reduction in pulmonary function. Administration of *T.occidentalis* and TPS by simultaneous modality could normalize the hydroxylproline content of the lung indicating the effect of these compounds on the inhibition of lung fibrosis. Simultaneous administration of *P.longum* reduced the hydroxylproline content of the lung but to a lesser extent.

The elevated levels of lung uronic acid and hexosamine contents were also significantly reduced by the simultaneous administration of these compounds In the tumor cells, oxidation of the primary alcohol group of aldoses of sugar derivatives occur, yielding uronic acid. This uronic acid leads to the formation of glucuronic acid lactone, which is an esterified form of uronic acid. In the presence of glucuronic acid lactone, prolyl hydroxylase enzyme converts the prohydroxyproline to hydroxyproline. Glucuronic acid lactone also activates fiber formation from collagen during fibrosis. Hexosamine is a significant compound in lung tumor cells. It plays an important role in the synthesis of N-acetyl neurominic acid (sialic acid), which is a component of glycolipids present on the surface of tumor cells (Voet and Voet, 1995). Therefore, total sialic acid levels in serum have been recognized as a valuable non-specific marker of Tumor burden in various diseases (Erbil, 1985) including melanoma (Donoso, 1985). Serum sialic acid level was drastically elevated in metastatic Tumor bearing animals. However, this elevated level of serum sialic acid was significantly reduced by

simultaneous administration of *T.occidentalis* followed by TPS and *P.longum*. Inhibition of all these parameters indicates a reduction in the tissue damage and lung fibrosis.

Metastatic property of tumor cells highly up regulated with the elevated levels of sialic acid content (Fernandes et al., 1991) Gamma glutamyl transpeptidase (GGT), a marker of cellular proliferation, was increased in the serum compared with its level in normal animals. GSH is synthesized intracellularly which provides energy to the tumor cells by gamma glutamyl cycle and GGT catalyses this GSH breakdown (Voet and Voet, 1995). Sialic acid and GGT level in the serum was significantly reduced by the treatment of *P.longum, T.occidentalis* and TPS. Histopathological analysis also correlated with the above results.

Administration of *P.longum*, *T.occidentalis* and TPS after B16F-10 melanoma cell induction increased the cell mediated immune response by promoting the NK- cell activity, ADCC, ACC and IL-2 production. The enhanced cell mediated immune responses and increased production of NK cells is the major protective measures of the immune system during metastasis. An effective enhancement in ADCC by the administration of *P.longum*, *T.occidentalis* and TPS were observed. The presence of humoral immunity is not consistently correlated with increased tumor resistance in the host. But antibodies can mediate cell destruction either via binding with complement or by acting as an opsonin to facilitate phagocytosis by macrophages or other phagocytic cells bearing Fc receptors and ADCC. ADCC is the co-operative interaction of humoral and cell

mediated immune effector mechanism. The expression of NK cell activity and ADCC activity at an early stage of tumor may lead to the exitration of tumor mass by enhancing the immunological status. *P.longum*, *T.occidentalis* and TPS were found to enhance ADCC and ACC activity in normal and tumor bearing animals and the maximum activity was observed much early compared to control animals.

The proinflammatory cytokines such as IL-Iß, IL-6, TNF-α and GM-CSF also directly correlates with the metastatic potential of several human carcinomas like colon, pancreas, prostate and brain (Huang et al , 2000; Yoneda et al 1998). IL- 2 plays a central role in the vertebrate immune response (Smith, 1998). It augments 'natural' immunity by stimulating natural killer (NK) cells (Trinchieri et al, 1984). Treatment with *P.longum*, *T.occidentalis* and TPS has stimulated the level of IL-2 production, when compared to the tumor bearing control animals. It could decrease the elevated level of proinflammatory cytokines in B16F-10 melanoma cell induced mice. TNF-α is among the most potent inducers of transcription factor NF-κB, which has been recognized as a major regulator of pathogen and inflammatory cytokine-inducible gene regulation (Pradeep and Kuttan, 2004). Administration of *P.longum*, *T.occidentalis* and TPS could effectively reduce the level of TNF-α in metastatic tumor bearing animals. IL-6 has been suggested to be a mediator of morbidity and mortality in patients with metastatic disease (Drachenberg et al 1999). We found that *P.longum*, *T.occidentalis* and TPS could inhibit the production and gene expression of proinflammatory cytokines such as IL-IB, IL- 6, GM- CSF, TNF-α and IL-12p40.

TIMP is a multifactorial inhihitor of tumor growth and tumor invasion. TIMP suppress the MMP and ECM turnover (Sakkoula et al, 19970). In addition it has a pluripotent effect on cell growth, apoptosis and differentiation. The extract of *P.longum*, *T.occidentalis* and TPS could significantly increase the level and gene expression of TIMP and could successfully shift the equilibrium towards an angiostatic condition when compared to the control animals.

Metastasis is a multistep process, which involves a series of steps, adhesion of the cancer cells to the basement membrane, invasion through the basement membrane, circulation, extravasation and proliferation at a new distant site Interruption of the metastatic cascade at any of these steps can prevent the production of clinically symptomatic metastasis. Our results demonstrate that these compounds could inhibit different stages of metastasis in a dose dependent manner *in vitro*. This inhibition helps to prevent the metastasis of tumor cells directly *in vivo*. Metalloproteinase have been implicated in the denaturation of the basement membrane during the metastatic invasion of tumor cells (Tryggvason, 1987). As shown in the zymographic and MMP expression analysis treatment of B16F-10 melanoma cells with *P.longum*, *T.occidentalis* and TPS could inhibit the production of matrix metalloproteinases effectively. This could inhibit the invasion of metastatic cells also.

Adhesion of tumor cells on the extracellular matrix play a vital role during trans-endothelial migration of tumor cells (Burrow, 1991) and have been implicated in metastasis. In this study we found that *T.occidentalis* and TPS could inhibit the adhesion of the metastatic of B16F10 melanoma cells which in turn

inhibit the metastasis *in vivo*. Tumor cell mobility is another accelerating step of metastasis which promotes the migration of cells through circulation.

Lysyl oxidase (LOX) expression contributes to cellular invasive ability. The data demonstrate that LOX specifically expressed by invasive/metastatic cancer cells compared with poorly invasive/nonmetastatic cancer cells, and that modulation of LOX expression affects *in vitro* cellular invasive activity. The prolyl hydroxylase enzyme plays a central role in the synthesis of all collagens, as the 4-hydroxyproline residues are essential for the folding of the newly synthesized collagen. The excessive collagen formation was found in patients with various fibrotic diseases (Kivirikko et al., 1992). Administration of *T.occidentalis* could significantly lower the expression LOX and prolyl hydroxylase, while by treating with *P.longum* and TPS the effect was not that much significant. Loss of nm23-H1 expression is associated with metastatic potential. Administration of *T.occidentalis* and TPS could effectively enhance the expression of nm23.

In this study we found the antimetastatic activity of some natural products such as *P.longum*, *T.occidentalis* and TPS *in vivo* and their related activity in the in *vitro* system using the same model of cell lines. From these studies it is indicated that *P.longum*, *T.occidentalis* and TPS possesses antimetastatic activity.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

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(FACULTY OF SCIENCE)

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Chapter 6

Antiangiogenic activity of Piper longum, Piperine, Thuja occidentalis and TPS fraction in in vivo as well as in vitro models.

Introduction

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100-200 μm of blood vessels. For multicellular organism to grow beyond this, size requires new blood vessels. Complex and cellular actions are implicated in angiogenesis, such as extracellular matrix degradation, proliferation and migration of endothelial cells to form tubes (Bussolino et al, 1997). The angiogenic process is tightly controlled by a variety of positive or negative regulators, which are composed of growth factors, cytokines, lipid metabolites and cryptic fragments of haemostatic proteins (Bussolino et al, 1997) and many of these factors are initially characterized in other biological activities. Altered levels of proinflammatory and proangiogenic factors are observed in various forms of cancer (Chen et al, 1999) including melanoma (Lazar et al., 2001). The proinflammatory cytokines such as IL-1ß, IL-6, TNF-α, GM-CSF act as autocrine growth factors for tumor cells. These cytokines could be prometastatic or pro-angiogenic and their deregulated expression directly correlates with the metastatic potential of several human carcinomas (Inser and Asahara, 1999). Among these molecules, vascular endothelial growth factor produced by many tumor and normal cells, plays a key role in regulating normal and abnormal angiogenesis. It binds to endothelial cell surface receptors and activates various functions of the cell including angiogenesis (Veikkoln et al, 2000). Since angiogenesis occurs on limited occasions, the side effects of antiangiogenesis treatment is expected to be very limited, and it also work on a broad spectrum of solid tumors because all these tumors need to induce

angiogenesis for their progression. Antiangiogenic therapy is a promising diversion in cancer treatment. Identification and purification of natural products or its derivatives will be highly relevant in this regard.

Materials and Methods

Animals

Four to six weeks old male C57BL/6 mice weighing 20-25g were used for this study.

Cells

B16F-10, a highly metastatic mice melanoma cell line and, Human umbilical vein endothelial cells (HUVECs) isolated from human umbilical cord veins by collagenase

In vivo antiangiogenic studies

Drug exposure

The animals were intraperitoneally treated with of alcoholic extract of *Piper longum* (10mg/dose/animal), Piperine (1.14mg/dose/animal), *Thuja occidentalis* (5mg/dose/animal) and TPS (1mg/dose/animal) on same day with B16F-10 melanoma cells. The drugs were continued for five consecutive days.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS in capillary formation

Angiogenesis was induced in five groups of C57BL/6 mice (6mice/group) by injecting B16F-10 melanoma cells (10^6cells/animal) intradermally on the shaven ventral skin surface of each mouse (Leyon and Kuttan, 2004). Group I

animals received PBS (Phosphate buffered saline) and served as vehicle control. Group II was treated with reference compound TNP-470 at a concentration 30mg/kg body weight as recommended by NCI. Group III-V animals were treated with *P.longum*, *T.occidentalis* and TPS as given above. After 9 days, all the animals were sacrificed, ventral skin cut removed, washed in PBS and the number of tumor directed capillaries per cm^2 around the tumor was counted using a dissection microscope (Kishi et al, 2000).

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on cytokine production in angiogenesis induced animals.

Four groups of C57BL/6 mice were induced angiogenesis as previous experiment. Group I was kept as untreated angiogenesis induced control animals. Group II-IV animals were treated intraperitoneally with *P.longum*, Piperine, *T.occidentalis* and TPS respectively. Three animals from each group were sacrificed at two different time points (24h and 9^{th} day after tumor injection). Blood was collected, serum separated and level of IL-1ß, IL-6, IL-2, TNF- , GM-CSF were detected using ELISA kits according to the manufacturer's instructions

In vitro antiangiogenic studies

Drug exposure

The B16F-10 melanoma cells were treated with non-toxic concentrations of *Piper longum* (2, 5 and10µg/ml), Piperine (2, 5 and10µg/ml), *Thuja occidentalis* (1, 2 and 5µg/ ml) and TPS (0.01, 0.05 and 1µg/ ml) for *in vitro* analysis.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS in aortic ring assay

As described in chapter 2 (Nicosia, 1998) aortas were harvested from 6 weeks old Sprague Dawley rats. 96-well flat bottom titre plate was coated with collagen, after gelling; ~1mm long aortic rings were placed in the wells and sealed in place with an overlay of 50µl of collagen. VEGF with or without condition medium and *P.longum*, Piperine, *T.occidentalis* and TPS was added to the wells in a final volume of 200µl of 199 medium with 10% FCS. As controls, medium alone was assayed. On day 6, the rings were analyzed by phase-contrast microscopy for micro vessel out growth.

Quantitaion of gene-specific mRNA of VEGF in B16F-10 melanoma cells

Quantikine mRNA is a novel method, which can be used to quantitate gene- specific mRNA at lower levels. Briefly B16F-10 melanoma cells ($1X10^6$) were plated in 30 mm petridish in DMEM with 10% FCS at 37^0C in $5\%CO_2$. Cells were then treated with *P.longum*, Piperine, *T.occidentalis* and TPS for 4h. After the incubation the cells were washed and mRNA preparations were made according to the manufacture's procedure. mRNAs were hybridized with gene-specific biotin-labeled detection probes and digoxigenin alkaline labeled detection probes in a microplate. Hybridization solution was then transferred to a streptavidin-coated microplate and the mRNA probe hybrid was captured. Following wash to remove the unbound conjugate, a substrate solution was added. An amplifier solution was then added and the developed colour was measured spectrophotometrically at 490nm.

Endothelial cell proliferation assay

[^{3}H]-Thymidine incorporation assay (Lee et al., 1999) was carried out as described in chapter 2. Briefly, HUVECs were seeded at a density of 5×10^4cells/well in gelatin coated 96-well plates. After 24h, various concentrations of *P.longum*, Piperine, *T.occidentalis* and TPS was added and stimulated by the addition of 2ng/ml VEGF for 30h, followed by the addition of 1μCi/well of [^{3}H] thymidine for 16-18h. After two washes with ice cold PBS, high molecular weight DNA was precipitated using 10% trichloro acetic acid at 4^0C for 15min. ^{3}H radioactivity was solublized in 0.5N NaOH and determined by Rack Beta liquid scintillation counter.

Endothelial cell migration assay

Motility assay (Gua et al., 2002) was carried out using Human endothelial cell as described previously in chapter 2. Briefly, 96-well titer plate was coated with type-I collagen and incubated overnight at 37^0C. HUVECs were seeded into the coated wells at a density of 2×10^5 cells/well and incubated for 24h. Scrape the monolayer cells to make a clear area with a narrow tip and wash with serum free medium. Various concentrations of *P.longum*, Piperine, *T.occidentalis* and TPS followed by VEGF (20ng/ml) were added into the wells and incubated for 24h. After incubation the cells were fixed and stained using crystal violet and photographed (10X).

Tube formation assay

Tube formation assay (Gupta et al., 2002) was performed as explained in chapter 2. Briefly, 30μl of growth factor reduced ice-cold matrigel was pipetted

into a 96-well flat bottom titre plate and kept for 30 minutes at 37^0C. HUVECs were seeded into the layer of matrigel at a density of 1×10^3 cells/well followed by the addition of 2ng/ml VEGF and FGF. Various concentrations of *P.longum* or Piperine, or *T.occidentalis* or TPS was added into the wells and incubated for 48h at 37^0C in the presence of 5% CO_2 atmosphere. After incubation the cells were fixed, stained using Diff Quick stain and photographed (20X).

Results

In vivo antiangiogenic studies

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS in capillary formation

The ability of *P.longum*, Piperine, *T.occidentalis* and TPS to inhibit *in vivo* tumor induced angiogenesis was examined by injecting B16F-10 melanoma (10^6cells/animal) cells intradermally on the shaven ventral side of animals. The number of tumor directed capillaries were significantly reduced in the *P.longum*, Piperine, *T.occidentalis* and TPS treated group (Fig. I). Control animals had an average number of 32.4 ±3.2 capillaries around the tumor whereas the *P.longum* or Piperine, or *T.occidentalis* or TPS treated animals had only 15.5±1.8, 11.8±1.9 and 13.4±1.0 capillaries respectively. The reference compound TNP-470 treated animals had only 3.5±1.0 capillaries showing 88.8% inhibition in neovessel formation when administered subcutaneously to the animals as recommended by NCI (Table I).

Table I

Effect of *P.longum*, *T.occidentalis* and TPS on tumor directed capillary formation

Treatment	No.of tumor directed Capillaries (cm^2)	% of inhibition
Control	31.4 ± 3.2	_
TNP 470	3.5±1.5	88.8
P longum	15.5 ± 1.8*	50.6
T.occidentalis	11.8±1.9*	62.4
TPS	13.4±1.0*	57.3

The values are mean ± SD

Angiogenesis was induced by injecting 10^6 B16F-10 melanoma cells and treated with *P.longum*, *T.occidentalis* and TPS intraperitoneally. All the animals were sacrificed 9 days after tumor challenge and number of tumor directed micro vessels per cm^2 around the tumor was counted using a dissection microscope.

*$P < 0.001$

Figure I

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on capillary formation in angiogenesis induced animals

a) Control

b) TNP 470 (standard)

c) Treated with *P.longum*

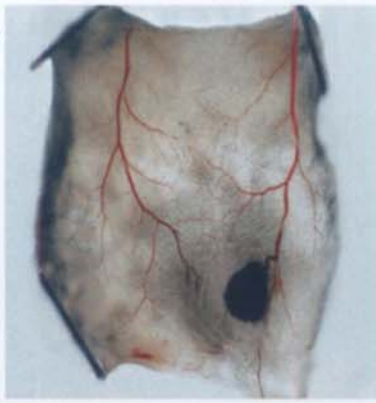
d) Treated with Piperine

e) Treated with *T.occidentalis*

f) Treated with TPS

Fig. I Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on capillary formation in angiogenesis induced animals

a)



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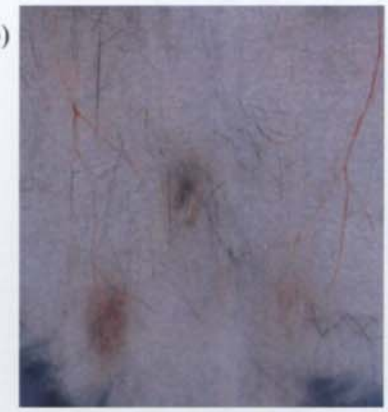

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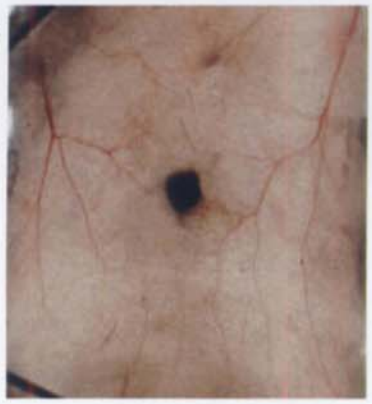
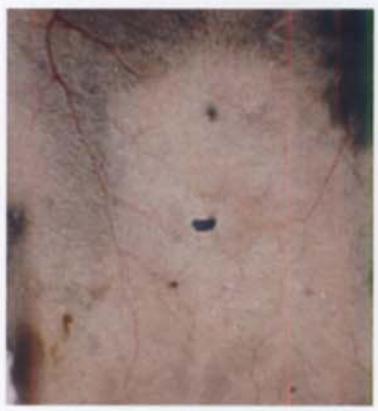
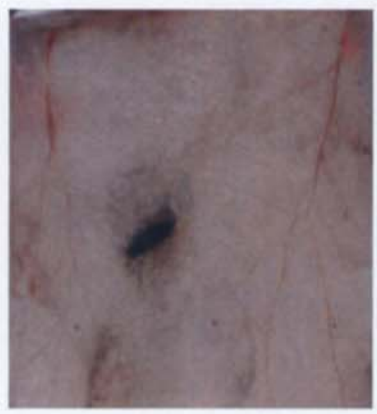


d)

e)



f)



Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on pro-inflammatory cytokines of angiogenesis induced animals

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of angiogenesis induced animals were showing a varying pattern during the period of study. In control animals, the level of IL-1β in the serum was doubled 24h after tumor cell inoculation (30±4.5pg/ml) compared to normal (16±3.5pg/ml) and maintained the same level even after 9 days. Even though the extract of *P.longum* did not affect this initial elevation of cytokine at 24 hour, but it effectively reduced to 21.7± 3.8pg/ml by day 9 after tumor induction. Similarly administration of *T.occidentalis* (18±3.2pg/ml) and TPS (21.2± 1.8pg/ml) showed decreased level of IL-1β on 9th day after angiogenesis induction (Table II).

TNF-α level was drastically elevated (630±8.5pg/ml) in the serum of control animals after 9th day of tumor induction compared to the normal level of TNF-α (20 ±3.2pg/ml). Administration of *P.longum*, Piperine, *T.occidentalis* and TPS could effectively down regulate the elevated level of TNF-α to 185.9±9.2pg/ml, 174±18.4pg/ml and 183.2±20.1pg/ml by 9th day of tumor induction.

Similarly administration of *P.longum*, Piperine, *T.occidentalis* and TPS could lower the elevated level of IL-6 (69.4±5.8pg/ml, 57±3.9pg/ml and 63.4±6.7pg/ml respectively), compared to the control animals (320±9.5pg/ml) after 9th day of tumor induction. The normal level of IL-6 is 35±6.5pg/ml (Table II).

Table II

Effect of *P.longum*, *T.occidentalis* and TPS on pro-inflammatory cytokine profile of angiogenesis-induced animals

	Normal	Control		*Piper longum*		Piperine		*T.occidentalis*		TPS	
Cytokines (pg/ml)	Day 0	Day 1	Day 9	Day 1	Day 9	Day 1	Day 9	Day1	Days9	Day1	Days9
IL-1β	16± 3.5	30± 4.5	30±3.7	27.5±4.1	21.7±3.8	28.2±0.8	15.5±0.9	26±4	18±3.2	27.4±3.2	21.2±1.8
IL-6	35± 6.5	35.3±5.6	320±29.5	39.4±4.6	69.4±5.8	34.0±1.2	67.0± 2.3	34.9±4.2	57±3.9	33.2±4.1	63.4±6.7
TNF-α	20± 3.2	180±16.3	630±58.5	178.4±6.7	185.9±9.2*	162.6±5.7	138.3±5.7*	181±16.7	174±18.4*	184.1±17.3	183.2±20.1*
GM-CSF	18± 0.94	70±5.6	30± 3.2	39.8 ±5.9*	20.1±2.5*	29.8±0.7*	17.8±0.7*	27±4.2*	17±2.9*	33.4±2.7*	19.4±1.5*
IL-2	23± 2.7	16±2.1	20±2.5	21.8±3.9	33±5.1*	26.1±0.5*	39.8±0.7*	27.8±3.8*	53±4.9*	23.6±1.7*	48.4±3.9*
VEGF	16±8	62±9	150±12	58±11.3	79±12.4*	37.7±0.6*	62.2±1.5*	33±9.9*	59±10.2*	57±6.3	63.7±7.8*
TIMP	600± 36	350± 29	360±22	943±48*	1002±37*	682.1±4.2*	794.0±5.4*	1010±42*	1100±39*	932±84.2*	1005±87.4*

All the values are mean ± SD. Blood was collected from the angiogenesis induced animals at the indicated time points after tumor challenge. The cytokine level was estimated by ELISA method. *p<0.001 compared to control.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the colony stimulating factor and vascular endothelial cell growth factor levels in B16F-10 melanoma cell injected animals

The level of growth factors such as GM-CSF and VEGF were determined using ELISA kits. In control animals, the level of GM-CSF (30±3.2pg/ml) and VEGF (150±12pg/ml) in the serum was elevated after 9th day of tumor induction compared to the normal levels of GM-CSF (18±3.1pg/ml) and VEGF (16±8pg/ml). Administration of *P.longum*, Piperine, *T.occidentalis* and TPS could effectively lower the elevated level of GM-CSF (20.1±2.5pg/ml, 17±2.9pg/ml and 19.4±1.5pg/ml respectively) and VEGF (79±12.4pg/ml, 59±10.2pg/ml and 63.7±7.8pg/ml respectively) after 9th day of tumor induction (Table II).

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the IL-2 and TIMP-1 profile of angiogenesis induced animals

The lowered levels of IL-2 in the control animals (16±2.1pg/ml) 24h after angiogenesis induction was normalized by the treatment with *P.longum*, Piperine, *T.occidentalis* and TPS (21.8±3.9pg/ml, 27.8±3.8pg/ml and 23.6±1.7pg/ml respectively). The IL-2 level in the extract-treated animals on day 9 was found significantly enhanced to (33.8±5.1pg/ml, 53±4.9pg/ml and 48.4±3.9pg/ml) by treating with *P.longum*, Piperine, *T.occidentalis* and TPS respectively) compared to the control (20±2.5pg/ml) as well as normal (23±3.2pg/ml) animals (Table II).

The tissue inhibitor of metalloprotease level in the serum of normal mice was 600±36pg/ml, which was reduced by the induction of tumor cell to 350±19pg/ml in the untreated control animals. But *P.longum*, Piperine,

T.occidentalis and TPS (943±28pg/ml, 1010±42pg/ml and 932±84.2pg/ml respectively) treatment could elevate the levels of this MMP inhibitor. Blood sample on 9th day also showed a similar TIMP-1 profile in angiogenesis induced animals. In control, it was only 360±22pg/ml and *P.longum*, Piperine, *T.occidentalis* and TPS administration could maintain the initial elevation thereby negatively contributing to the formation of neovessels (Table II).

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the rat aortic ring assay

The sprouting vessels from aortic rings were investigated to determine whether *P.longum*, Piperine, *T.occidentalis* and TPS inhibit VEGF-induced angiogenesis *ex vivo.* VEGF (20ng/ml) significantly stimulated vessel sprouting in conditioned medium alone (Fig II). The presence of *P.longum*, Piperine, *T.occidentalis* and TPS resulted in a significant reduction of vessel sprouting induced by VEGF, and its inhibitory activity was dose dependent.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the VEGF mRNA expression

The level of VEGF mRNA expression was highly elevated in the untreated B16F-10 melanoma cells (27.65±1.21attomoles). Treatment with *P.longum*, Piperine, *T.occidentalis* and TPS for 4h could significantly reduce the elevated level of VEGF to 11.6±0.32, 9.01±0.22 and 10.3±0.27attomoles respectively (Table III).

Figure II

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on aortic ring assay

a) Control

b) Normal

c) Treated with *P.longum* (2µg/ml)

d) Treated with *P.longum* (5µg/ml)

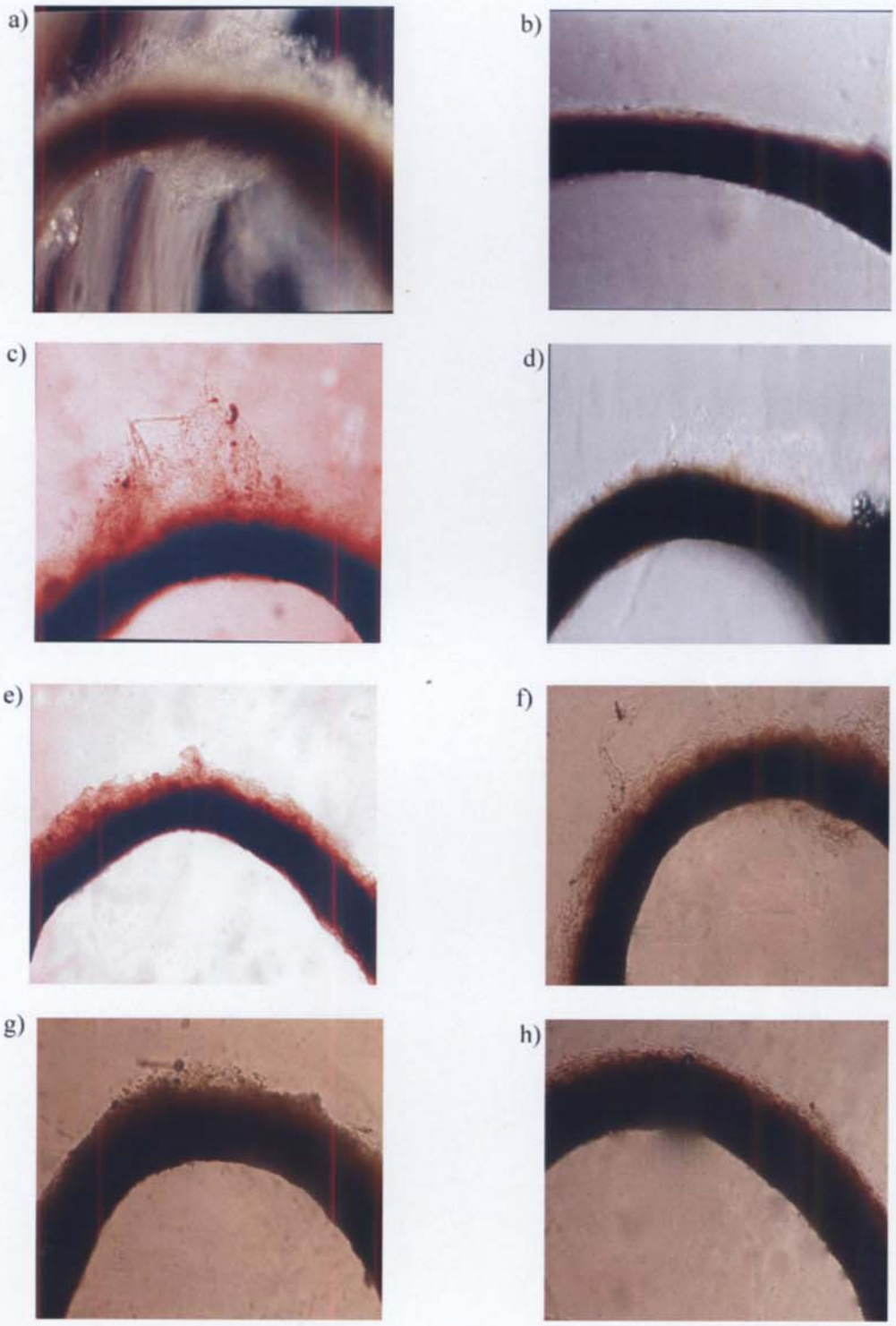
e) Treated with *P.longum* (10µg/ml)

f) Treated with Piperine (2µg/ml)

g) Treated with Piperine (5µg/ml)

h) Treated with Piperine (10µg/ml)

Fig. II Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on aortic ring assay



i) Treated with *T.occidentalis* (1µg/ml)

j) Treated with *T.occidentalis* (2µg/ml)

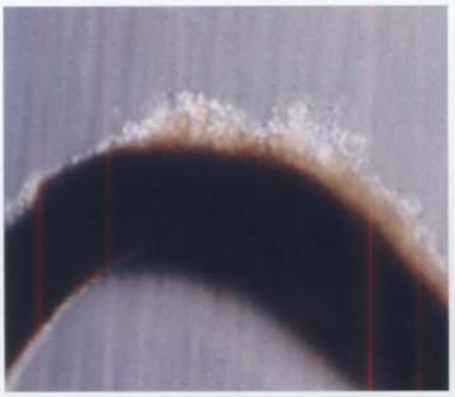
k) Treated with *T.occidentalis* (5µg/ml)

l) Treated with TPS (0.01µg/ml)

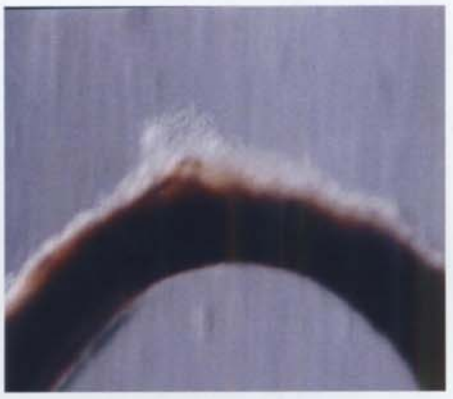
m) Treated with TPS (0.05µg/ml)

n) Treated with TPS (0.1µg/ml)

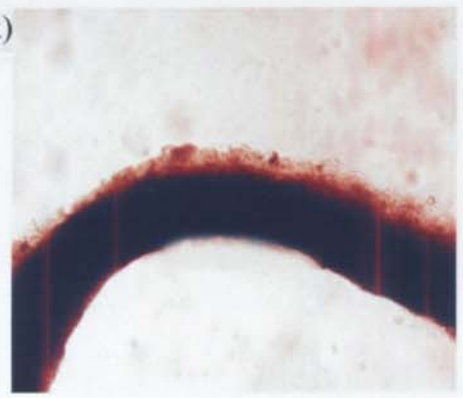
i)



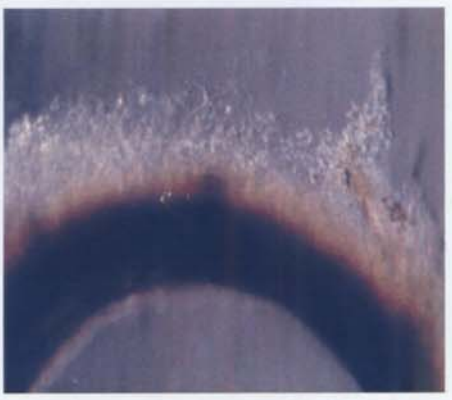
j)



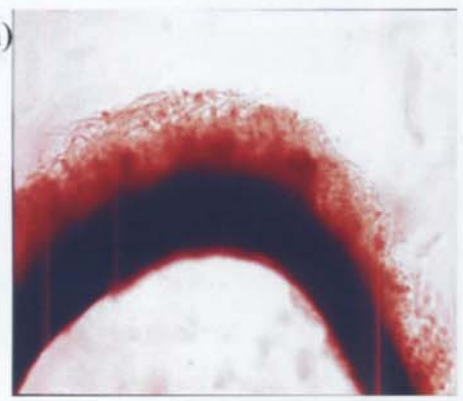
k)



l)



m)



n)

Table III

Effect of *P.longum*, *T.occidentalis* and TPS on VEGF-mRNA level

Treatment	VEGF-mRNA level (attomoles/ml)
Control	27.6 ±1.21
P longum	11.6±0.32*
T.occidentalis	9.0±0.22*
TPS	10.3±0.27*

The values are mean ± SD.B16F-10 melanoma cells ($1X10^6$) were treated with *P.longum*, *T.occidentalis* and TPS for 4h. mRNA was prepared and hybridized with gene-specific biotin-labeled detection probes and digoxigenin alkaline labeled detection probes according to the manufactures procedure. OD was measured spectrophotometrically at 490nm.

*$P < 0.001$

***P.longum*, Piperine, *T.occidentalis* and TPS inhibits VEGF induced proliferation of endothelial cells.**

To determine antiangiogenic activity of extract of *P.longum*, Piperine, *T.occidentalis* and TPS *in vitro*, its inhibitory effect on VEGF induced proliferation of endothelial cells was evaluated. *P.longum*, Piperine, *T.occidentalis* and TPS inhibited VEGF induced HUVEC proliferation in a dose dependent manner (Table IV). These inhibitory effects were not due to cytotoxicity of *P.longum*, Piperine, *T.occidentalis* and TPS in endothelial cells. The effect of these compounds on DNA synthesis of HUVECs was monitored by [^{3}H] thymidine incorporation assay. VEGF (20ng/ml) significantly increased DNA synthesis of HUVECs, and this effect was completely blocked by *P.longum*, Piperine, *T.occidentalis* and TPS.

***P.longum*, Piperine, *T.occidentalis* and TPS inhibits VEGF induced migration of endothelial cells.**

The effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the inhibition of migration was analyzed. The endothelial cell migration was drastically enhanced upon the addition of VEGF to the culture medium. *P.longum*, Piperine, *T.occidentalis* and TPS significantly inhibited the VEGF induced migration of endothelial cells at a concentration of 10μg/ml, 10μg/ml, 5μg/ml and 1μg/ml respectively (Fig. III).

***P.longum*, Piperine, *T.occidentalis* and TPS inhibits VEGF induced tube formation of endothelial cells.**

The effect of *P.longum*, Piperine, *T.occidentalis* and TPS on

Table IV

Effect of *P.longum*, *T.occidentalis* and TPS on proliferation of HUVECs

Treatment		% of proliferation
P.longum	10μg/ml	20.5
	5μg/ml	11.6
	1μg/ml	3.7
T.occidentalis	5μg/ml	24.7
	2μg/ml	18.3
	1μg/ml	5.9
TPS	1μg/ml	23.1
	0.5μg/ml	15.4
	0.25μg/ml	6.2

HUVECs were seeded at a density of 5×10^4cells/well in 96-well plates. After 24h, various concentrations of *P.longum*, *T.occidentalis* and TPS was added and stimulated by the addition of 2ng/ml VEGF for 30h, followed by the addition of 1μCi/well of [^{3}H] thymidine for 16-18h. CPM was determined by Rack Beta liquid scintillation counter.

Figure III

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the inhibition of VEGF induced migration of endothelial cells.

a) '0' hour after making a clear area

b) Control

c) Treated with *P.longum* (10µg/ml)

d) Treated with Piperine (10µg/ml)

e) Treated with *T.occidentalis* (5µg/ml)

f) Treated with TPS (1µg/ml)

Fig. III Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on migration assay

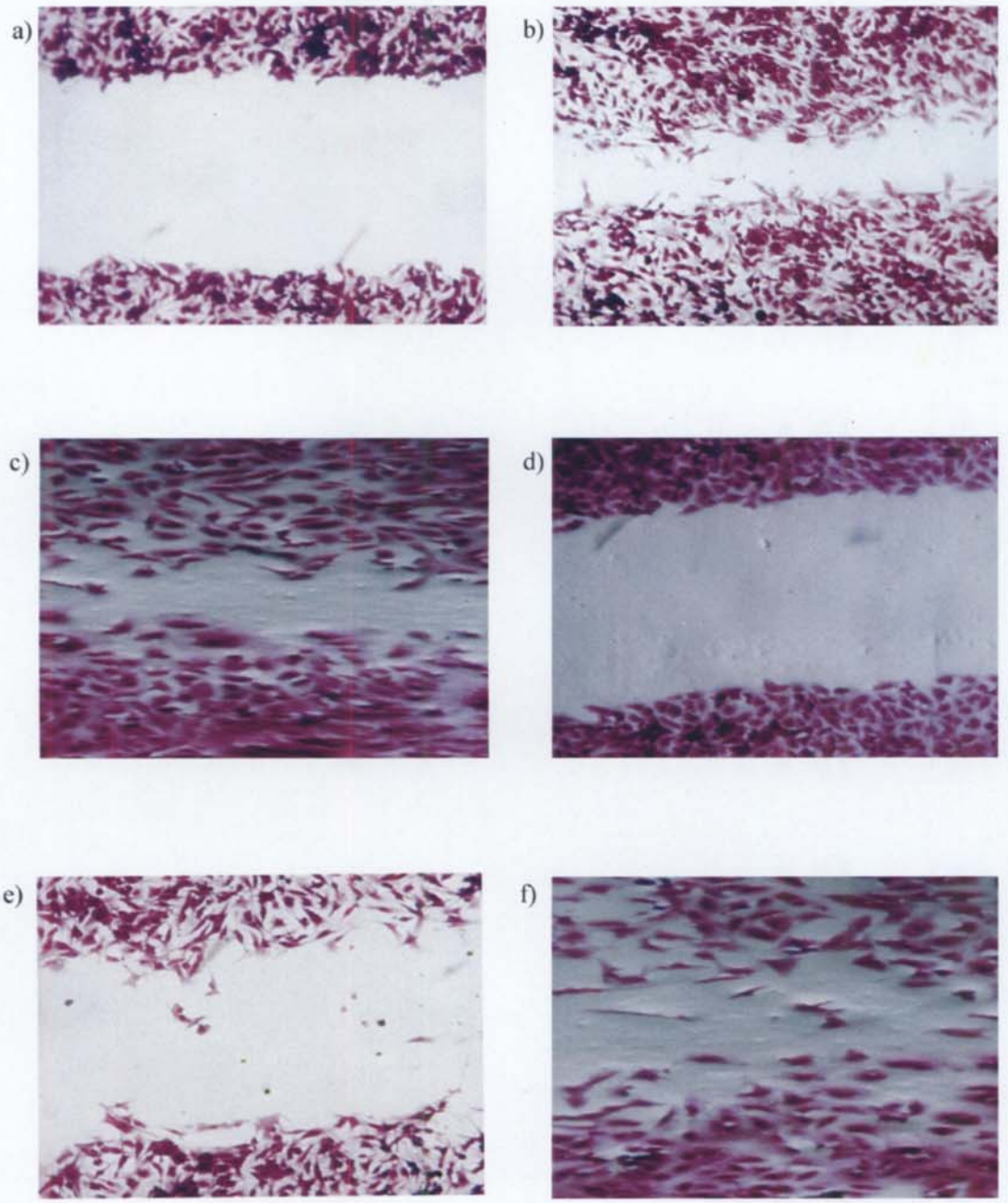


Figure IV

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the inhibition of VEGF induced tube formation of endothelial.

a) Control

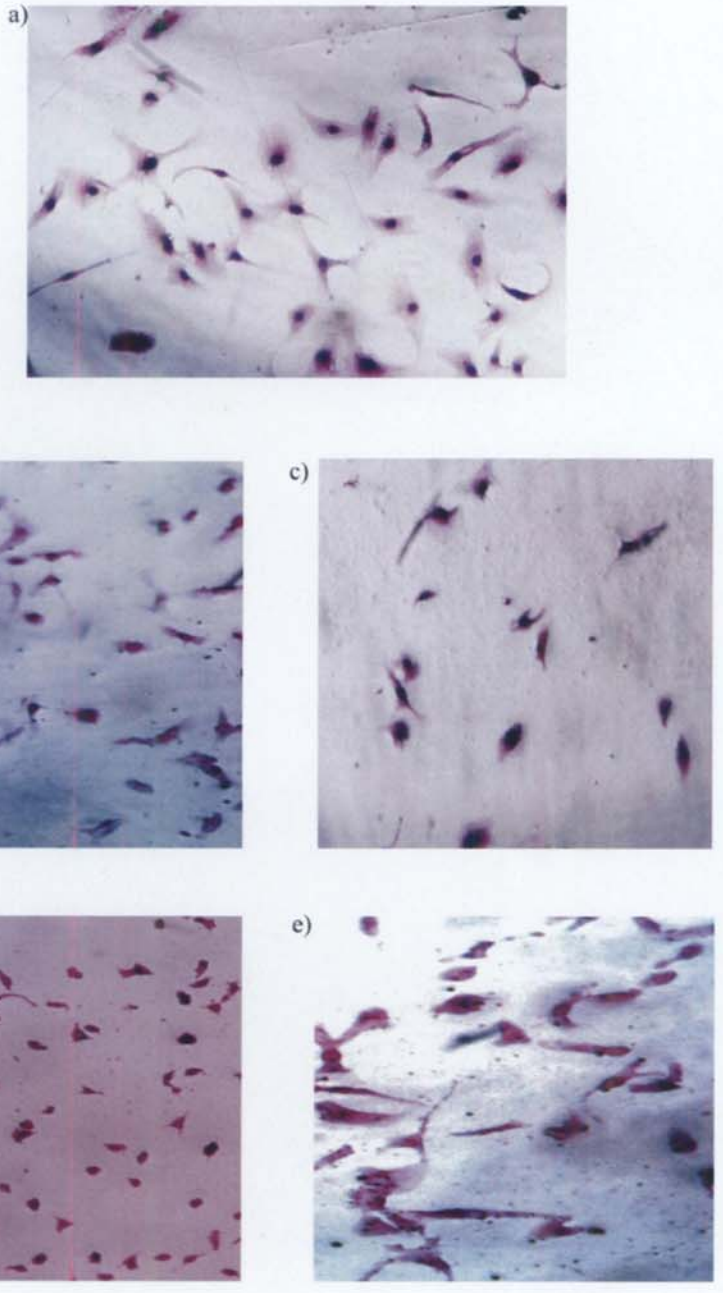
b) Treated with *P.longum* (10µg/ml)

c) Treated with Piperine (10µg/ml)

d) Treated with *T.occidentalis* (5µg/ml)

e) Treated with TPS (1µg/ml)

Fig. IV Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the tube formation assay



morphological differentiation of endothelial cells was investigated using matrigel assay. When HUVECs were placed on growth factor-reduced matrigel in the presence of VEGF, VEGF lead to the formation of elongated and tube-like structures, which were organized by much larger number of cells compared with the control. *P.longum*, Piperine, *T.occidentalis* and TPS effectively abrogated the width and length of endothelial tubes in a concentration-dependent manner (Fig. IV). Half maximal inhibition was seen at a concentration of 10μg/ml, 10μg/ml 5μg/ml and 1μg/ml respectively. These results demonstrate that *P.longum*, Piperine, *T.occidentalis* and TPS have the ability to block VEGF-induced *in vitro* angiogenesis.

Discussion

Angiogenesis plays an important role in embryogenesis and tumorigenesis (Folkman, 1995). This is underscored by the fact that solid tumor growth beyond a few mm^2 in diameter requires new blood vessel growth (Folkman, 1971). In the present study, we provide direct evidences that *P.longum*, Piperine, *T.occidentalis* and TPS has a potent antiangiogenic activity in the *in vitro* and *in vivo* models that can support the antitumor action of these compounds. Tumor vessel count has been correlated with lymph node status and survival (Risau, 1997)). *P.longum*, Piperine, *T.occidentalis* and TPS inhibited tumor induced angiogenesis in C57BL/6 mice when they were injected with B16F-10 melanoma cells. In this study it is notable that *P.longum*, Piperine, *T.occidentalis* and TPS selectively inhibited tumor induced capillary formation without any visible effect on the pre-existing blood vessels.

The level of some of the proinflammatory cytokines such as IL-1ß, IL-6, TNF-α, GM-CSF evaluated in this study are also important in the process of angiogenesis. This also directly correlates with the metastatic potential of several human carcinomas like colon, pancreas, prostate, brain (Yoneda et al 1998; Huang et al, 2000). IL-6 is involved in the angiogenic switch during cervical oncogenesis, by inducing VEGF via the STAT3 pathway (Yoneda et al, 1998). TNF-α is among the most potent inducers of transcription factor NF-κB, which has been recognized as a major regulator of pathogen and inflammatory cytokine-inducible gene regulation (Pradeep and Kuttan, 2004). Even though GM-CSF did not modulate endothelial functions related to inflammation (Bussolino et al, 1991), it induces endothelial cells to proliferate and migrate. Administration of *P.longum*, Piperine, *T.occidentalis* and TPS significantly lowered the elevated serum level of IL-1ß, IL-6, TNF-α, GM-CSF. We found that treatment with these natural products could decrease the elevated level of pro-inflammatory cytokines in B16-F10 melanoma cells induced mice, and thus could inhibit the angiogenesis. IL-2 promotes the proliferation and differentiation of helper T-cells, Cytotoxic T-cells and B- cells. It also augments innate or 'natural' immunity by stimulating natural killer cells (Caligiuri et al., 1993). The level of IL-2 was significantly increased by the administration of *P.longum*, Piperine, *T.occidentalis* and TPS extract, when compared to the control animals.

TIMP is a multifactorial inhibitor of angiogenesis, tumor growth and tumor invasion (Stetler-Stevenson, 1990). TIMP suppress the MMP and ECM turnover. In addition it has a pluripotent effect on cell growth, apoptosis and

differentiation (Jiang et al, 2002). It inhibits angiogenic factor induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo* independent of MMP inhibition. The extract of *P.longum*, Piperine, *T.occidentalis* and TPS could significantly increase the production TIMP-1 and could successfully shift the equilibrium towards an angiostatic condition when compared to the control animals.

VEGF is the important growth factor proven to be specific and critical for a mitogen for vascular endothelial cells derived from arteries, veins and lymphatic but it is devoid of consistent and appreciable mitogenic activity for other cell types (Griffin et al, 2002). VEGF promotes angiogenesis in three-dimensional *in vitro* models inducing confluent microvascular endothelial cells to invade collagen gels and form capillary like structures (Stacker et al, 2002). Even though VEGF is a potent mitogenic stimulation of endothelial cells, several studies have demonstrated the ability of VEGF to function as a survival factor for endothelial cells (Benjamin et al, 1999). Since VEGF is generated from a variety of tumors, it is the most important angiogenic factor associated closely with induction and maintenance of the neovasculature in human tumors (Veikkola and Alitalo, 1999; McMahon, 2000). In this study we found that treatment with *P.longum*, Piperine, *T.occidentalis* and TPS could inhibit the level of VEGF in the serum of animals, which were induced angiogenesis with B16F-10 melanoma cells. Supporting to this, VEGF mRNA level of B16F-10 melanoma cells was also reduced by the treatment of *P.longum*, Piperine, *T.occidentalis* and TPS.

Angiogenesis is composed of several processes; dissociation of pericytes

from preexisting vessels, digestion of extracellular matrix with proteases, proliferation, migration and invasion of endothelial cells, tube formation, then finally remodeling occurs. VEGF is considered to be secreted from tumor cells in a paracrine fashion to induce blood vessel growth. *P.longum*, Piperine, *T.occidentalis* and TPS almost completely suppressed VEGF induced endothelial cell proliferation, migration and tube formation in a dose dependent manner. The antiangiogenic activity of *P.longum*, Piperine, *T.occidentalis* and TPS was supported by its remarkable suppression in sprouting of endothelial cells in rat aorta.

These antiangiogenic activities of *P.longum*, Piperine, *T.occidentalis* and TPS *in vivo* may be explained by its inhibitory action on proliferation, migration and differentiation of endothelial cells in response to angiogenic growth factors such as VEGF. The *in vitro* experiments have established the antiangiogenic activity of the compound. It is suggested that *P.longum*, Piperine, *T.occidentalis* and TPS may possess novel molecular properties that interfere with common angiogenic signaling pathways triggered on growth factor stimulation in endothelial cells. By using *ex vivo* and *in vivo* angiogenesis models, the antiangiogenic activities of *P.longum*, Piperine, *T.occidentalis* and TPS were evaluated. Taken together these results states that *P.longum*, *T.occidentalis* and TPS may be inhibiting tumor progression via its antiangiogenic activity

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

for the fulfillment of the degree of

Doctor of Philosophy In Immunology

(FACULTY OF SCIENCE)

By

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Chapter 7

Radioprotective and chemoprotective activity of Piper longum, Piperine, Thuja occidentalis and Thuja polysaccharide fraction

Introduction

Ionizing radiation is toxic to organisms since it induces deleterious structural changes in essential macromolecules (Navaro et al, 1971). Agents that protect normal tissues against radiation damage can increase patient tolerance to radiotherapy. Several synthetic compounds have been found to provide good radioprotection in experimental animals, but their clinical utility is limited by their expensive cost and their toxicity on repeated administration (Uma Devi, 1998). Drugs such as amifostine produce side effects like nausea, vomiting and hypotension (Foster et al, 1997). Therefore, there is a need to find nontoxic and less expensive drugs for clinical radioprotection. Recent studies in our laboratory show that commonly used medicinal plants and herbal preparations (Kuttan, 1996; Rekha et al, 2000) are a good source of radioprotectors in experimental models, as well as in patients receiving radiotherapy.

Cyclophosphamide (CTX) is a widely used alkylating agent for antineoplastic therapy. It is a chemical derivative of mechlorethamine and functions as an alkylating agent that alkylates or interacts with formed DNA. It is one of the commonly used drugs in oncology. CTX is extensively metabolized *invivo* to active and inactive metabolites (Sladek, 1988). Initially CTX is transformed in the liver by cytochrome p450 mixed function oxidase enzymes to 4-hydroxy cyclophosphamide and aldophosphamide. Then it enters to the cells and activated to intracellular alkylating metabolites such as acrolin and phosphoramide mustard (Clavin, 1997). CTX administration causes cardiac toxicity, hematopoietic depression, hemorrhagic cystitis, gonadal dysfunction, nausea, gastrointestinal toxicity, vomiting, alopecia renal toxicities etc (Slavin, 1975). Most chemotherapeutic drugs cause dose limiting

toxicity to bone marrow elements, hence bring about immonosuppression (Cheson et al, 1999). Herbal drugs offer distinct advantages over currently available immunostimulating agents as they are capable of minimizing the toxicity induced by radiotherapy and chemothapy to normal with out comparing its antineoplastic activity.

Materials and methods

Animals

Inbred strains of male Swiss albino mice (6-8 weeks old) and BALB/c mice were used for this study.

Drug administration

The animals were treated with 10 doses of *P.longum* (10mg/animals; ip), Piperine (1.14mg/dose/animal), *T.occidentalis* (5mg/dose/animal) and TPF (1mg/dose/animal) intraperitoneally for 10 consecutive days.

I. Radioprotective effect of *Piper longum*, Piperine, *Thuja occidentalis* and Thuja polysaccharide fraction

Irradiation protocol

In each experiment, animals were treated with a single dose of radiation of 6 Gy. The source of radiation was a ^{60}Co teletherapy unit (Theratron 780, Canada). Animals were restrained in specially designed, well-ventilated cages and exposed to whole body radiation at a rate of 1.4 Gy/minute. Simultaneously after the radiation dose, treatment was begun

of the effect of *P. longum,* Piperine, *T. occidentalis* and TPS on hematological parameters

Swiss albino mice were divided into five groups (6animals/group). All animals received a single dose of whole body radiation (6 Gy/animal). Group I served as untreated control and group II-V were simultaneously treated with the extract of *P. longum,* Piperine, *T. occidentalis* and TPS for five consecutive days. Blood was collected from caudal vein, and total leukocyte count was determined using a hemocytometer. Differential count, hemoglobin content (Chessbrough and Mac Arthur., 1976) and weight of the animals were recorded prior to radiation exposure and continued on every third day for 30 days.

Determination of the effect of *P. longum,* Piperine, *T. occidentalis* and TPS on bone marrow cellularity and α-esterase activity.

Six groups of male Swiss albino mice (18mice/group) were used for the experiment. Group I was kept as a non-irradiated normal control. Animals in groups II, III, IV, V and VI were exposed to a single dose (6 Gy/animal) of whole body radiation. Group II was served as the irradiated control and in-group III-VI were treated with *P. longum,* Piperine, *T. occidentalis* and TPS simultaneously after the radiation exposure for five consecutive days. Six mice from each group were sacrificed on day 2, 7 and 11 for analysis of bone marrow cellularity and α-esterase activity.

Bone marrow cellularity was determined using haemocytometre according to the method of Sredni et al (Sredni et al, 1992). Bone marrow was collected from femur and made into single cell suspension. The number of cells was determined using a hemocytometer and expressed as total live cells (trypan blue exclusion

method) per femur.

Bone marrow cells from the above preparations were smeared on clean glass slides and stained with p-rosaniline and Harris hematoxylin to determine the non-specific α-esterase activity by simultaneous azo dye coupling method (Bancroft and Cook HF, 1984).

Determination of the effect of *P. longum*, Piperine, *T. occidentalis* and TPS on spleen nodule formation.

BALB/c mice (4-6 weeks old) were used to study spleen nodule formation assay. thirteen groups (6mice/group) of animals were exposed to whole body radiation (4Gy/animal). Group I received bone marrow cells ($1x10^6$cells/animal) from normal mice through tail vein and kept as untreated control. Group II, III IV and V received bone marrow cells from *P. longum,* Piperine, *T. occidentalis* and TPS (5 doses) treated animals. Group VI, VII, VIII and IX were received bone marrow cells as above and the animals were treated with *P. longum,* Piperine, *T. occidentalis* and TPS for 5 more consecutive days. Group X-XIII was treated with bone marrow cells from untreated animals and 5dose of compound. After 7 days the animals were sacrificed and the spleen were excised. The number of spleen colonies were counted using colony counter and the relative spleen weight was calculated.

Determination of the effect of *P. longum,* Piperine, *T. occidentalis* and TPS on scavenging of radiation induced free radicals, oxidative damages and normal cell proliferation

Swiss albino mice were divided into six groups (18mice/group). Group I was kept as a non-irradiated normal control. Animals in groups II to VI were exposed to a

single dose (6Gy/animal) of whole body radiation. Group II was served as the irradiated control and in-group III to VI *P. longum,* Piperine, *T. occidentalis* and TPS were administered simultaneously after the radiation exposure for ten consecutive days.

Six animals from each group were sacrificed after 2, 7 and 11 days by cervical dislocation. Blood was collected by heart puncture immediately following sacrifice and serum was separated. The liver and small intestine were collected to evaluate radiation-induced toxicities; total protein was estimated by Lowry's method (Lowry et al, 1951). GSH levels in liver homogenate and intestinal mucus were estimated by the method of Moron et al (Moron et al, 1979), based on the reaction with DTNB.

Activities of liver homogenate and serum pathophysiological enzymes such as alkaline phosphatase (King 1965) and glutathione pyruvate transaminases (Bergmeyer and Bernt, 1980) were determined. The lipid peroxidation level in liver and serum was measured using thiobarbituric acid-reactive substances (TBARS) by previously published method (Ohkawa et al, 1979).

Histopathological studies

The jejunal portion of the small intestine from the previous experiment was excised and fixed in 10% formaldehyde. Sections (4 μm) were stained with eosin and hematoxylin.

Results

Radioprotective effect of *Piper longum,* Piperine, *Thuja occidentalis* and Thuja polysaccharide fraction

Effect of *P.longum,* Piperine, *T.occidentalis* and TPS on hematological parameters

Irradiation significantly reduced total WBC in control mice to 1900 cell/mm^3 after 3rd day of radiation exposure and gradually increased up to 5200 cells/mm^3 by day 30 (Fig.I). The *P. longum,* Piperine, *T. occidentalis* and TPS treated group had lower WBC count initially, however the values increased significantly, relative to untreated controls, up to 7883.3 cells/mm^3, 8443.4 cells/mm^3, 7558.3 cells/mm^3, 6932 cells/mm^3 respectively on 21st day. There was no significant effect on the differential count, hemoglobin level and body weight (data not shown).

Effect of *P. longum,* Piperine, *T. occidentalis* and TPS on bone marrow cellularity and α-esterase activity.

There was also a drastic reduction in bone marrow cellularity in irradiated control animals ($4.4x10^6$cells/femur), compared to normal animals ($14.5x10^6$cells/femur) 48h after radiation. Treatment with the *P. longum,* Piperine, *T. occidentalis* and TPS significantly increased the bone marrow cellularity relative to untreated controls, to $8.03x10^6$cells/femur, $9.31x10^6$cells/femur, $7.7x10^6$cells/femur and $6.8x10^6$cells/femur, respectively after 48h, which increased gradually to $16.4x10^6$cells/femur, $16.9x10^6$cells/femur, $6.9x10^6$cells/femur and $15.3x10^6$cells/femur respectively on day 11 (Table I).

The effect of *P. longum,* Piperine, *T. occidentalis* and TPS on α-esterase

Fig II. Effect of *P. longum* , Piperine, *T. occidentalis* and TPS on total WBC count in irradiated mice

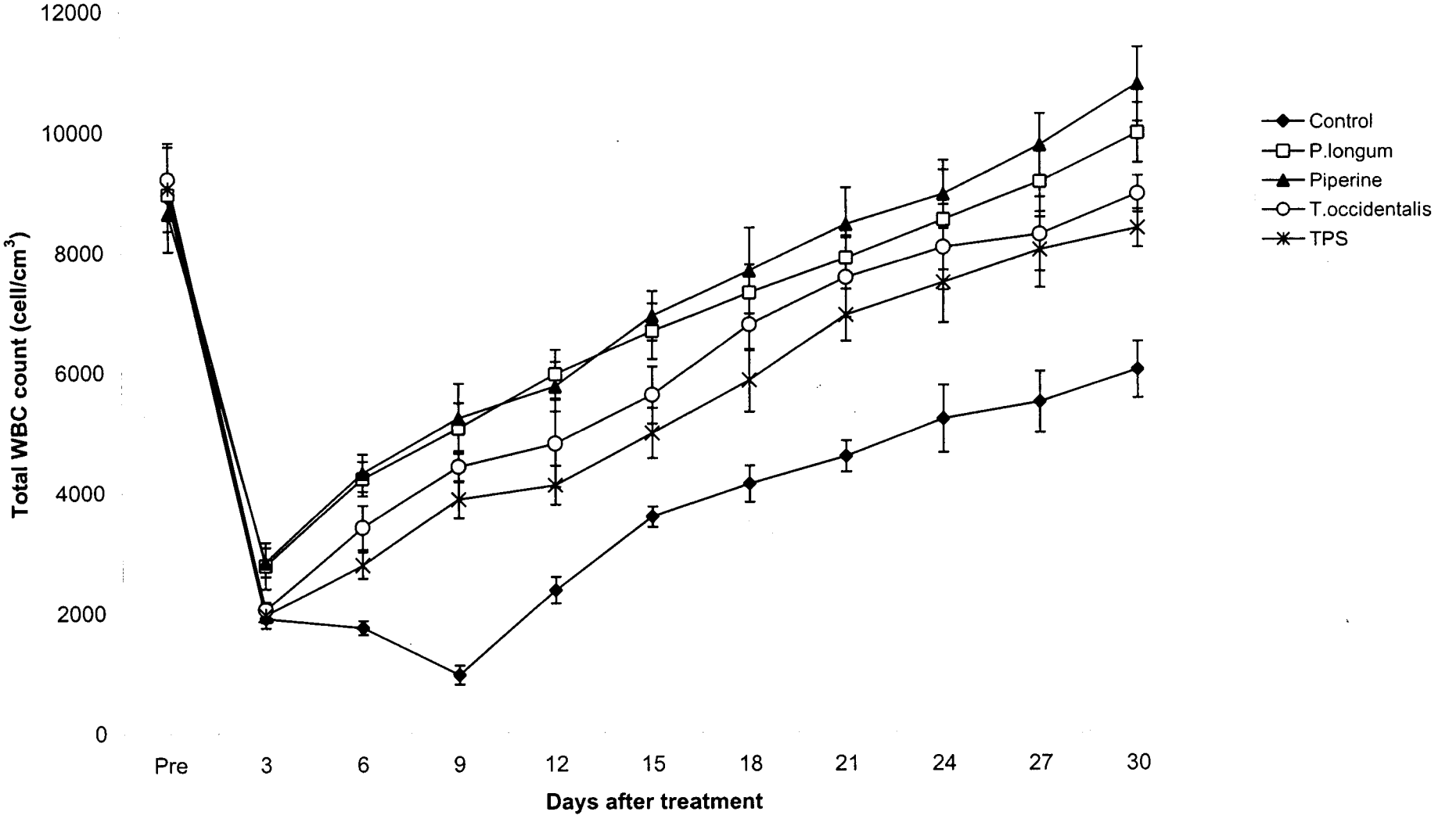


Table 1

Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on bone marrow cellularity and α-esterase activity in mouse treated with radiation

Treatment	Bone marrow cellularity (cells/femur) x10^6			No. of α-esterase positive cells/4000 cells		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	14.5± 0.7	-	-	992± 71.4	-	-
Control	4.4± 0.5	7.4± 1.2	12.2± 1.2	200± 39.4	443± 22.9	693.5± 29.0
Piper longum + Radiation	8.0± 0.4*	14± 1.1*	16.7± 1.2*	323.2± 36.2*	620± 56.7*	946.5± 94.9*
Piperine + Radiation	9.31± 0.7*	15.7± 1.2*	16.9± 1.3*	401.3± 39.6*	678± 60.4*	1042.1± 92.7*
T.occidentalis + Radiation	7.7± 0.6*	13.5± 1.4*	16.9± 1.6*	270.8± 27.7*	606± 65*	940± 106.8*
TPS + Radiation	6.8± 0.5*	12.4± 1.1*	15.3± 1.4*	243.2± 24.2*	574± 50.2*	912± 82.3*

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS after exposing to γ-radiation (6Gy). Bone marrow cells were collected from femur. Values are the mean ± SD. Statistically significant from untreated control.

* $P < 0.001$

positive cells is presented in Table I. The number of α-esterase positive cells in bone marrow of irradiated control animals was dramatically low (200cells/4000 cells) at 48h and did not reach the normal level (992cells/4000cells) even after 11 days. In the case of *P. longum,* Piperine, *T. occidentalis* and TPS treated irradiated animals, there was a significant increase in α-esterase positive cells (323.2cells/4000cells, 401.3cells/4000cells, 270.8cells/4000cells and 243.2cells/4000cells respectively) at 48h compared to the control and after day 11, the levels of α-esterase positive cells were nearly normal.

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on spleen nodule formation.

Effect of *P. longum,* Piperine, *T. occidentalis* and TPS on spleen colony formation was shown in Table II. Maximum number of spleen colonies was observed when compound was given prior and after the radiation exposure (14.8, 16.5, 15.2and 14.0). But in radiation alone treated control animals, the total number of spleen colonies was only (6±1.1).

Effect of *P. longum,* Piperine, *T. occidentalis* and TPS on scavenging of radiation induced free radicals, oxidative damages and normal cell proliferation

The animals exposed to radiation had a lowered level of GSH in intestinal mucosa (6.6nmol/ml) and liver homogenate (2.23nmol/ml) after 48h. A significantly higher GSH content was observed in *P.longum* (intestinal mucosa 14.8nmol/ml, and liver homogenate 3.96nmol/ml), Piperine (intestinal mucosa 15.9nmol/ml, and liver homogenate 4.2nmol/ml), *T.occidentalis* (intestinal mucosa 15.3nmol/ml, and liver homogenate 4.02nmol/ml) and TPS (intestinal mucosa 14.1nmol/ml, and liver homogenate 3.5nmol/ml) treated animals relative to untreated controls after 48h

Table II

Effect of *Piper longum* on spleen nodule formation in radiation exposed mice.

Treatment	No. of spleen colonies
Normal BM (control)	6±1.1
P.longum BM	9.2±1.5
Piperine BM	10.7±0.9
T.occidentalis BM	13.5±1.7
TPS BM	12.1±1.0
Normal BM + *P.longum*	10.8±1.1
Normal BM + Piperine	12.1±0.9
Normal BM + *T.occidentalis*	14.6±1.5
Normal BM + TPS	12.7±0.9
P.longum BM + *P.longum*	14.8±2.3
Piperine BM + Piperine	16.5±1.2
T.occidentalis BM + *T.occidentalis*	15.2±1.9
TPS BM + TPS	14.0±1.3

BALB/c mice were exposed to single dose of whole body irradiation and treated with different doses of compounds. Animals were injected with bonemarrow (BM) cells from both treated and untreated donor mice. Recepient mice were sacrificed on 7^{th} day and number of spleen colonies was counted.

(Table III).

In irradiated control animals, the liver ALP was 15.2KA units at 48 h after radiation exposure, and the level was elevated to 16.1KA units on day 7 of radiation exposure, while in the *P. longum,* Piperine, *T. occidentalis* and TPS treated group the ALP level was decreased to 11.7KA units, 12.3KA units, 12.2KA units and 11.5KA units at 48h (Table IV). The normal ALP level is 14KA units. On day 11 after radiation exposure, the liver ALP was decreased to normal levels in all cases.

In the case of serum ALP, the irradiated control animals showed an elevated level of 15.8 U/ml at 48 h and 20.2 U/ml on day 7 however, the administration of *P. longum,* Piperine, *T. occidentalis* and TPS decreased the elevated level of serum ALP to 13.5U/ml, 12.1U/ml 15.2U/ml and 14.3U/ml on day 7, when compared to the control animals. The normal level of serum ALP is 13 U/ml.

Serum and liver GPT, which were increased after radiation (120U/ml and 89.7U/ml respectively), were significantly lower in the *P. longum,* Piperine, *T. occidentalis* and TPS treated group (serum 80.5U/ml, 77.2U/ml, 92.2U/ml and 94.7U/ml; liver 84.8U/m, 75.4U/m, 84.1U/m, and 85.6U/mTable V) after 48 h than in untreated control animals. On day 11 after radiation exposure, the liver and serum was decreased to normal levels in all cases.

Whole body γ-irradiation caused an increase in the amount of lipid peroxidation products (Table VI). An increase in the thiobarbituric acid-reactive substances level in liver (4.5nmol/mg protein formed/min/mg protein) and serum (2.96nmol/ml) was evident in untreated control animals 48h after the radiation exposure. In *P. longum,* Piperine, *T. occidentalis* and TPS treated, irradiated animals

Table III

Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on GSH (reduced glutathione) in mice treated with radiation

Treatment	Intestine (nmol/mg protein)			Liver (nmole/mg)		
	48th h.	7th day	11th day	48th h.	7th day	11th day
Normal	17.2± 0.27	-	-	6.5± 0.3	-	-
Control	6.6± 1.3	8.1± 0.6	12.7± 0.4	2.2± 0.2	5.1± 1.8	6.1± 0.2
Piper longum + Radiation	14.7± 1.5*	16.8± 1.8*	18.2± 2.2*	3.9± 0.5*	6.1± 0.2*	6.2± 0.2*
Piperine + Radiation	15.9± 1.4*	17.4± 1.3*	18.5± 2.1*	4.2± 0.4 *	6.0± 0.2*	7.2± 0.6*
T.occidentalis + Radiation	15.3± 1.9*	16.2± 1.1*	16.7± 1.3*	4.0± 0.3*	5.7± 0.2*	5.9± 0.3*
TPS + Radiation	14.1± 0.9*	15.9± 1.4*	16.0± 1.0*	3.5± 0.3*	5.5± 0.6*	5.6± 0.7*

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS after exposing γ-radiation (6Gy). Values are the mean ± SD. Statistically significant from untreated control.

* $P < 0.001$

Table IV

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on ALP (Alkaline Phosphatase) in mice treated with radiation

Treatment	Serum (U/ml)			Liver (KA)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	13.0± 0.4	-	-	14.0± 0.26	-	-
Control	15.8± 0.57	20.2± 0.84	14.7± 1.3	13.2± 0.15	16.1± 1.3	14.7± 0.47
Piper Longum + Radiation	12.4± 1.2	13.5± 0.72	7.5± 0.44	11.7± 0.95	13.7± 0.6	13.8± 1.0
Piperine+ Radiation	11.3± 0.9	12.1± 0.9	8.4*± 1.0	12.3± 1.0	13.9*± 0.9	14.4± 1.1
T.occidentalis + Radiation	13.1*± 1.5	15.2± 1.7	10.5± 1.0	12.2± 1.2	14.3*± 1.5	12.7± 1.3
TPS + Radiation	13.7± 1.2	14.3*± 1.3	10.9± 1.2	11.5± 1.0	13.2± 1.1	13.5± 1.2

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS after exposing γ-radiation (6Gy). Values are the mean ± SD. Statistically significant from untreated control.

* $P < 0.001$

Table V

Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on GPT (Glutathione pyruvate transaminase) in mice treated with radiation

Treatment	Serum (U/ml)			Liver (U/ml)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	61± 2.0	-	-	50.3± 4.0	-	-
Control	120± 9.0	98.5± 4.7	76.6± 3.9	89.8± 3.3	72.1± 5.3	56.9± 2.3
P. longum + Radiation	80.5± 6.1*	74.4± 5.9*	64.0± 5.5*	84.8± 7.9*	63.2± 2.6*	50.8± 2.7*
Piperine + Radiation	77.2± 6.3*	70.3± 6.6*	59.3± 4.3*	75.4± 6.3*	60.1± 5.7*	49.2± 3.8*
T.occidentalis + Radiation	92.2± 4.6*	79.2± 5.8*	69.4± 4.2*	84.1± 2.3*	63.9± 2.9*	52.9± 2.4*
TPS + Radiation	94.7± 5.9*	83.2± 7.3*	72.1± 6.5*	85.6± 8.1*	65.2± 6.0*	55.9± 4.7*

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS after exposing to γ-radiation (6Gy). Values are the mean ± SD. Statistically significant from untreated control. * $P < 0.001$

Table VI

Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on lipid peroxidation (LPO) level in mice treated with irradiation

Treatment	Serum (nmol/ml)			Liver (nmol/mg protein/ min/mg.progein)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	1.42± 0.2	-	-	1.26± 0.1	-	-
Control	2.9± 0.1	3.5± 0.5	1.6± 0.2	4.5± 0.4	3.04± 0.7	1.9± 0.03
Piper longum + Radiation	1.37± 0.1*	2.58± 0.8*	1.55± 0.2*	3.2± 0.3*	1.87± 0.6*	0.96± 0.1*
Piperine + Radiation	2.31± 0.1*	2.12± 1.0*	1.40± 0.5*	2.83± 0.1*	1.51± 0.5*	1.20± 0.9*
T.occidentalis + Radiation	1.58± 0.2*	2.83± 0.5*	1.68± 0.2*	3.43± 0.3*	2.48± 0.3*	1.23± 0.1*
TPS + Radiation	1.41± 0.1*	2.58± 0.7*	1.71± 0.6*	3.51± 0.2*	2.73± 0.1*	1.37± 0.9*

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS after exposing to γ-radiation (6Gy). Values are the mean ± SD. Statistically significant from untreated control. * $P < 0.05$

at the same time point, the level of lipid peroxidation products was significantly reduced to 3.1nmol/mg protein formed/min/mg protein, 2.8nmol/mg protein formed/min/mg protein, 3.4nmol/mg protein formed/min/mg protein and 3.5nmol/mg protein formed/min/mg protein in liver and it was 1.3nmol/ml, 2.3nmol/ml, 1.5nmol/ml and 1.4nmol/ml in serum. At day 11 after radiation exposure, the liver lipid peroxoide (LPO) level reached normal levels (serum 1.68nmol/ml; liver 1.23nmol/mg protein formed/min/mg protein) in treated animals.

Effect of *P. longum,* Piperine, *T. occidentalis* and TPS on radiation induced damage in mouse intestine

Complete crypt recovery was not seen during the observation period in irradiated control group, while in the irradiated Thuja-treated group, the normal crypt number was restored by 7^{th} day after radiation exposure (Figure III).

II. Protective effect of *P. longum,* Piperine, *T. occidentalis* and TPS against Cyclophosphamide induced toxicity in mice

Cyclophosphamide treatment

In each experiment, the animals were injected intraperitoneally with Cyclophosphamide (25mg/kg body weight/dose) for ten consecutive days. The animals were treated with compounds along with CTX on consecutive days.

Determination of the effect of *P. longum,* Piperine, *T. occidentalis* and TPS on haematological changes after CTX administration

Five groups of male Swiss albino mice (6mice /group) were used in this study. Group I animals were treated with 10 doses of CTX on consecutive days. Group II-V animals were treated with 10 doses of *P. longum,* Piperine, *T. occidentalis* and TPS

Figure III

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the histopathology of small intestine of radiation exposed mice.

a) Normal (non-irradiated)

b) Control (radiation alone)

c) Treated with *P. longum* after radiation exposure.

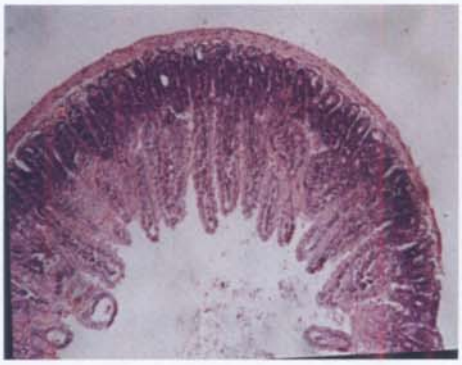
d) Treated with Piperine after radiation exposure.

e) Treated with *T.occidentalis* after radiation exposure.

f) Treated with TPS after radiation exposure.

Fig. III Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the histopathology of radiation exposed mice.

a



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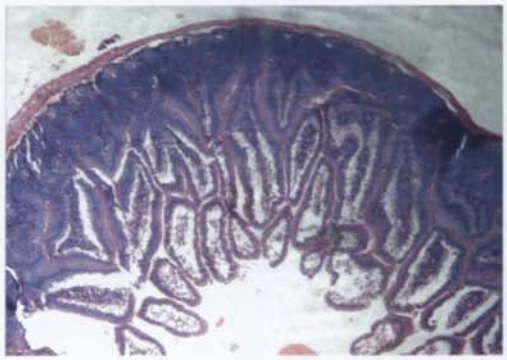

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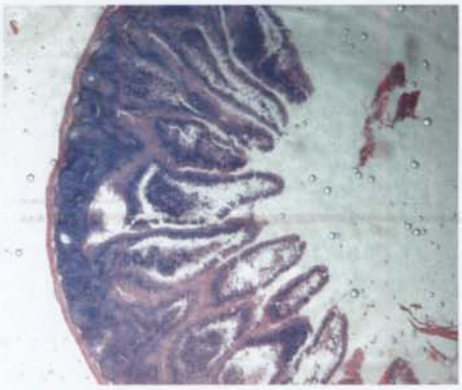

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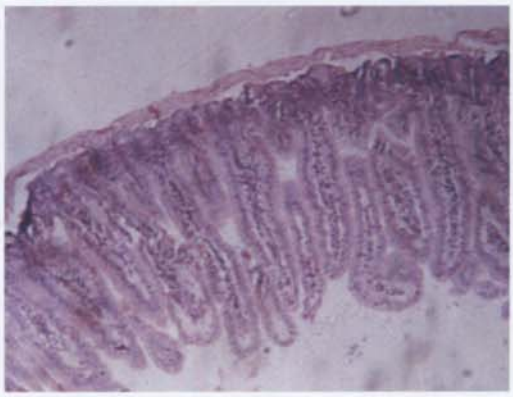

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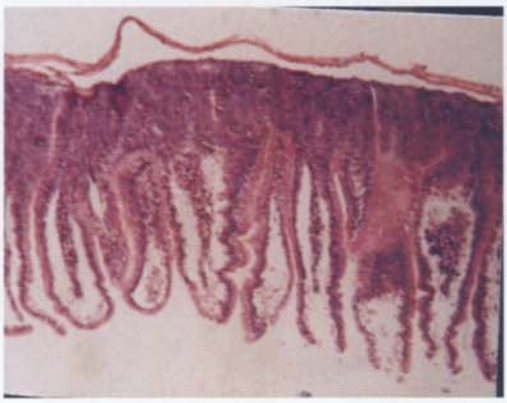

respectively along with CTX on consecutive days.

Blood was collected from the caudal vein and parameters such as total WBC count (Haemocytometer method), differential count (Leishman's stain), haemoglobin content (cyanmethemoglobin method) (Chessbrough and Mac Arthur., 1976) as well as body weight were recorded prior to the drug treatment and every third day for one month.

Determination of the effect of *P. longum*, Piperine, *T. occidentalis* and TPS on the bone marrow cellularity and α-esterase positive cells after CTX administration

Bone marrow cellularity was determined by the method of Sredni et al (Sredni et al., 1992). The animals were divided in to six groups (18mice/group). Group I was kept as untreated control. Group II was treated with five doses of CTX (25mg/kg body weight/dose;ip) on consecutive days. Group III-IV animals were treated with *P.longum,* Piperine, *T. occidentalis* and TPS respectively for five consecutive days along with CTX. Six animals from each group were sacrificed by cervical dislocation on 2^{nd}, 7^{th} and 11^{th} day after CTX administration. Bone marrow cells from femur was collected and made into sing le cell suspension and the cell number was determined using haemocytometre and the number of α-esterase positive cells were determined (Bancroft and Cook HF, 1984) as explained in chapter- 2.

Determination of the effect of *P. longum*, Piperine, *T. occidentalis* and TPS on scavenging of CTX induced free radicals, oxidative damages and normal cell proliferation

A similar set of experiment was conducted as above and treated for 10 consecutive days. Six animals from each group were sacrificed after 2, 7 and 11 days

by cervical dislocation. Blood was collected by heart puncture immediately following sacrifice and serum was separated. The liver and small intestine were collected to evaluate radiation-induced toxicities; total protein was estimated by Lowry's method (Lowry et al, 1951). GSH levels in liver homogenate and intestinal mucus were estimated by the method of Moron et al (Moron et al, 1979) based on the reaction with DTNB.

Activities of liver homogenate and serum pathophysiological enzymes such as alkaline phosphatase (King 1965) and glutathione pyruvate transaminases (Bergmeyer and Bernt, 1980) were determined. The lipid peroxidation level in liver and serum was measured using thiobarbituric acid-reactive substances (TBARS) by previously published method (Ohkawa et al, 1979).

Histopathological studies

The jejunal portion of the small intestine from the previous experiment was excised and fixed in 10% formaldehyde. Sections (4μm) were stained with eosin and hematoxylin as explained in chapter 2.

Results

Effect of *P. longum*, Piperine, *T. occidentalis* and TPS on haematological changes after CTX administration

CTX administration significantly reduced the total WBC count in mice (Fig.IV). Myelosuppression as seen from the WBC count was observed through out the period of CTX administration. Administration of *P.longum* could significantly increase the WBC count from day 12 onwards. On day 18 the CTX alone treated group showed a count of 3560.3 ± 302.4 while it was significantly increased by the

Fig. III Effect of *P. longum* , Piperine, *T. occidentalis* and TPS on total WBC count in ctx treated mice

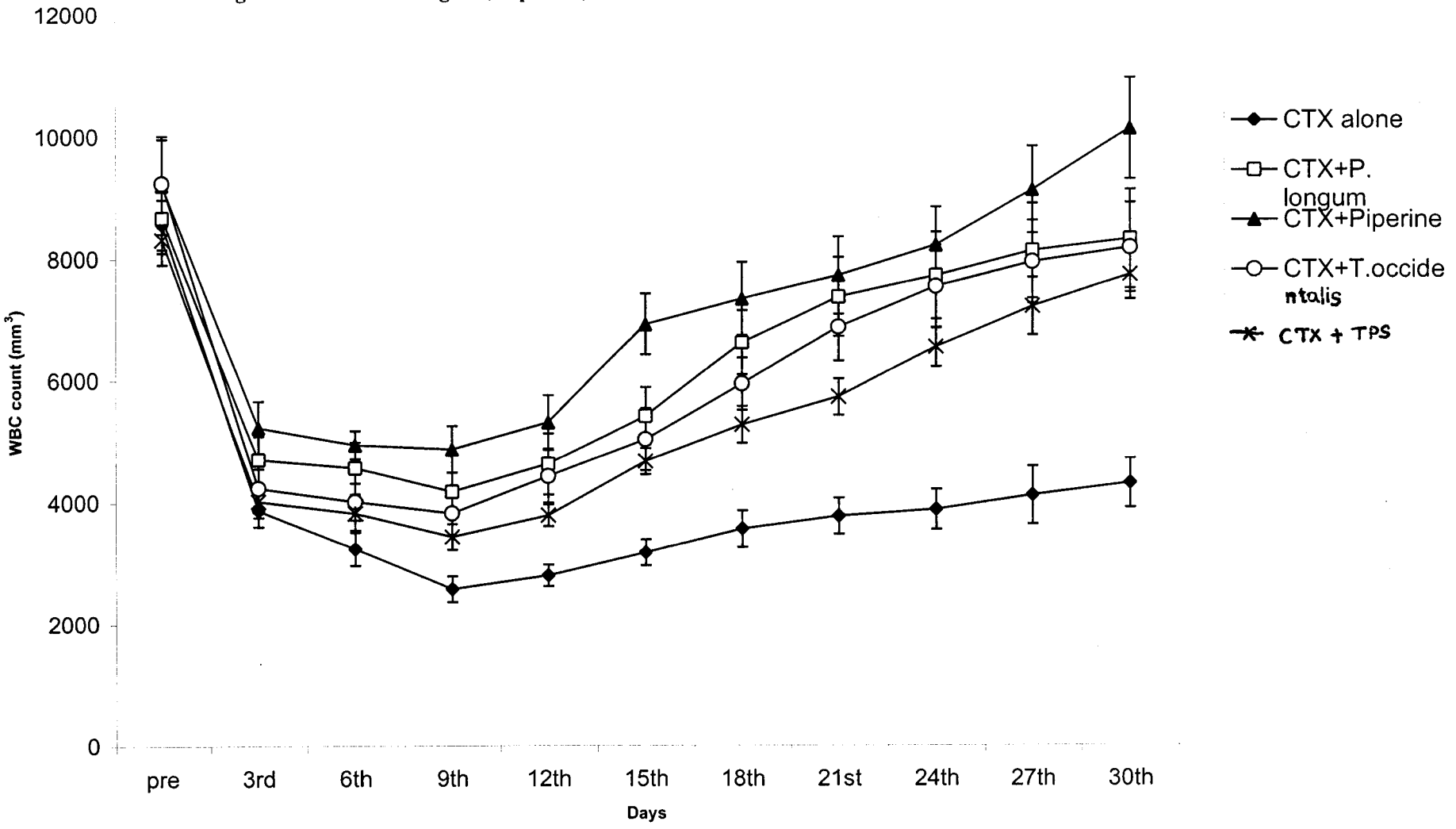

Table VII

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on bone marrow cellularity and α-esterase activity in CTX administered animals

Treatment	Bone marrow cellularity (cells/femur) x10^6			No. of α-esterase positive cells/4000 cells		
	48th h	7th day	11th day	48th h	7th day	11th day
Normal	14.9± 0.7	-	-	992± 71.4	-	-
CTX alone (control)	4.8± 0.37	6.9± 0.58	11.7± 1.10	107.4± 9.6	384.4± 30.4	637.8± 74.9
CTX+ *P.longum*	8.7± 0.7*	10.1± 0.9*	14.9± 1.5*	336.9± 30.12*	670.3± 52.43*	913.5± 72.84*
CTX+ *Piperine*	9.2± 0.8*	12.4± 1.1*	14.5± 1.6*	402.7± 19.4*	723.1± 43.2*	1012.5± 67.4*
CTX+ *T.occidentalis*	7.9 ± 0.6*	9.8± 0.8*	14.2± 1.3*	293.4± 28.1*	623.7± 58.7*	874.9± 64.3*
CTX+ TPS	6.7± 0.4*	8.2± 0.5*	12.8± 0.9*	253.6± 20.2*	547.7± 65.7*	749.4± 39.9*

Animals were treated with 5 doses of *P. longum,* Piperine, *T.occidentalis* and TPS followed by CTX administration. Bone marrow cells were collected from femur. Values are the mean ± SD. Statistically significant from untreated control. * $P < 0.001$

treatment with *P.longum* (6618± 534.2), Piperine (6923.5±503.4), *T.occidentalis* (5937.2±427.5) and TPS (5372.2±235.3). There was no significant effect on the differentia count, hemoglobin level and body weight (data not shown).

Effect of *P. longum*, Piperine, *T. occidentalis* and TPS on the bone marrow cellularity and α-esterase positive cells after CTX administration

There was also a drastic reduction in bone marrow cellularity in CTX treated control animals (4.8±0.37X10^{6}cells/femur) after 48h, when compared to normal animals (14.9X10^{6}cells/femur). Treatment with *P.longum* (8.78±0.74X10^{6}cells/femur), Piperine (9.23±0.85X10^{6}cells/femur), *T. occidentalis* (7.93±0.X10^{6}cells/femur) and TPS (6.78±0.43X10^{6}cells/femur) could significantly increase the bone marrow cellularity on the same day. It was increased gradually; clearly indicating that bone marrow cell proliferation is significantly increased after treatment (Table VII).

The effect of *P.longum* extract on a-esterase positive cells is presented in table-VII. The number of α-esterase positive cells was significantly decreased after CTX administration. *P.longum,* Piperine, *T. occidentalis* and TPS treatment elevated the α-esterase activity of maturing monocytes. On day 11 *P.longum,* Piperine, *T. occidentalis* and TPS treated group had 913.5±72.8cells/4000 cells, 1012.5±67.4cells/4000 cells, 874.9±64.3cells/4000 cells, and 749.4±39.9cells/4000 cells, while CTX alone administered group had only 637.8± 74.9cells/4000 cells respectively.

Effect of *P. longum*, Piperine, *T. occidentalis* and TPS on scavenging of CTX induced free radicals, oxidative damages and normal cell proliferation

The CTX alone treated mice had a lowered level of GSH in intestinal mucosa (5.90±1.24nmol/ml) and liver homogenate (2.40±0.28nmol/ml) after 48h. A significantly higher GSH content was observed in *P.longum* (intestinal mucosa 16.2±1.40nmol/ml, and liver homogenate 5.72±0.41nmol/ml) Piperine (intestinal mucosa 16.2±1.40nmol/ml, and liver homogenate 5.72±0.41nmol/ml) *T. occidentalis* (intestinal mucosa 16.2± 1.40nmol/ml, and liver homogenate 5.72±0.41nmol/ml) and TPS (intestinal mucosa 16.2±1.40nmol/ml, and liver homogenate 5.72±0.41nmol/ml) treated animals relative to control animals on day 11. The normal level of GSH in liver is (6.5±0.33nmol/ml) (Table VIII).

In the case of serum ALP, the CTX alone treated control animal showed an elevated level of 16.3±1.5U/ml at 48 hours and 17.1±2.09U/ml on day 7. However the administration of *P.longum* (13.9±1.80KAunits) Piperine (12.6±1.80KAunits), *T. occidentalis* (15.1±1.80KAunits) and TPS (14.9±1.0KAunits) decreased the elevated level of serum ALP on day 7. The normal level of serum ALP is 13.0±0.40U/ml (Table IX).

In CTX alone treated control animals, the liver ALP was 17.3±2.04KA units after 48h and the level was elevated to 21.43±2.40KAunits on day 7 after CTX administration. The elevated level of liver ALP was significantly decreased by the treatment with *P.longum* (14.0±2.02U/ml) Piperine (14.8±1.2U/ml), *T. occidentalis* (15.1±2.1U/ml) and TPS (15.3±1.0U/ml) on the same day (Table IX).

The increased level of serum GPT level in CTX alone treated group

Table VIII

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on GSH (reduced glutathione) in CTX administered animals

Treatment	Intestine (nmol/mg protein)			Liver (nmole/mg)		
	48th h.	7th day	11th day	48th h.	7th day	11th day
Normal	17.2± 0.2	-	-	6.5± 0.3	-	-
CTX alone (Control)	5.9± 1.2	7.8± 0.6	11.4± 0.9	2.4± 0.2	3.2± 0.9	4.2± 0.3
CTX+ *P.longum*	14.2± 1.83*	15.48± 1.62*	16.2 ± 1.40*	3.7± 0.3*	4.8± 0.4	5.7± 0.4
CTX+ *Piperine* +	15.9± 1.4*	17.4± 1.3*	18.5± 2.1*	4.0± 0.3 *	5.2± 0.4*	6.4± 0.4*
CTX+ *T.occidentalis* +	15.3± 1.9*	16.2± 1.1*	16.7± 1.3*	3.0± 0.2*	4.1± 0.2*	5.3± 0.5*
CTX+ TPS +	14.1± 0.9*	15.9± 1.4*	16.0± 1.0*	3.2± 0.3*	3.8± 0.2*	4.7± 0.2*

Animals were treated with 10 doses of *P. longum,* Piperine, *T.occidentalis* and TPS followed by CTX administration. Intestinal mucosa and liver were collected. Values are the mean ± SD. Values are the mean ± SD. Statistically significant from untreated control. * P < 0.001

Table IX

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on ALP (Alkaline Phosphatase) in CTX administered animals

Treatment	Serum (U/ml)			Liver (KA)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	13.0± 0.4	-	-	14.0± 0.26	-	-
CTX alone+ (Control)	16.3± 1.5	17.1± 2.1	15.9± 1.9	17.3± 2.0	21.4± 2.4	17.5± 1.3
CTX+ *P.longum*	14.8± 1.4*	14.0± 2.0*	13.0± 1.9*	14.4± 1.7*	13.9± 1.8	13.1± 1.7*
CTX+ *Piperine*	13.2± 0.9	14.8± 1.2	13.0± 0.97	14.5± 1.0	12.6± 1.0	14.2± 1.2
CTX+ *T.occidentalis*	15.6± 1.3	15.0± 1.4	14.6± 1.2	16.4± 1.1	15.1± 2.0	14.2± 1.5
CTX+ TPS	16.8± 1.0	15.9± 1.6	14.9± 1.3	15.7± 1.1	14.9± 1.0	13.1± 0.9

Animals were treated with 10 doses of *P. longum,* Piperine, *T.occidentalis* and TPS followed by CTX administration. Intestinal mucosa and liver were collected. Values are the mean ± SD. Values are the mean ± SD. Statistically significant from untreated control. * P < 0.001

(101.21±9.84U/ml) was lowered by the treatment with *P. longum* (82.46±7.89U/ml) Piperine (78.5±6.9U/ml), *T. occidentalis* (84.3±7.4U/ml) and TPS (87.1±8.7U/ml) on day 7 (Table X). Similarly CTX alone treated group showed an elevated level of liver GPT level (77.08±3.35U/ml) on day 7, when compared to the normal animals (50.3±4.0U/ml). These increased levels of liver GPT level were lowered by the administration of *P.longum* (63.55±4.43U/ml) Piperine (57.4±4.1U/ml), *T. occidentalis* (69.6±4.6U/ml) and TPS (73.2±6.2U/ml) on 7th day (Table X).

CTX administration caused an increase in the amount of lipid peroxidation products (Table XI). An increase in the thiobarbituric acid reactive substance level in the serum (3.0±0.53nmol/ml) and liver (4.09±0.49nmol/mg/protein formed/min/mg protein) was evident in the CTX alone administered animals after 48h. The level of serum lipid peroxidation where also significantly reduced by treating with *P.longum* (1.65±0.15nmol/ml), Piperine (1.50±0.11nmol/ml), *T.occidentalis* (1.75±0.15nmol/ml), and TPS (2.1±0.31nmol/ml) after 48 hours. Similarly the level of liver lipid peroxidation products was significantly reduced by the administration of *P.longum* (1.91±0.33nmol/mg/protein formed/min/mg protein), Piperine (1.44±0.57nmol/mg/protein formed/min/mg protein), *T. occidentalis* (2.50±0.29nmol/ mg/protein formed/min/mg protein) and TPS (2.61±0.34nmol/mg/protein formed/min/mg protein) after 48 hours. 11 days after CTX administration the lipid peroxide level reached normal in *P.longum,* Piperine, *T. occidentalis* and TPS treated animals.

Effect of *P. longum,* Piperine, *T. occidentalis* and TPS on CTX induced damage in mouse intestine

Compete crypt recovery was not found during the observation period in the

Table X

Effect of *P. longum,* Piperine, *T.occidentalis* and TPS on GPT (Glutathione pyruvate transaminase) in CTX administered animals

Treatment	Serum (U/ml)			Liver (U/ml)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	61± 3.0	-	-	50.3± 4.0	-	-
Control (CTX alone)	125.3± 11.4	101.2± 9.8	83.4± 7.6	83.5± 6.4	77.0± 3.3	62.8± 5.7
CTX+ *P.longum*	96.7± 8.7*	82.4± 7.8*	68.4± 7.2*	78.4± 5.5*	63.5± 4.4*	51.5± 4.1*
CTX+ *Piperine*	90.4± 8.0*	77.3± 6.2*	64.4± 5.2*	69.2± 5.8*	57.4± 4.1*	49.4± 3.8*
CTX+ *T.occidentalis*	100.5± 11.4*	87.9± 7.1*	71.6± 6.3*	80.7± 4.3*	69.6± 4.6*	53.1± 3.8*
CTX+ *TPS*	105.4± 10.1*	93.4± 8.0*	77.4± 6.1*	84.3± 8.1*	73.2± 6.2*	56.4± 4.2*

Animals were treated with 10 doses of *P.longum,* Piperine, *T.occidentalis* and TPS followed by CTX administration. Values are the mean ± SD. Values are the mean ± SD. Statistically significant from untreated control. * $P < 0.001$

Table XI

Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on lipid peroxidation (LPO) level in CTX administered animals

Treatment	Serum (nmol/ml)			Liver (nmol/mg protein/ min/mg.progein)		
	48h.	7th day	11th day	48 h.	7th day	11th day
Normal	1.42± 0.2	-	-	1.26± 0.1	-	-
Control	3.0± 0.5	2.3± 0.3	1.9± 0.3	4.09± 0.49	3.44± 0.46	3.10± 0.45
Piper longum + Radiation	2.27± 0.42*	1.65± 0.15	1.42± 0.23	3.73± 0.37*	1.91± 0.33	1.12± 0.18*
Piperine + Radiation	1.8± 0.08*	1.50± 0.11*	1.43± 0.31*	2.53± 0.42**	1.44± 0.57*	1.25± 0.08*
T.occidentalis + Radiation	2.55± 0.36*	1.75± 0.15*	1.50± 0.18*	4.26± 0.37*	2.50± 0.29*	1.42± 0.30*
TPS + Radiation	2.92± 0.42*	2.10± 0.31*	1.73± 0.40*	4.52± 0.39*	2.61± 0.34*	0.96± 0.08*

Animals were treated with 10 doses of *P. longum,* Piperine, *T.occidentalis* and TPS followed by CTX administration. Values are the mean ± SD. Values are the mean ± SD. Statistically significant from untreated control. * $P < 0.001$,** $P < 0.05$

CTX alone treated group, while in *P.longum,* Piperine, *T. occidentalis* and TPS treated group the normal crypt number was observed by day 7.

Discussion

Radioprotection may include scavenging of free radicals and protection of cellular and sub-cellular entities against oxidative damage as well as restoration of normal cell proliferation. Compounds having such properties can offer protection against radiation damage in the clinical setting. Major side effects of radiotherapy comprise tissue injury in target and non-target cells, especially cells of the immune system. This study was carried out mainly to determine the immunomodulatory activity of *P.longum*, Piperine, *T. occidentalis* and TPS and its potential to reduce toxicity induced by radiation and cyclophosphamide in order to evaluate its use as an adjuvant during radiotherapy and chemotherapy.

Administration of *P.longum*, Piperine, *T. occidentalis* and TPS significantly reduced the leucopenia induced by sublethal dose of radiation therapy and cyclophosphamide .The *P.longum*, Piperine, *T. occidentalis* and TPS treated mice attained normal WBC counts at the end of the treatment, whereas the regenerative capacity in control animals was very low and did not regain a normal level. This indicates that treated group stimulated the hemopoietic system, which is highly sensitive to ionizing radiation and chemotherapy owing to its high proliferation rate. In irradiated and cyclophosphamide treated mice, *P.longum*, Piperine, *T.occidentalis* and TPS treatment significantly increased the bone marrow cellularity and α-esterase positive cells, which indicated proliferation of stem cells and their differentiation.

Glutathione metabolism is often correlated with cellular sensitivity to

anticancer agents. The GSH detoxification system is an important part of cellular defense against a large array of injury agents. GSH offers protection against oxygen-derived free radicals and cellular lethality following exposure to ionizing radiation. (Biaglow et al, 1987) and metabolism of CTX. GSH is a versatile protector and executes its radioprotective and chemoprotective function through free radical scavenging, restoring the damaged molecule by hydrogen donation, reducing peroxides and maintaining protein thiols in a reduced state (Bump et al, 1990). *P.longum*, Piperine, *T. occidentalis* and TPS was found to significantly enhance the level of GSH, which is involved in detoxification.

The decreased serum enzyme activities observed in the irradiated CTX treated group could be due to the increased formation of hydroxyl radicals. It has been reported that hydroxyl radicals inhibits alkaline phosphatase activity (Ohyashiki et al, 1994) and that oxygen radicals generated during the peroxidation reaction will attack the protein molecules in membranes (Fox, 1975). The liver enzymes, which moderately increased in irradiated and CTX administered group by day 7, were lowered by the treatment with *P.longum*, Piperine, *T. occidentalis* and TPS. In treated groups the level of ALP was decreased and then retained the normal level. Similarly, serum and liver GPT levels, which were increased after radiation, were significantly lower in *P.longum*, Piperine, *T. occidentalis* and TPS treated groups.

The free radicals generated during the metabolism of CTX, and radiolytic products, including hydroxyl and hydroperoxyl radicals can initiate lipid peroxidation (Releigh, 1989). In the present study it was observed that irradiation and CTX administration increased the serum and liver lipid peroxide (LPO) level, but the

P.longum, Piperine, *T. occidentalis* and TPS treated group had a significantly lower LPO level than the control group. Inhibition of LPO in biomembranes can be caused by antioxidants (Hendry et al, 1982).

The small intestine has been reported to have a remarkable capacity for repair (Hendry et al, 1982). A delay in the rate of recovery after high doses of radiation and CTX administration could be explained on the basis of high stem cell depletion by apoptotic as well as mitotic death. In the present study, the rate of recovery was faster in *P.longum*, Piperine, *T. occidentalis* and TPS treated group.

These results indicate that administration of alcoholic extract of *P.longum*, Piperine, *T. occidentalis* and TPS protected mice from toxic effects of radiation and Cyclophosphamide and promoted the recovery of bone marrow cells and leukocytes. One of the radioprotective mechanisms may be by scavenging free radicals. It is proposed that *P.longum*, Piperine, *T. occidentalis* and TPS mediated protection against the CTX could be due to its immunomodulatory activity and by the induction of phase II enzymes involved in the detoxification pathways. These results thus give some insight into the mechanisms involved in the radioprotective and chemopreventive action of *P.longum*, Piperine, *T. occidentalis* and TPS.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

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(FACULTY OF SCIENCE)

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Chapter 8

Induction of apoptosis in B16F-10 melanoma cells by Piper longum, Piperine, Thuja occidentalis and Thuja polysaccharide fraction

Introduction

Cancer development is known to be associated with increased cell proliferation and decreased apoptosis (Lowe et al., 2000). The apoptotic elimination of drug resistant cells amplifies the chemotherapeutic effects in responsive cells. Apoptosis is instrumental in destroying the developing neoplasms and facilitating the tumor regression. Apoptotic cells are characterized by cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and ultimate fragmentation of cell as membrane enclosed vesicles designated as apoptotic bodies (Takashi, Hiroshi, 2005).

Caspases are expressed and activated by proteolytic cleavage after death stimulus. Caspases function in both in initiation of apoptosis in response to apoptotic signals and in the subsequent effector pathway to disassemble the cells (Thornbery and Lazebnick, 1998). Bcl-2 family genes are important group that regulate apoptosis,. which include bcl-2, bcl-x, bax, mcl-1, bak-2 and bak-3. Among them bcl-2 is an antiapoptotic which promotes the chemoresistance but increases the recurrence rates enhances radioresistance and tumor malignant potential (Hockenbery et al, 1990; Gazzaniga et al, 1996).

Nuclear Factor-κB (NF-κB) participates in the regulation of diverse biological processes including immune and inflammatory responses, cell growth and apoptosis (Albert and Baldwin, 2001). Activation of AP-1 has been shown to play an important role on cell proliferation (Bharat, 2000). CREB is cyclic AMP response element binding protein which is a transcription factor that down stream in the protein kinase-A (PKA) signaling pathway. We have reported the effect of

Piperine on the activation of transcription factors previously (Pradeep and Kuttan, 2004). In this chapter we are demonstrating the effect of *T.occidentalis* on the activation of transcription factors in B16F-10 melanoma cells *in vitro.* In the present study we investigated whether the natural products such as *P.longum,* Piperine, *T. occidentalis* and TPS could modulates apoptosis of B16F-10 melanoma cells *in vitro.*

Materials and Methods

Cell line

B16F-10 melanoma cells were used for the *in vitro* study

Chemicals and Kits used

Cells to cDNA kits, Mouse bcl-2, caspase-3, GAPDH and Erk primer sets were used for this study.

Morphological analysis and DNA ladder studies

One million B16F-10 melanoma cells were treated with different concentrations of *P.longum* (2μg/ml, 5μg/ml and 10μg/ml), Piperine (2μg/ml, 5μg/ml and 10μg/ml), *T.occidentalis* (1μg/ml, 2μg/ml and 5μg/ml), and TPS (0.01, 0.05 and 1μg/ ml), and incubated further for 48h in serum free DMEM at 37°C in the presence of 5% CO_2. After that the cells were washed three times with PBS, centrifuged and the cell pellet was separated. To detect apoptotic morphology, a small portion of the cell pellets were suspended in PBS; smear was prepared and stained with hematoxylin and eosin. Apoptosis was characterized by the morphological changes such as chromatin condensation, nuclear condensation and apoptotic bodies.

Determination of the effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the DNA fragmentation

The rest of the pellet was suspended in 1ml of cytoplasm extraction buffer (10mM Tris-HCL buffer, pH 7.5 containing 150mM NaCl, 5mM MgCl2 and 0.5 % Triton X-100). DNA was extracted using phenol-chloroform method and resolved on 1.5% agarose gel containing ethidium bromide. The bands were visualized and recorded using gel-documentation system.

RT-PCR and Gene expression studies

B16F-10 melanoma cells were grown in serum free DMEM for 4h in the presence and absence of *P.longum* (10µg/ml), Piperine (10µg/ml), *T.occidentalis* (5µg/ml), and TPS (1µg/ ml). Total RNA was extracted from B16F-10 cells and cDNA was synthesized, using moloney murine leukemia virus reverse transcriptase. Gene expression analysis was done by PCR analysis. The mouse bcl-2, p53 and caspases-3 genes were amplified against GAPDH standard. The target PCR products generated from a positive control cDNA is included in the kit. PCR products were analyzed by agarose gel electrophoresis and visualized using gel documentation system as explained in chapter 2.

Preparation of nuclear extracts

Nuclear extracts were prepared by slightly modified previously published methods (Dignam, Lebovitz and Roeder, 1983). B16F-10 cells were grown in $25cm^2$ culture flask. When the cells were getting subconfluent, cells were treated with *T.occidentalis* (5µg/ml) for 2h at 37° in 5% CO_2 in serum free medium. The cells were washed with PBS twice and incubated with TNF-α (10ng/ml) for 30 min at 37° in 5% CO_2. The nuclear extracts were prepared by the method

described in Chapter-2. Protein concentrations of the nuclear extracts were estimated using standard Bradford method (Bradford, 1976) and stored at $^{-}70^{0}$C.

Transcription factor profiling

Each transcription factor profiling kit was provided in a 96 well format with consensus DNA binding sequence. When nuclear extracts added to the well, DNA will bind to their consensus sequences in the well. Bound transcription factors in the DNA were detected by specific primary antibody towards NF-ĸBp65, NF-ĸBp50, NF-ĸB c-Rel, c-Fos, ATF-2 and CREB. A horse radish peroxidase conjugated secondary antibody is then used to detect the bound primary antibody. The enzymatic product was measured with standard microtiter plate reader at 655nm. Percentage inhibition was calculated by the formula,

100-([OD of treated / OD of control]X 100).

Results

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the apoptotic morphology in B16F-10 melanoma cells

During apoptotic condition the cells under go morphological changes and cellular content reorganization. Untreated control cells did not show any morphological modifications or reorganizations (Fig-Ia). *P.longum* did not show effect at lower concentration (2µg/ml, 5µg/ml) but treatment with higher concentration (10µg/ml) displayed the stages of apoptosis as chromatin condensation or nuclear condensation (Fig-Ib). Piperine at a concentration of 2µg/ml did not give any apoptotic changes but treatment with 5µg/ml and

Figure I

Effect of *P. longum*, Piperine, *T. occidentalis* and TPS on the apoptotic morphology in B16F-10 melanoma cells

a) Untreated control with out apoptosis.

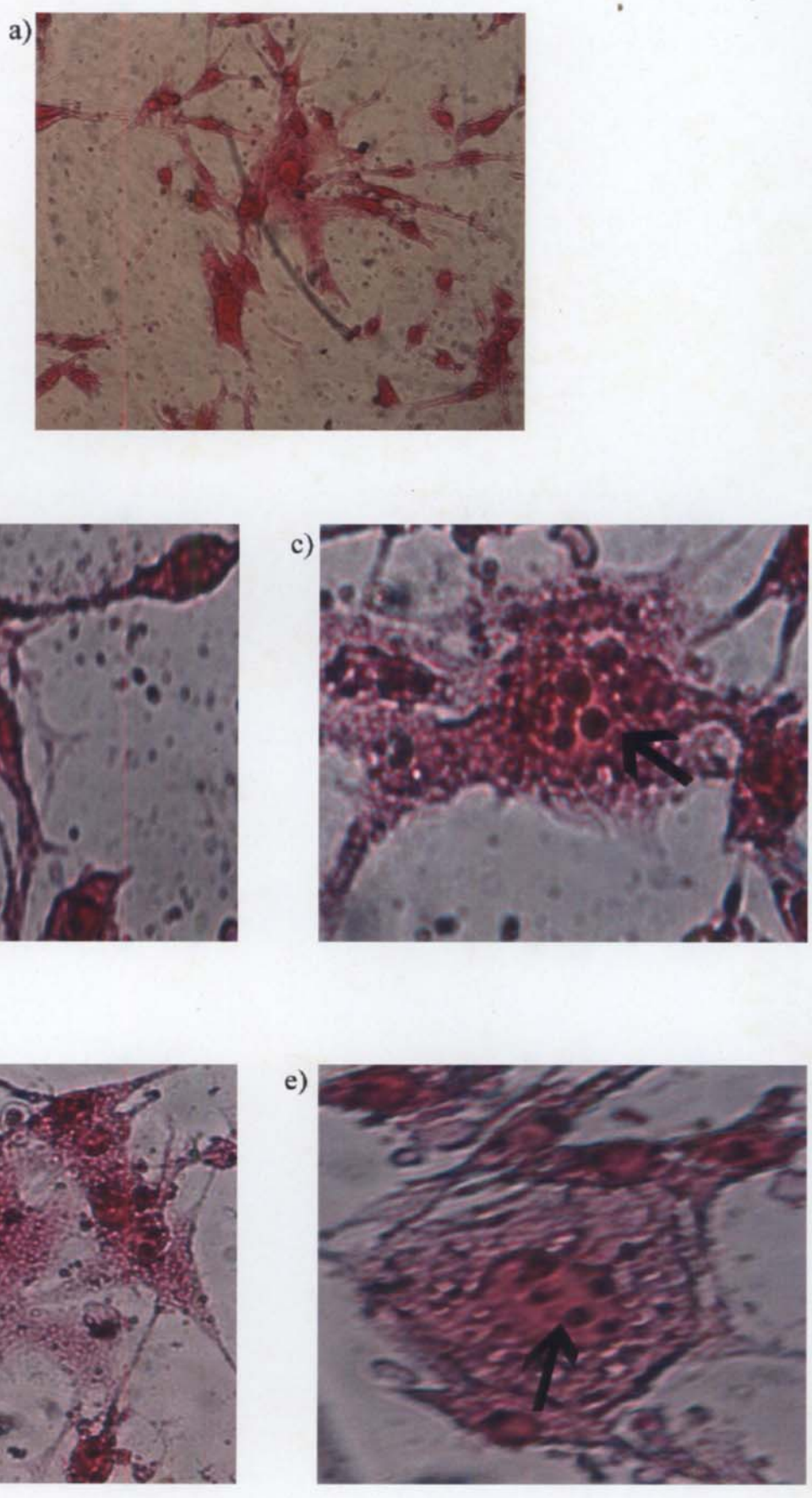
b) B16F-10 cells treated with *P.longum* (10μg/ml).

c) B16F-10 cells treated with Piperine (10μg/ml).

d) B16F-10 cells treated with *T.occidentalis* (5μg/ml).

e) B16F-10 cells treated with TPS (1μg/ml).

Fig. Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the apoptotic morphology in B16F-10 cells



10μg/ml produced the formation of apoptotic bodies and chromatin condensation (Fig-Ic). *T.occidentalis* at a concentration of 1μg/ml did not cause any apoptosis but 2μg/ml and 5μg/ml concentrations gave the cytoplasmic and nuclear changes related to apoptosis (Fig-Id). Lower concentrations of TPS such as 0.01μg/ml and 0.0510μg/ml did not produce any morphological changes but higher concentration of TPS (1μg/ml) displayed apoptotic characters (Fig-Ie).

Effects of *P.longum*, Piperine, *T.occidentalis* and TPS on the DNA ladder formation

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the DNA ladder pattern is shown in Fig-II. DNA of untreated control cells did not show any ladder pattern. *P.longum*, Piperine and *T.occidentalis* extract produced DNA ladder pattern in a dose dependent manner. Even though TPS at the concentration of 1μg/ml showed morphological changes it did not produce any DNA ladder formation.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the bcl-2 gene expression in B16F-10 melanoma cells

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS is shown in Fig-III. Lane 1 represents the molecular weight markers. Lane 2 shows the amplified bcl-2 positive control cDNA (235 bp) included in the kit. Lane 3 shows bcl-2 gene in the untreated B16F-10 melanoma cells. Bcl-2 level was significantly down regulated by the treatment with *P.longum*, Piperine, *T.occidentalis* and TPS. Lane 4 to lane 7 represents the *P.longum* (10μg/ml), Piperine (10μg/ml), *T.occidentalis* (5μg/ml) and TPS (1μg/ml) treatment respectively. Lane 8 represents the GAPDH

Figure II

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the DNA ladder Formation

Lane1, Marker

Lane2, Untreated control

Lane3, B16F-10 cells treated with *P.longum* (2μg/ml).

Lane4, B16F-10 cells treated with *P.longum* (5μg/ml).

Lane5, B16F-10 cells treated with *P.longum* (10μg/ml).

Lane6, B16F-10 cells treated with Piperine (2μg/ml).

Lane7, B16F-10 cells treated with Piperine (5μg/ml).

Lane8, B16F-10 cells treated with Piperine (10μg/ml).

Lane9, B16F-10 cells treated with *T. occidentalis* (1μg/ml).

Lane10, B16F-10 cells treated with *T. occidentalis* (2μg/ml).

Lane11, B16F-10 cells treated with *T. occidentalis* (5μg/ml).

Lane12, B16F-10 cells treated with TPS (0.01μg/ml).

Lane13, B16F-10 cells treated with TPS (0.05μg/ml).

Lane14, B16F-10 cells treated with TPS (1μg/ml).

177B 89

Fig. V Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the ladder formation

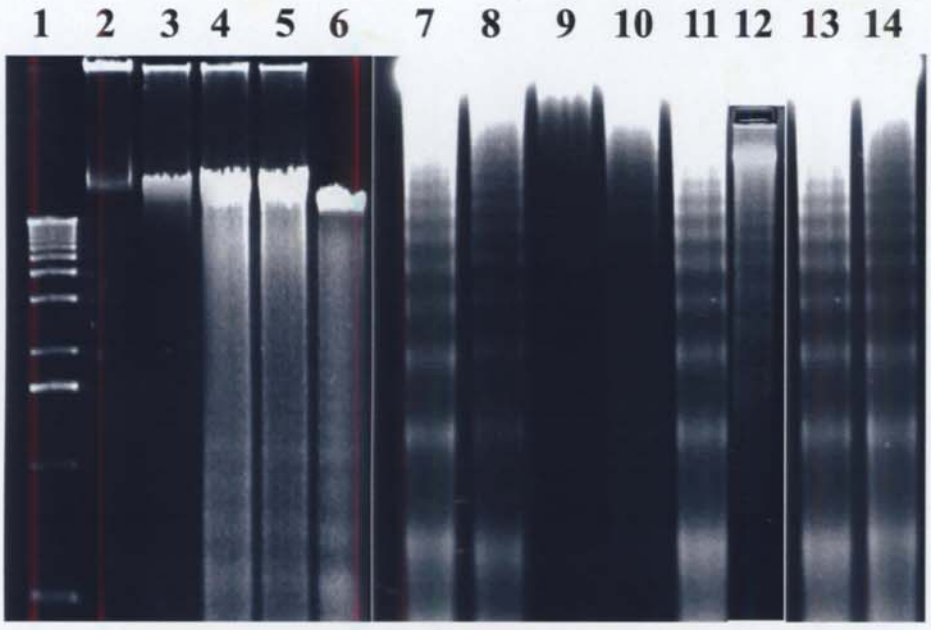


Figure III

Effect of *P.longum,* Piperine, *T.occidentalis* and TPS on the bcl-2 gene expression in B16F-10 melanoma cells

Lane1, Marker

Lane2, bcl-2 Positive control (235 bp)

Lane3, Untreated control

Lane4, *P.longum* treated (10µg/ml)

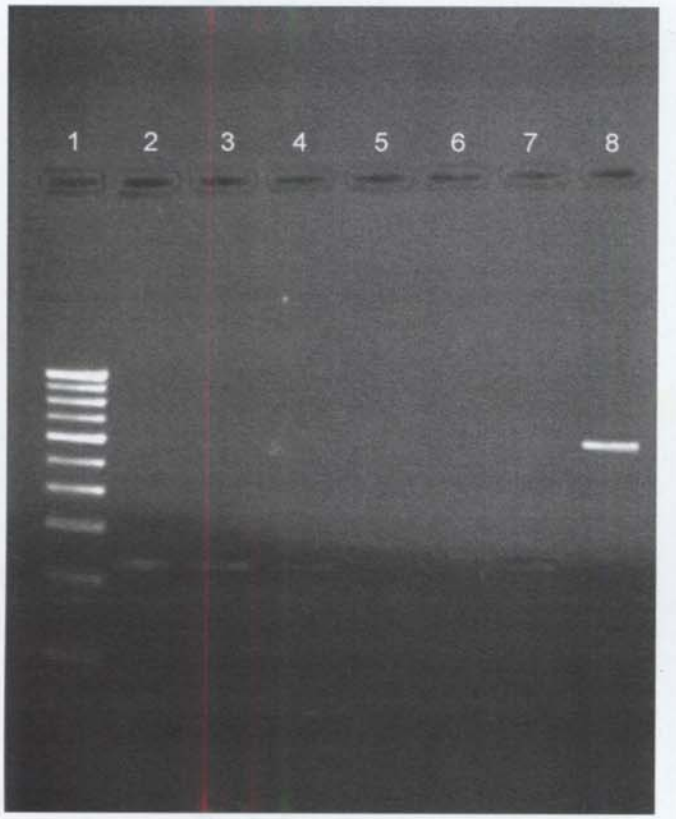
Lane5, Piperine treated (10µg/ml)

Lane6, *T.occidentalis* treated (5µg/ml)

Lane7, TPS treated (1µg/ml)

Lane8, GAPDH (527 bp)

Fig. III Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the bcl-2 expression in B16F-10 cells



(527bp).

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the caspase-3 proapoptotic gene expression in B16F-10 melanoma cells

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the caspase-3 gene expression in B16F-10 melanoma cells is shown in Fig-IV. Mouse caspase-3 gene was amplified against mouse GAPDH standard. Lane 1 shows the molecular weight markers.

Lane 2 shows the amplified caspase-3 positive control cDNA (414 bp) included in the kit. Lane3 shows the caspase-3 gene in the untreated control B16F-10 melanoma cells. Caspase-3 level was very low in the untreated group. Treatment of *P.longum* (10μg/ml), Piperine (10μg/ml), *T.occidentalis* (5μg/ml) and TPS (1μg/ml) could up-regulate the Caspase-3 genes (lane 4 to lane 7) respectively. Lane8 shows the GAPDH (527bp) standard.

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on p53 gene expression

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the expression of p53 gene is shown in Fig-V. *P.longum* (10μg/ml), Piperine (10μg/ml), *T.occidentalis* (5μg/ml) and TPS (1μg/ml) did not produce any change in the p53 expression. Fig III.b lane 1 shows the molecular weight markers. Lane 2 shows the amplified positive control p53 gene (205 bp) included in the kit. We did not get any prominent expression of P53 gene in the untreated control group (Fig V, lane 3) treatment with *P.longum*, Piperine, *T.occidentalis* and TPS could not up regulate the p53 expression (Fig IV. b lane 4-7). Lane8 shows the GAPDH (527bp) standard.

Figure IV

Effect of *P.longum,* Piperine, *T.occidentalis* and TPS on the caspase-3 proapoptotic gene expression in B16F-10 melanoma cells

Lane1, Marker

Lane2, Caspase-3 Positive control (414bp)

Lane3, Untreated control

Lane4, *P.longum* treated (10µg/ml)

Lane5, Piperine treated (10µg/ml)

Lane6, *T.occidentalis* treated (5µg/ml)

Lane7, TPS treated (1µg/ml)

Lane8, GAPDH (527 bp)

Fig. IV Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the caspase-3 expression in B16F-10 cells

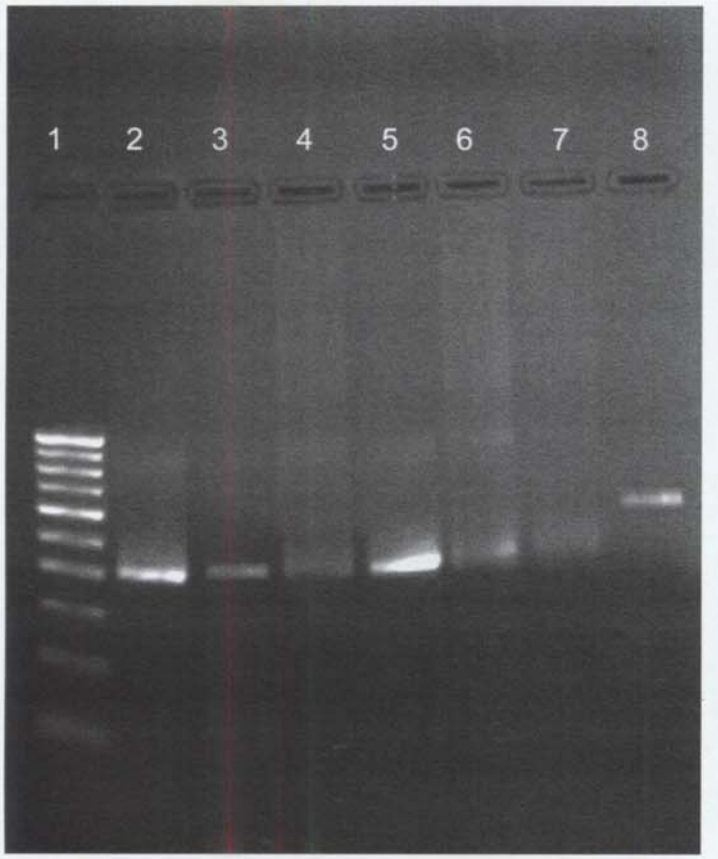


Figure V

Effect of *P.longum*, Piperine, *T.occidentalis* and TPS on the p53 gene expression in B16F-10 melanoma cells

Lane1, Marker

Lane2, p53 Positive control (205bp)

Lane3, Untreated control

Lane4, *P.longum* treated (10µg/ml)

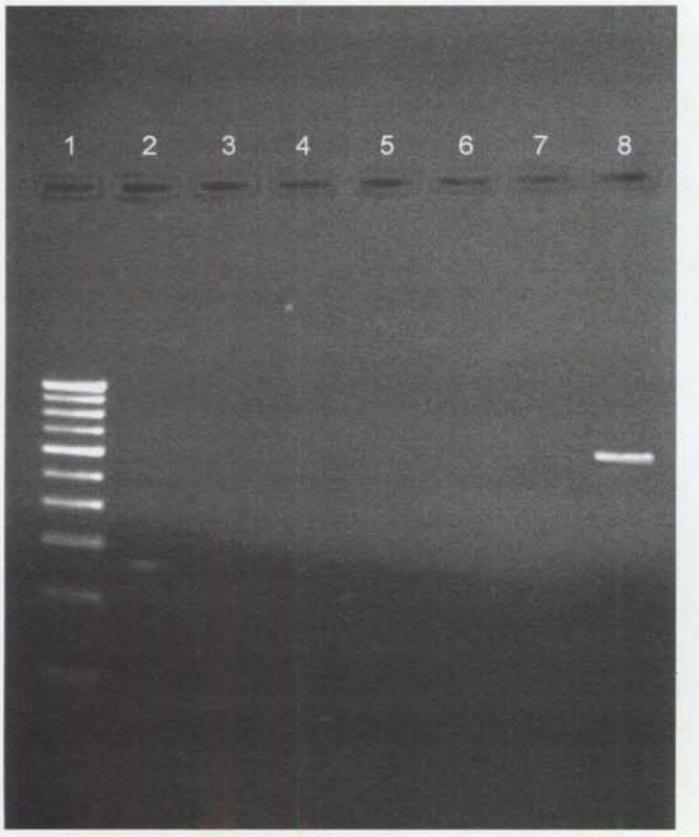
Lane5, Piperine treated (10µg/ml)

Lane6, *T.occidentalis* treated (5µg/ml)

Lane7, TPS treated (1µg/ml)

Lane8, GAPDH (527 bp)

Fig. V Effect of *P. longum*, Piperine, *T.occidentalis* and TPS on the p53 expression in B16F-10 cells



Effect of *T.occidentalis* on the nuclear translocation of transcription factors

Effect of *T.occidentalis* on the nuclear translocation of transcription factors is shown in Table I. We expressed the activation level as optical density at 655nm and % inhibition of each transcription factor by the treatment of *T.occidentalis*. It inhibited the nuclear translocation of NF-ĸB p65 by 65.42%. Percentage inhibition in the translocation of NF-ĸB p50 with the treatment of *T.occidentalis* was 62.18%. It could inhibit the translocation of NF-ĸB c-Rel by 76.95%. Translocation of c-Fos and ATF-2 were also inhibited by the treatment of *T.occidentalis*. Percentage inhibition of c-Fos with the treatment of *T.occidentalis* was 87.94%. Percentage inhibition of ATF-2 was 75.68% by *T.occidentalis*. These compounds could inhibit the translocation of cyclic AMP response element binding protein (CREB) effectively (79.62%).

Discussion

In this study we found that treatment of B16F-10 melanoma cells with *P.longum*, Piperine, *T.occidentalis* and TPS could produce the apoptotic reorganization such as the chromatin condensation, cell shrinkage and formation of the membrane bound apoptotic bodies. Cleavage of DNA into 50-300 bp length fragments, giving a ladder pattern, is a biochemical hallmark of apoptosis (Walker et al, 1991). As an evidence of apoptosis *P.longum*, Piperine, and *T.occidentalis* treatment could induce the DNA fragmentation.

High expression of the bcl-2 genes frequently altered in cancers, by its ability to contributing neoplastic cell expansion by prolonging cell survival (Ottavi et al., 1993). Since bcl-2 is a blocker of programmed cell death, over

expression of bcl-2 or its related antiapoptotic homologs prevents or markedly delays the normal turn over of cells in vivo, helps neoplastic growth prolonging cell survival rather than cell division (Vaux et al., 1988). In this study we found that treatment of B16F-10 melanoma cells with *P.longum*, Piperine, *T.occidentalis* and TPS down regulated the expression of bcl-2gene.

Caspase-3 is the important executioners of apoptosis being responsible either partially or totally for the proteolytic cleavage of many key proteins. Activation of caspases initiates a proteolytic cascade that allows rapid transmission and exponential amplification of a death stimulus. Increased caspase-3 expression in the *P.longum*, Piperine, *T.occidentalis* and TPS treated cells is correlated with its apoptotic property.

Many findings proved that cell lines derived from p53 deficient animals fail to undergo apoptosis in response to DNA damaging agents (Clarke et al., 1994). Apart from p53 dependent apoptosis, some cell lines showed interesting features of several p53 independent pathways of apoptosis. In this study *P.longum*, Piperine, *T.occidentalis* and TPS treatment could not enhance the p53 expression in B16F-10 melanoma cells and we assume that the apoptosis induced by these compounds is independent of p53 pathway. This study suggests that these compounds could induce apoptosis may have good therapeutical value.

The prototypical NF-κB is a heterodimer composed of p50 and p65 subunits, which are the most frequent component of active NF-κB. Similar to NF-κB, AP-1 and its components regulating gene expression have also been shown to play an important role in cell proliferation, cell cycle regulation and tumor

promotion (Karin, Liu and Zandi, 1997). In the present study we found that the pretreatment of B16F-10 cells with *T.occidentalis* could inhibit the activation or nuclear translocation of transcription factors such as NF-κB p65, NF-κB p50, NF-κB c-Rel, c-Fos, ATF-2 and CREB.

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(FACULTY OF SCIENCE)

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Summary and Conclusion

Metastatic spread of tumor cells is the main cause of death in cancer patients. Formation of metastatic foci is a continuous process that can begin early in the growth of the primary tumor and increases with tumor duration and tumor burden. Thus design of more effective therapies to treat metastatic cancer requires better understanding of the molecular events and cellular processes that are involved in the processes of metastases formation.

The metastatic cascade consists of several highly complex and interdependent steps. Each step is rate limiting so that failure to complete any of these events completely disrupts metastases formation. All standard combinations of currently available antimetastatic therapy have low efficiency, low response rate and produce a number of side effects. The development of an efficient therapy, which is equally effective, and less toxic to prevent the metastatic process represents a major advance in cancer treatment.

In the present study we have evaluated the inhibitory effect of some medicinal plants *Piper longum*, *Thuja occidentalis* and its active component Thuja polysaccharide fraction on experimental metastasis. The antimetastatic activity of Piperine was done in our laboratory previously (Pradeep and Kuttan, 2002). We have also tried to elucidate the mechanism of action of these natural products in the inhibition of metastasis. Immunomodulatory, anticarcinogenic, antiangiogenic, apoptotic, chemo protective and radio protective an activity of the medicinal plant extracts *Piper longum*, *Thuja occidentalis* and its active components Piperine, and Thuja polysaccharide fraction (TPS) were also

investigated.

One of the major objectives of immunotherapy is to modulate immune responses such as augmentation of humoral and cell- mediated immunity. All these natural products used in this study augmented the humoral and cell mediated immunity as there is a marked increase in the production of circulating antibodies, antibody producing cells and enhancement in ADCC and ACC activity in normal and tumor bearing animals. There is a marked enhancement in the activity of non-specific immune cells such as NK cell after the administration of these compounds, compared to tumor bearing control animals. The early activation of NK cell will contribute to defense against diseases especially during cancer development. The increase in the total WBC count and bone marrow cells after the administration of these natural products indicated 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 these medicinal plant extracts and its active compounds might play an important role as immunostimulant and could be used as adjuvant during immunotherapy. Administration of *P.longum*, Piperine, *T.occidentalis* and TPS could significantly reduce tumor formation induced with DLA cells and EAC cells. These compounds also could reduce the sarcoma formation induced with 3-methyl cholanthrene and increased the life span.

A crucial step during invasion and metastasis is the destruction of biological barriers, such as the basement membrane, which requires activation of proteolytic enzymes (Jasti et al, 2005). Many studies have shown that enhanced

production of members of matrixmetalloproteinase (MMP) family contributes to tumor invasion, metastasis, and angiogenesis (Jens et al, 2002).Administration of *Piper longum*, *Thuja occidentalis* and TPS inhibited the metastatic tumor colony formation in the lungs after 21 days of tumor induction. There was also a corresponding increase in the life span of metastatic tumor bearing animals, treated with these compounds. Administration of *P.longum*, *T.occidentalis* and Thuja polysaccharide fraction could also normalize the hydroxyproline content of the lungs indicating that these compounds could inhibit lung fibrosis produced during lung metastasis. There was an increased expression of surface sialic acid on circulating tumor cells, which facilitates their invasive behaviour. In melanoma, both secretion of glycoproteins and shedding of gangliosides have been demonstrated (Hersey, 1985) and the metastatic property of tumor cells highly up regulated with the elevated levels of sialic acid content (Fernandes et al, 1991). The elevated level of serum sialic acid was significantly reduced by administration of *P.longum*, *T.occidentalis* and TPS. The elevated levels of lung uronic acid and hexosamine content also significantly were reduced by the simultaneous administration of these compounds indicating a reduction in the lung fibrosis and metastasis. Serum gamma glutamyl transpeptidase, a marker of cell proliferation, was found to be increased in metastatic tumor bearing animals. Increase of GGT has been reported during the growth of tumor cells (Hanigan, 1994). But treatments with these compounds could reduce the serum gamma glutamyl transpeptidase (GGT) level. The host defense response has been enhanced by the administration of these products as demonstrated by the

enhanced level of IL-2, which in turn stimulate NK cell activity. *P.longum* and *T.occidentalis* could effectively reduce the level of TNF-α in metastatic animals, which will result in the improvements of cancer cachexia. GM-CSF level was also found to be lowered in drug treated tumor bearing animals compared to control animals. It has been reported that in malignant conditions GM-CSF may promote metastasis by the production of IL-1 and TNF (Orosz, 1993). Inhibition of these cytokines may interrupt the metastatic process.

Adhesion and Invasion are two major steps in the process of metastatic dissemination. Metastatic tumor cells disseminating through the blood must penetrate the capillary basement membrane, which is a mechanical barrier for invasion. Tumor has to attach to the extra cellular matrix by the help of their surface receptors that bind to specific adhesion molecules in the matrix which is a primary step of invasion and the adhesion molecules play a vital role in the trans-endothelial migration of tumor cells (Burrow, 1991). *Thuja occidentalis* inhibited the adhesion of B16F-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 molecules, but *P.longum* and TPS did not inhibit the adhesion of B16F-10 melanoma cells. *P.longum*, *T.occidentalis* and TPS inhibited the invasion and motility of B16F-10 melanoma cells in a dose dependent manner.

Proteases contribute to each step beginning with the first break down of the basal membrane of a primary tumor to the extended growth of established metastases. Several studies of the tumor progression is positively correlated with the expression of MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) which are

degrading the type IV collagen of the basement membrane (Zucker et al, 1993; Bernhard et al, 1994). Treatment of *T.occidentalis* inhibited the production of type IV collagenase as seen from zymographic analysis but *P.longum*, and TPS could inhibit the production of type IV collagenase only at higher concentration. The secretion and activation of metalloproteinases are not enough to ensure that they will degrade the target matrix substrate (Jon et al, 2003). This is because, natural proteinase inhibitor proteins, such as TIMPs function as metastasis suppressor proteins which act to inhibit tumor cell invasion of the extracellular matrix (Gottesman, 1990). Treatment with Piperine, *T.occidentalis* could enhance TIMP levels and expression in metastatic tumor bearing animals, which may be one of the reasons for the inhibition of invasion by these compounds

It has been demonstrated that nm23 gene may act as metastatic suppressor gene in experimental models (Henderson, 1993). Evidences suggest that patients whose tumor showed reduced nm 23H1 expression had a higher rate of lymph node metastasis and reduced survival (Bevilaqua et al, 1989). Treatment with Piperine, *T.occidentalis* could enhance the level of nm23 in B16F10 melanoma cells *in vitro.* Administration of Piperine, *T.occidentalis* significantly reduced the elevated expression of K-ras, prolyl hydroxylase and lysyl oxidase in the metastatic tumor bearing animals.

Cytokines could be prometastatic or proangiogenic molecule and their deregulated expression directly correlates with the metastatic potential of several carcinomas such as those of ovary, prostate, colon, pancreas and brain (Zerbin et al, 2003; Andela et al, 2000). It has been reported that in malignant conditions

GM-CSF may promote metastasis by stimulating the production of IL-1 and TNF (Orosz et al, 1993). It has been reported that IL-6 level is high in cancer, which promote the production of other cytokines (Dendorfer et al, 1995). IL-12 may also increase the secretion of interferons, which may enhance the TNF production and subsequent cachexia (Uno et al, 1998) IL-12 is a dimeric form of IL-12p40 and IL-12p35. The cells, which produce IL-12 cytokine contain large amount of IL-12p40 compared to IL-12p35. IL-12p40 can acts as a proinflammatory cytokine, which can activate the tyrosine kinase-2 and STAT proteins (Oppmann et al, 2002). Treatment of B16F-10 cells with *P.longum*, Piperine, *T.occidentalis* and TPS inhibited the expression of proinflammatory cytokines such as IL-1ß, IL-6, TNF-∝ GM-CSF and IL-12p40. Treatment with these compounds such as *P.longum*, Piperine, *T.occidentalis* and TPS significantly inhibited the production of proinflammatory cytokines such as IL-1 ß, IL-6, GM-CSF and TNF-∝.

P.longum, Piperine, *T.occidentalis* and TPS inhibited VEGF induced proliferation, migration and tube formation of endothelial cells *in vitro.* More over, this extract markedly inhibited capillary formation towards the tumor in mice. *P.longum*, Piperine, *T.occidentalis* and TPS also caused a significant reduction in the VEGF mRNA level. Our present data demonstrate a possible role of *P.longum*, *T.occidentalis* and TPS in preventing cancer from becoming malignant, presumably via selective curb of neovessel formation in the tumor site.

The treatment with the extract of these medicinal plants and their active compounds are found to enhance GSH levels in intestinal mucosa and liver of irradiated animals, which offer protection to the irradiated and cyclophosphamide

treated animals possibly by eliminating toxic metabolites and scavenging free radicals, thus by fascilitating the animals to tolerate the insult of radiation and CTX damage. The immunopotentiating activity of these compounds may also contribute to the radioprotective and chemoprotective effects.

The hallmark of the programmed cell death on apoptosis process is the fragmentation of genetic DNA, an irreversible event that commits in plasma and internal membrane permeability (Bowen and Wockshin, 1981). The expression of certain genes frequently becomes altered in human cancer. Among these gene Bcl-2 gene is a blocker of programmed cell death or apoptosis (Tsujimoto, 1985) and caspases are large family of proteases whose members function in the promotion of apoptosis. Activation of upstream caspases initiates a proteolytic cascade that allows rapid transmission and exponential amplification of a death stimulus. Effector caspases such as caspase-3 are activated, culminating in the destruction of cell by apoptosis (Wilson et al, 1994). Compounds such as *P.longum*, Piperine, *T.occidentalis* and TPS were found to inhibit the expression of antiapoptotic bcl-2 gene in B16F-10 melanoma cells. These compounds were shown to promote the expression of proapoptotic gene caspase-3. Supporting the above properties, membrane blebbing and DNA fragmentation has been found when the B16F-10 melanoma cells were subjected to treat with these compounds. The expression of the Erk is also up regulated by the treatment with Piperine and *T.occidentalis*. Transcription factors that participate in the regulation of diverse biological processes including immune and inflammatory responses, cell growth and apoptosis of cells (Karin and Delhase, 2002). We could find that

T.occidentalis could inhibit the nuclear translocation of subunits of nuclear factor-κB (NF-κB) such as p65, p50, c-Rel and subunits of activated protein (AP-1), such as c-Fos and ATF-2 (activated transcription factor-2) and cyclicAMP response element binding protein (CREB) in B16F-10 metastatic model. NF-κB and AP-1have selectively regulated the expression of proinflammatory cytokines such as IL-1, IL-6, GM-CSF, TNFα and IL-12p40 as well as the expression of degradative enzymes such as matrix metalloproteinases (MMPs).

In summary this study is focused on the effect of some medicinal plants and its active component such as *P.longum*, Piperine, *T.occidentalis* and TPS which are found in the indigenous system of medicine and the diet. The result not only indicated the effectiveness of these natural products in the inhibition of metastasis and the promotion of apoptosis; it also revealed the mechanisms how these compounds inhibit metastasis and how these compounds promote apoptosis. One interesting feature of our study is about the availability of these compounds, because some of the compounds are abundantly present in the spices. Thus these compounds either alone or in combination with radiotherapy or with any chemotherapeutical agent may help to prevent metastasis or tumor progression in clinical patients.

Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

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Anticancer, antimetastatic and immunomodulatory activity of medicinal plants- Piper longum and Thuja occidentalis

Thesis submitted to

UNIVERSITY OF CALICUT

for the fulfillment of the degree of

Doctor of Philosophy In Immunology

(FACULTY OF SCIENCE)

By

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Abbreviations

ACC	**- Antibody-dependent complement mediated**
ADCC	**- Antibody-dependent cell-mediated cytotoxicity**
ATF-2	**- Activator of transcription factor**
BAD	**- Bcl-2 associated death promoter**
COX	**- Cyclooxygenase**
CRB	**- CRE binding protein**
CTLs	**- Cytotoxic-T lymphocytes cytotoxicity**
ECM	**- Extracellular matrix**
EGF	**- Epidermal growth factor receptor**
EGTA	**-Ethylene glycol-bis (2aminoethylether)tetra acetic acid**
ERK	**-Extracellular regulated kinase factor**
FGF	**- Fibroblast growth factor**
GM-CSF	**-Granulocyte monocyte colony stimulating factor**
HIF	**- Hypoxia-inducible factor**
IFN	**- Interferon**
IL	**- Interleukin**
IKK	**- Inhibitory kappa B kinase**
LOX	**- Lysyl oxidase**
MAPK	**- Mitogen activated protein kinase**
M-CSF	**- Macrophage-Colony stimulating factor**
MEK	**- MAPK/ERK kinase**
MMPs	**- Matrix metalloproteinases**

CTX	**- Cyclophosphamide**
LPO	**- Lipid peroxide**
MTT	**- 3-(4, 5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium**
NF-κB	**- Nuclear factor-kappaB**
NK cells	**- Natural killer cells**
PIGF	**- Placental growth factor**
RHD	**- Rel homology domain**
TIMP	**- Tissue inhibitor metalloprotease**
TNF	**- Tumour necrosis factor**
TPS	**- Thuja polysaccharide**
VCAM	**- Vascular cell adhesion molecule**
VEGF	**- Vascular endothelial growth factor**

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List of Publications

1. **E.S. Sunila**, G. Kuttan, Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. ***Journal of Ethnopharmacology***. 90 (2004) 339-346.
2. **E.S. Sunila**, G. Kuttan, *Piper longum* inhibits VEGF and proinflammatory cytokines and tumor-induced angiogenesis in C57C/6 mice. ***International immunopharmacology*** 6 (2006) 733-741.
3. **E.S. Sunila**, G. Kuttan, Protective effect of *Piper longum* fruit ethanolic extract on radiation induced damages in mice a preliminary study. ***Fitoterapia.*** 76 (2005) 649-655
4. **E.S. Sunila**, G. Kuttan, Protective effect of *Thuja occidentalis* against radiation-induced toxicity in mice. ***Integrative Cancer Therapies*** 4(4) (2005) 322-328.
5. C.R. Pradeep, **E.S. Sunila**, G. Kuttan, Expression of vascular endothelial growth factor (VEGF) and VEGF receptors in tumor angiogenesis and malignancies. ***Integrative Cancer Therapies*** 4(4) (2005) 315-321.
6. **E.S. Sunila**, G. Kuttan, A preliminary study on antimetastatic activity of *Thuja occidentalis* L. in mice model. ***Immunopharmacology and immunotoxicology.***28 (2006).269-280.