Effect of natural products on the activation of NF-κB and proinflammatory cytokine gene expression during metastasis

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By
C.R. PRADEEP M.Sc

AMALA CANCER RESEARCH CENTRE,
AMALA NAGAR, THRISSUR-680555
KERALA, INDIA
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"To accomplish great things, we must not only act but also dream, not only plan, but also believe"

- Anatole France

To

Loved ones

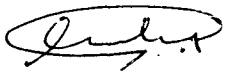
Who let me believe in my dreams

DECLARATION

I, Pradeep. C.R 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

1st June 2005



Signature of the candidate

CERTIFICATE

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

Thrissur

1st June 2005

Dr.Girija Kuttan, Ph.D

Professor and Supervising Guide

Department of Immunology

Amala Cancer Research Centre

Thrissur-680555

Dr. G. Kuttan, Ph.D.
Professor
Amala Cancer Research Centre
Thrissur-680 555

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CONTENTS

Introduction

"Death of cells is an essential part of life" is often a philosophical assertion in order to describe and define cancer. 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 this 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 consequences of these alterations leads to the formation of pathological overgrowth known as a tumour (Bishop,1991).

There are considerable variations occurs among tumours, 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 tumours and the latter as malignant tumours or cancers. Malignant tumours are more aggressive and have characteristics of immortalization, transformation and metastasize (Sugarbaker and Ketchem, 1997).

Majority of cancer patients who succumb to the disease die from metastasis. In most cases, primary tumour can be removed by surgery or local irradiation, but cells disseminated in the body may give rise after variable time periods, to metastases formation, unless they can be completely eradicated by treatments. 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.

The metastatic process has many different facets. Malignant cells acquire the potential to leave the primary tumour, invade surrounding structures and tissue by an active process, disseminate in the vascular system, withstand adverse physical, cellular and humoral factors during the period, adhere to and emigrate by active motility from vessels and infiltrate a target organ to give rise to metastases in a highly selective pattern (Fidler and Hurt, 1982; Travali et al,1990).

Invasive cancer cells degrade natural tumour barriers, ie the basement membrane and connective tissues (Pierce, 1970). Specific proteolytic enzymes produced by tumour 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 (Cazorla, Hernandez and Nadal, 1998). 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 (Zucker et al, 1993; Bernhard, Graber and Muschel, 1994). Several experimental observations suggest that MMPs are not only breaking down the physical barrier of extracellular matrix but also modulating the growth factors and cytokines stored in extracellular matrix, which may promote the neoplastic progression (Chamber and Matrisian, 1991).

Survival of the tumour cells and successful formation of a secondary tumor require the manipulation of environment in and around the tumour cells

(Zucker et al, 1993). Several substances such as proinflammatory cytokines have been identified to act as prometastatic, proangiogenic and antiapoptotic agents of tumour cells. Pleiotropic cytokines such as IL-Iβ, IL-6, GM-CSF, TNF-α and IL-8 can act as autocrine growth factors for tumour cells. Expression level of these proinflammatory cytokines directly correlates with the metastatic potential of several human carcinomas (Huang et al, 2000; Yoneda, Kuniyasu and Crispens, 1998). These proinflammatory cytokines has an important role in tissue repair and tumour progression (Aggarwal and Natarajan, 1996). Certain of these factors activates the silent transcription factors and enhance the proliferation, motility and metastasis of tumour cells, others exert their influence indirectly by inducing the mobilization of growth factors from other sources such as macrophages, monotypes and vascular endothelial cells (Karin et al, 2002; Hanahan, 1997).

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. Activated or nuclear translocated NF-κB proteins have been implicated as playing a role in cellular transformation by either inhibiting the apoptotic pathways or providing continued positive growth stimuli (Siebenlist, Franzoso and Brown, 1994). A progressive activation of NF-κB

and AP-1 has recently been correlated with progression of breast cancer, melanoma and juvenile myelomonocytic leukemia (Nakshatri et al, 1997; Van Dam and Castellasi, 2001).

The molecular signaling mechanisms that lead to the induction of NF-κB in response to various external stimuli have not been fully clarified. One of the most extensively studied intracellular signaling cascades involved in proinflammatory responses is the mitogen-activated protein (MAP) kinase pathway. Three distinct groups of well characterized major MAP kinase subfamily members include extracellular - regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK) /stress activated threonine protein kinases (Wang et al, 2001). The activated form of protein kinases phosphorylates and activates other kinases or transcription factors, thereby altering the expression of the target genes (Jaffee et al, 2000). All these proinflammatory, prooxidant and transcriptional factor activation are closely linked to tumour promotion.

Conventional modalities of cancer therapy such as radiotherapy, chemotherapy and immunotherapy often cause serious damage to the immune system. Anticancer drug discoveries now an increasing their set of targets to treat cancer due to the ~~because~~ ~~of~~ the of molecular alterations in cancer cells occurs spontaneously and also with the expansion of knowledge of tumour progression mechanisms. But it is probably more important to know the order or pattern of the molecular alterations and how each of these affect the tumor cell phenotype or tumour cell progression.

There is good evidence that several class of compounds in spices, fruits and vegetables exhibit anti-carcinogenic effects mediated by different mechanisms

(Surh, 2002; Johnson, Williamson and Musk, 1994). Carcinogenesis is a multistage process that consists of at least three separate but closely linked process beginning with initiation, in which unrepaired or misrepaired DNA damage is replicated followed by promotion, which facilitates the transformation and clonal expansion of preneoplastic cells and neoplastic cells. There are number of stages at which the possibility of antitumour activity can be envisaged. Of the multistage carcinogenesis, promotion is closely linked to oxidative and inflammatory tissue damage. 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 (Surh et al, 2001). Conversely, a substance with pronounced anti-oxidant and anti-inflammatory effects is anticipated to act as an antitumour promoter. The present study is aimed to evaluate the effects of certain plant products, such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, β-Carotene and 13cis-Retinoic acid on the metastasis based on the inhibition of transcriptional factor's activation and the transcriptional factors mediated prometastatic factor's expression. We are trying to enter the anticancer agent discovery, and our focus is expanding to include not only agents cytotoxic to toward the malignant cells, but agents that may be growth controlling, growth inhibitory or deactivating the malignant cells through the control of proinflammatory or prometastatic factors mediated by the regulation of transcription factors.

Chapter-1

Review of Literature

Review of Literature

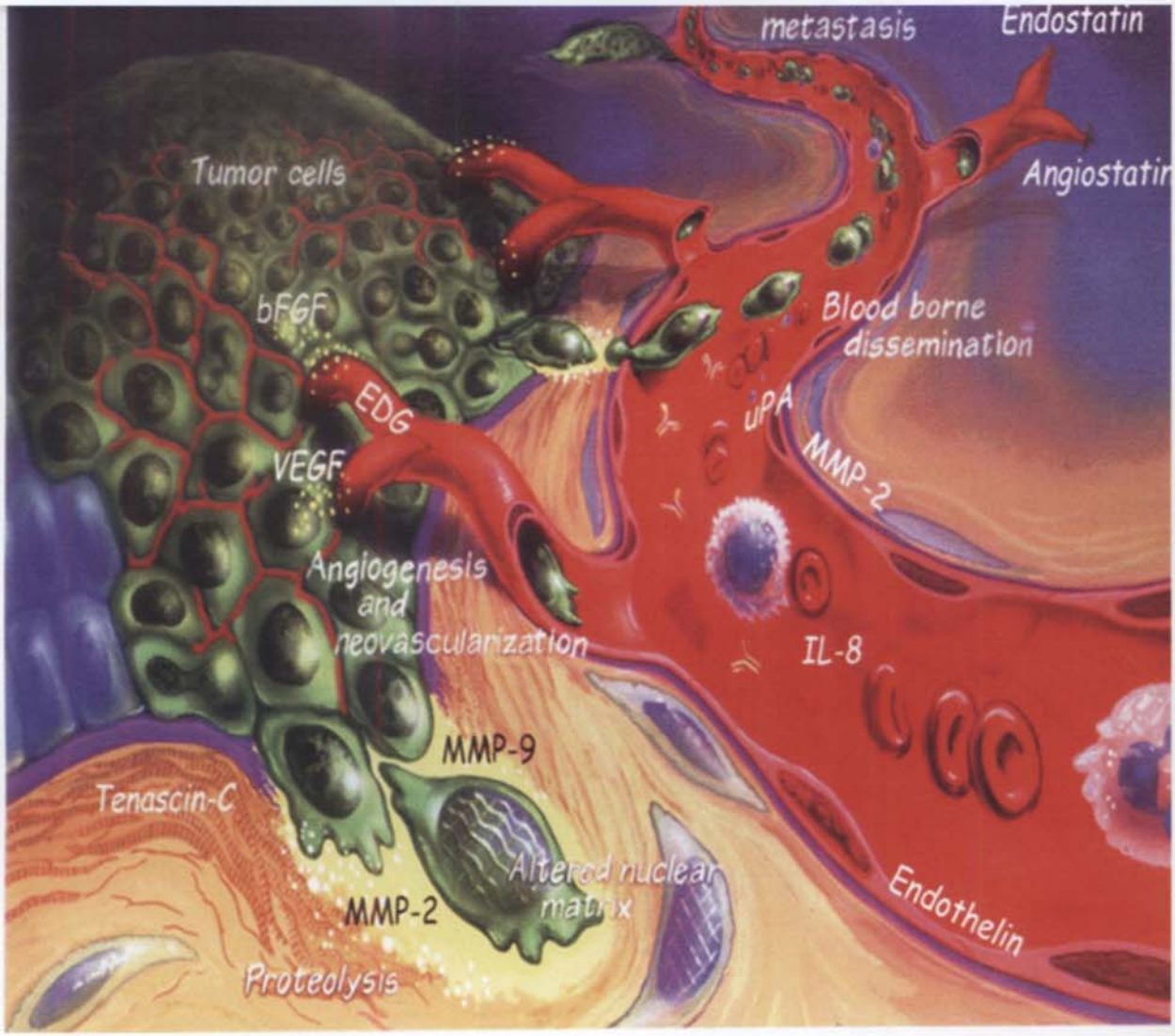
The past 10 years have seen heightened activity in studies of signal-transduction pathways, which has resulted in the development of important new molecular therapeutics such as selective tyrosine kinase inhibitors for different cancers. It is inevitable that this approach will have important implications for chemo-prevention of cancer, particularly as many inhibitors of signal transduction seen to be relatively free of the undesirable toxicities of classical chemotherapeutic agents (Danielsen and Maihle, 2002).

There are new chemopreventive drugs developed which have target activity on different signal transduction cascades such as epidermal growth factor (EGF) / ERBB2 receptor signaling cascades. (Raben et al, 2002). Such drugs might also be useful for cancer prevention. Further more, signal transduction cascades converge on many transcription factors such as NF-κB (Karin et al, 2002) or other regulatory molecules such as the cyclins and their respective kinases. (Turkson and Jove, 2000). Signal transduction targeting drugs prevent the activation of transcriptional factor's activation and corresponding gene expression of pro inflammatory cytokines, adhesion molecules and proteases (Danielson and Maihle, 2002). The individual activities of all these regulators of cell function might represent potentially useful targets for new preventive agents.

Metastasis

Metastasis is the dissemination of tumour cells from the primary site to the distant sites. The dynamic cellular heterogeneity of primary and metastatic tumours as well as their dispersion within various organ sites often prevents effective therapeutic treatment by standard anticancer clinical modalities.

Figure 1
The process of Metastasis



Multiple investigations of the biology of metastatic cancer cells have documented that subsequent spread of tumours from the primary site to distant sites, these newly established metastases have the capacity to undergo subsequent metastatic spread to form tertiary metastases (Crissman and Honn, 1986). The clinical realities of metastatic tumour spread coupled with the lethality often associated with progressive growth of metastatic tumours underscore the compelling requirement for the development of modalities for the treatment of cancer metastases.

Tumour Invasion and Tumour Neovascularization

The destructive penetration of tumour cells is an important facet of the metastasis (Goldfarb and Brunson, 1992). Invasive tumour cells must penetrate a number of extracellular matrix barriers in the host, including basement membranes, at several stages of tumour metastasis (Nicolson,1989). Investigation of the biochemical mechanism involved in tumour invasion and degradation of host extracellular matrices have documented a crucial role of proteolytic enzymes in these processes (Stettler-Stevenson et al, 1993).

Neovascularization is also recognized as being critical for the growth of tumours including cancer metastases (Folkman,1992). It is well recognized that the tumour vasculature is highly heterogeneous and distinct when compared to normal vascular organization (Jain,1990). Microvasculature towards tumours are dilated, saccular and tortuous and may contain tumour cells with in the microvascular lining of the vessel wall.

Matrix Matalloproteinases

Matrix metalloproteinases (MMPs) are a family of secreted of transmembrane proteins that are capable of digesting extracellular matrix and basement membrane components. Several experimental observations suggest the existence of a direct association between MMPs and neoplastic progression that probably is related to the capacity of breaking down the physical barrier of extracellular matrix (Jain, 1990). MMPs are a family of over 20 enzymes that are characterized by their ability to degrade the extracellular matrix (ECM) and their dependence up on Zn^{2+} binding for proteolytic activity (Kupfer et al; 1998). MMPs can be divided into four categories based on substrate preference such as collagenases, gelatinases, stromelysins and membrane associated MMPs (Stetler-Stevenson et al, 1996). Among 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 the basement membrane (Zucker et al, 1993). Expression levels of MMP-2 and MMP-9 are associated with tumour metastasis for various cancers (Waus et al, 2002). The regulation of MMPs occur at three levels such as regulation of gene expression, regulation of proenzyme processing and regulation of the action of enzymatic activity. Tissue inhibitors of metalloproteinases (TIMPs) are a family of secreted proteins produced by different cells of defense mechanism such as normal cells and endothelial cells and in their action TIMPs selectively but reversibly inhibit metalloproteinases (Stenlicht and Werb, 2001).

Role of Metalloproteinases in Tumour progression.

Because of their extracellular matrix (ECM) degrading activity and the correlation between high levels of their activity, MMPs have an important role in

the increased tumour metastasis. MMPs facilitate tumour cell metastasis by destroying the basement membrane and other components of the ECM (Sun and Helmer, 2001). This process helps the intravasation and extravasation of tumour cells finally promotes the metastasis. MMPs and TIMPs are regulating the angiogenesis by their effects on proangiogenic molecules (eg. members of the VEGF and angiopoetin family). A recent study has demonstrated that the role of MMP-9 in angiogenesis which regulates angiogenic islets by releasing VEGF-A from an extracellular reservoir (Bergers et al, 2000). Because MMPs degrade proteins in the extracellular matrix, their primary function has been presumed to be remodeling of the extracellular matrix. However, MMPs also acts on the non-matrix substrates, including cell surface and matrix bound growth regulators. *In vitro* studies shows that MMP-9 and MMP-2 can proteolytically cleave latent TGF-β providing a novel and potentially important mechanism for TGF-β activation, which is important for osteoblastic differentiation and tumour angiogenesis (Vu, 2001). In addition, MMPs accelerate the ectodomain shedding of surface tumour antigens, which enhances the escape mechanism.

Cytokines

The complex cellular interactions involving cells of the immune, inflammatory and haematopoietic systems are mediated by low molecular weight regulatory proteins known as Cytokines. Cytokines are small, structural proteins with molecular weights ranging from 8 KD to 40 KD. They have essential roles in control of cell proliferation and differentiation during embryonic development and later life. Cytokines are involved in the complex cellular interactions involving cells of the immune, inflammatory and hematopoietic systems. Cytokines bind to specific receptors on the membrane of target cells eliciting

biochemical changes that result in signal transduction. In most cases signals are sent to the nucleus and cause changes in gene expression. Cytokines act in three different ways, (a). autocrine action occurs when a particular cytokines binding to receptors on the membrane of the same cell that secreted it. (b). paracrine action occurs binding to the different target cells near to the producer cell and (c). endocrine action occurs binding to target cells in distant parts of the body (Kuby, 1994).

Cytokines are involved in regulating the immune response to foreign antigens and invading organisms and they are important in mounting other forms defense against infection by viruses and other pathogens. Essential processes of cellular functions, such as cellular renewal and wound healing, development of cellular and humoral immunity and inflammatory responses require participation of a range of cytokines (Fridman and Tartour, 1997) and many disease involving disruption of these processes is associated with altered regulation of cytokine, production such as cancer AIDS and autoimmune diseases (Meager, 1990).

General Properties of Cytokines

1. Cytokines are produced during the activation and effector phases of innate and specific immunity and serve to mediate and regulate immune and inflammatory responses.
2. Cytokine secretion is a brief and self-limited event.
3. Many individual cytokines are produced by multiple diverse cell types.
4. Cytokines act upon many different cell types. This property is called pleiotropism.

5. Cytokines often have multiple different effects on the same target cell.

6. Cytokines actions are often redundant and two or more cytokines mediate similar type of function.

7. Cytokines often influence the synthesis of other cytokines, leadings to cascades in which a second or third cytokine may be induced that may mediate the biologic effects of the first cytokine. The ability of one cytokine to enhance or support positive and negative regulatory mechanisms of other cytokines have a role in the immune and inflammatory responses.

8. The expression of many cytokine receptors is regulated by specific signals. The signals may be another cytokines or even the same cytokine that binds to the receptor, permitting positive or negative feedback.

9. Most cytokine genes are not expressed particularly at the translational level unless specifically stimulated by noxious events. In fact it has become clear that the protein kinases involved in triggering cytokine gene expression are activated by a variety of cell stressors.

10. For many target cells, cytokines act as regulators of cell division or growth factors (Kuby,1994; Fridman and Tartour, 1997; Meager, 1990; Abbas,Litchman and Pober, 1997).

Classification of Cytokines

The cytokines are broadly categorized into six different categories (Kuby, 1994; Fridman and Tartour, 1997; Meager, 1990; Abbas, Litchman and Pober, 1997).

1. Growth factors

The cytokines that are able to promote growth and division of various cell types is called growth factors.

2. Interleukin

The cytokines which are designated as interleukins is in reference to their role in cellular communication among leukocytes. It is produced by activated T-lymphocytes, monocytes, macrophages and tumour cells that act on other leukocytes population (eg. Monocytes, Neutrophils on Eosinophils). These molecules are sometimes called interleukins.

3. Hematopoietic colony stimulating factors

These cytokines regulate growth and differentiation of specific lineages of blood cells. The name colony stimulating factors (CSF) is conceptually useful since this group of cytokines function mainly to regulate proliferation and differentiation of various types of hematopoietic cells. (eg. granulocytes, macrophages and erythroid cells).

4. Transforming Growth Factors (TGF)

Two cytokines which promote proliferation of certain cells in culture have been named transforming growth factor α and β (TGF-α and β). TGF-α stimulates the growth of tissue such as placenta and kidney during embryonic development whereas TGF-β acts as growth inhibitor other than mitogen for most cell types.

5. Tumour Necrosis factor (TNF)

The name tumour necrosis factor reflects the history of these agents which was shown to cause tumour regression. There are two types at tumour necrosis factors

such as TNF-α and TNF-β. Tumour necrosis factors are produced mainly by activated macrophages and monocytes. TNF-β has also called "cachectin" because of its role in inducing cachexia.

Interferons (IFN)

Interferons were originally identified as agents produced by virus infected cells which can protect cells against further viral infections. It can elicit many other changes in cellular behavior including effects on cell growth, differentiation and modulation of the immune system. IFNs are divided into three groups α, β and γ on the basis of their distinct properties.

Cytokine Receptors

The principal function of cytokine receptors is to convert an extracellular signal into an intracellular signal such as activation of an enzyme or transcription factor that can trigger a target cell response. All known cytokine receptors are transmembrane proteins and the extracellular domains bind cytokine thereby providing the means of detection of the extracellular signal. Signal transduction usually involves the intracellular portions of the cytokine receptor. Cytokine receptors have grouped into five large families based up on the presence of conserved folding motifs on sequence homologies.

Ig superfamily and IL-1 Receptors

The first motif to be noted in cytokine receptors was the Ig domain and certain cytokine receptors like type-I and type-II interleukin-1 receptors. The receptors contain several extracellular domains that belong to the Ig superfamily. This motif is common for mesenchymal cell growth factor receptors and certain

receptors of colony stimulating factors such as c-kit ligand. (Fridman and Tartour, 1997; Abbas,Litchman and Pober, 1997).

Type I family cytokine receptors

All cytokine receptors bearing the two cysteine or tryptophan-serine- X - tryptophan serine (WSXWS) motifs are said to belong to the type I family cytokine receptors. The prototypic molecule for this family was growth hormone and this structure is shared by IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-13, IL-15, granulocyte-monocyte colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF). Interestingly, the IL-6 receptor contains both an Ig domain and the two cysteine WSXWS motif (Meager,1990; Abbas, Litchman and Pober,1997).

Interferon (IFN) receptors and TNF receptors family

Interferon receptors define the type-II family of cytokine receptors. These receptors are defined by the nucleotide sequences.

The fourth structural motif identified in cytokine receptors is a cysteine rich domain first identified in the two tumour necrosis factor (TNF) receptors (TNF-RI and TNF-RII) which have been called Type III cytokine receptors (Meager,1990; Abbas, Litchman and Pober,1997).

Seven membrane-α helical receptors

These are very large family of molecules that includes the receptors for the chemokines. This motif was originally found in β-adrenergic receptors and retinal rhodopsin and is common to all receptors that are coupled to heterodimeric guanosine triphosphate (GTP) binding signaling proteins (Meager,1990; Abbas, Litchman and Pober,1997).

Switching of Cytokines and Regulation of Cytokines During Tumour Progression

NF-κB is a transcription factor, key regulator of the inducible expression of many genes associated with immune function. For instance, NF-κB plays an essential role in the transcriptional regulation of many cytokine genes such as IL-1, IL-6, IL-8, IL-12p40, TNF-α and GM-CSF. Stimulation of T cells via the CD28 pathway leads to activation of NF-κB and subsequent binding to the CD28 response element (CD28 RE) of the IL-2 promoter (Ghosh, Tan and Rice, 1993). CD28 mediated activation of NF- κB were also trend upstream of the IL-8 expression (Stein and Baldwin, 1993; Wechsler, Gorden and Dendorfer,1994). This suggests a common pathway of CD-28 stimulated cytokine expression in T cells, mediated by NF-κB.

After Mosman hypothesis that cytokines released by CD4 T-cells could be divided into two groups such as Th1 cytokines include IL-2, IFN-γ, TNF- β or Th2 cytokines such as IL-4, IL-5, IL-6, IL-10. Different studies in oncology suggest that during tumour progression there is a switch over of immune response from Th1 to Th2 phenotype take place. IL-4, IL-5 and IL-10 are the dominant cytokines found in cutaneous basal cell (Meyes et al, 1997). Cytokines may directly influence carcinogenesis and metastasis by modifying the tumour phenotype. (Tartour and Fridman, 1998).

Role of Cytokines in the Tumour Progression

Interleukin-1 (IL-1)

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). Another important proinflammatory property of IL-1 is its ability to increase the expression of adhesion molecules such as intercellular adhesion molecule-1(ICAM-1) on mesenchezmal cells and vascular cell adhesion molecule-1(VCAM-1) on endothelial cells (Watanabe, Kawayashi and Kobayashi, 1998). IL-1 is also an angiogenic factor and plays a role in tumour metastasis and blood vessel supply (Horai, Saijo and Tanoiko, 2001). IL-1 also stimulates the production of other proinflammatory cytobines like IL-6 and TNF -α.

Interleukin – 6 (IL-6)

IL-6 is a growth factor for cervical cancer, Kaposis sarcoma derived cells myeloma, renal cell carcinoma and prostate carcinoma cell lines (Damian et al,1995). IL-6 cytokines also act as autrocrine or paracrine way. For instance IL-6 function as an *in vitro* growth factor of renal carcinoma and other hand IL-6 increases the proliferation of ovarian carcinoma cells (Reijo et al,1995; Van Meir,1990).

Interleukin-8 (IL-8)

IL-8 is member of the α-chemokine subfamily and IL-8 is produced by many cell types such as macrophages, T cells, neutrophils, fibroblasts, endothelial cells, neoplastic cells and hepatocytes. IL-8 is a potent chemoattractant and activator of neutrophils, basophils, T-cells and eosinophils (Tartour and Fridman,1998). It is a proangiogenic and tumorigenic factor for both *in vivo* and *in vitro* tumour models, which increases the expression of adhesion molecules of tumour cells and endothelial cells. Subsequent data demonstrated that IL-8 acts as an autocrine growth factor for tumour cells and as a proangiogenic molecule. IL-8

has shown to play a key role in melanoma progression and metastasis (Bar-Eli,1999).

Granulocytes-Monocyte Colony Stimulating Factors GM-CSF

GM-CSF is a pleiotropic cytokine produced by a number of different cell types such as macrophages, T cells, granulocytes, fibroblasts and endothelial cells. In addition, various carcinoma cell lines and myeloblastic leukemia cells can express GM-CSF constitutively (Bendtzen et al,2001). GM-CSF has an important role in tissue repair and tumour progression. It can activate different transcription factors such as nuclear factor-κB (NF-κB) and activated protein (AP-1) (Nuerath, Becker and Barbulescu, 1998).

Tumour necrosis factor - α (TNF-α)

Tumour necrosis factor-α (TNF-α) was originally identified as a cytokine responsible for endotoxin induced necrosis. TNF-α is one of the most important and potent inflammatory mediators (Aggarwal and Natarajan, 1996) with a wide range of biological activities. Continued, overexpression of TNF-α can result in the chronic inflammation which is characterestic of immune disorders such as rheumatoid arthritis and cancer (Hensel et al, 1987). TNF-α can induce different genes including transcription factors such as (a) NF-κB and c-jun/AP-1 (b)adhesion molecules, such as E-selectin and intercellular adhesion molecule (ICAM-1) (c). cytokines such as IL-1, IL-6, IL-8 (d) cytokine receptors such as IL-1 receptor and IL-6 receptor and (e) various inflammatory mediators such as stromelysin, collagenase, C3 complement protein and nitric oxide synthase (Hensel et al, 1987, Vandenabeele,1995).

Nuclear Factor -κB (NF - κB)

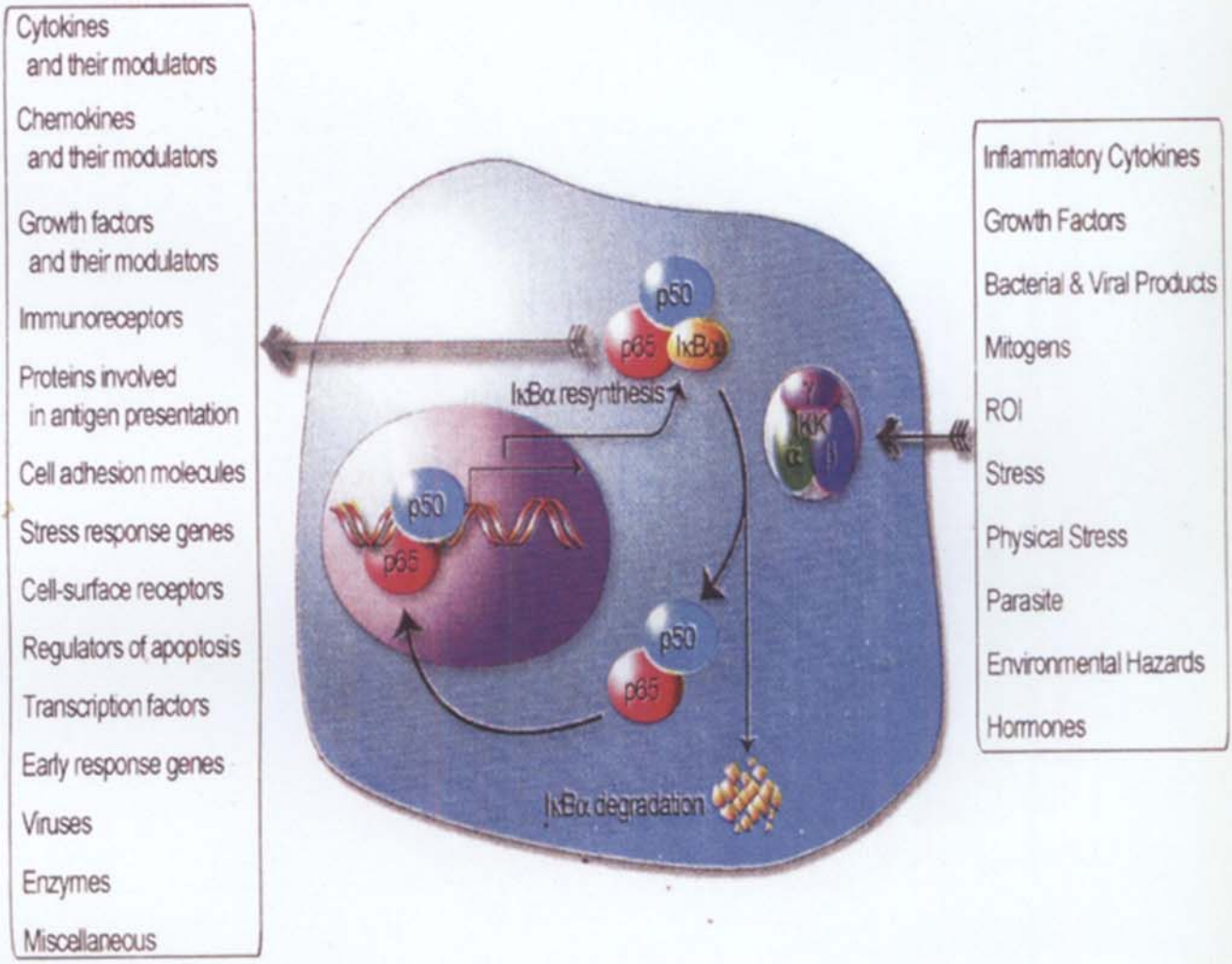
Nuclear factor- κB (NF-κB) represents a family of eukaryotic transcription factors that participates in regulation of diverse biological process, including immune and inflammatory responses, cell growth and apoptosis (Karin and Delhase, 2000; Silverman and Maniatis, 2001). In mammalian cells, the NF-κB family is composed of five members, p50, p52, Rel A (also termed p65), Rel B and c-Rel. The different NF-κB members share strong homology in their N-terminal Rel-homology domain (RHD) that is responsible for DNA binding, dimerization and nuclear translocation functions. The NF-κB proteins can form homo and heterodimers, although typical NF-κB complexes are heterodimers composed of a Rel member and p50 and p52. The Rel proteins possess both the N-terminal DNA binding domains and a C-terminal transactivation domain, while p50 and p52 contain only a DNA binding domain and appear to function by modulating the DNA-binding affinity of the Rel proteins. A characteristic of p50 or p52 is that they are produced as large precursor proteins, p105 and p100 respectively. Since the genes encoding p105 and p100 are named nfκb1 and nfκb2, these precursor proteins are often referred to NF-KB 1/p105 and NF-KB2/p100. Generation of p50 and p52 is mediated through proteasome-catalyzed processing of their precursors (Fan and Maniatis, 1991).

The function of NF-κB is regulated by a family of inhibitory proteins, known as inhibitor κB (IκB) (Baldwin,1996). A common structural feature of IκB is the presence of an ankyrin repeat domain (ARD) that mediates their association with NF-κB. Interestingly, the two NF-κB precursor proteins, p105 and p100, also contain the ARD in their C-terminal portion and function as IκB molecules (Rice,

Table-1
Stimuli that Activate NF-κB (Barnes and Karin, 1997)

Cytokines	Tumour necrosis factor- α Interleukin- 1β Interleukin- 17
Protein Kinase C activators	Phorbol esters Platelet activating factor
Oxidants	Hydrogen peroxide
Viruses	Rhinovirus Influenza virus Epstein-Baar virus Cytomegalovirus Adenovirus
Immune stimuli	Phytohaemagglutinin Anti-CD3 antibodies
Other agents	Lipopolysaccharide Ultraviolet radiation

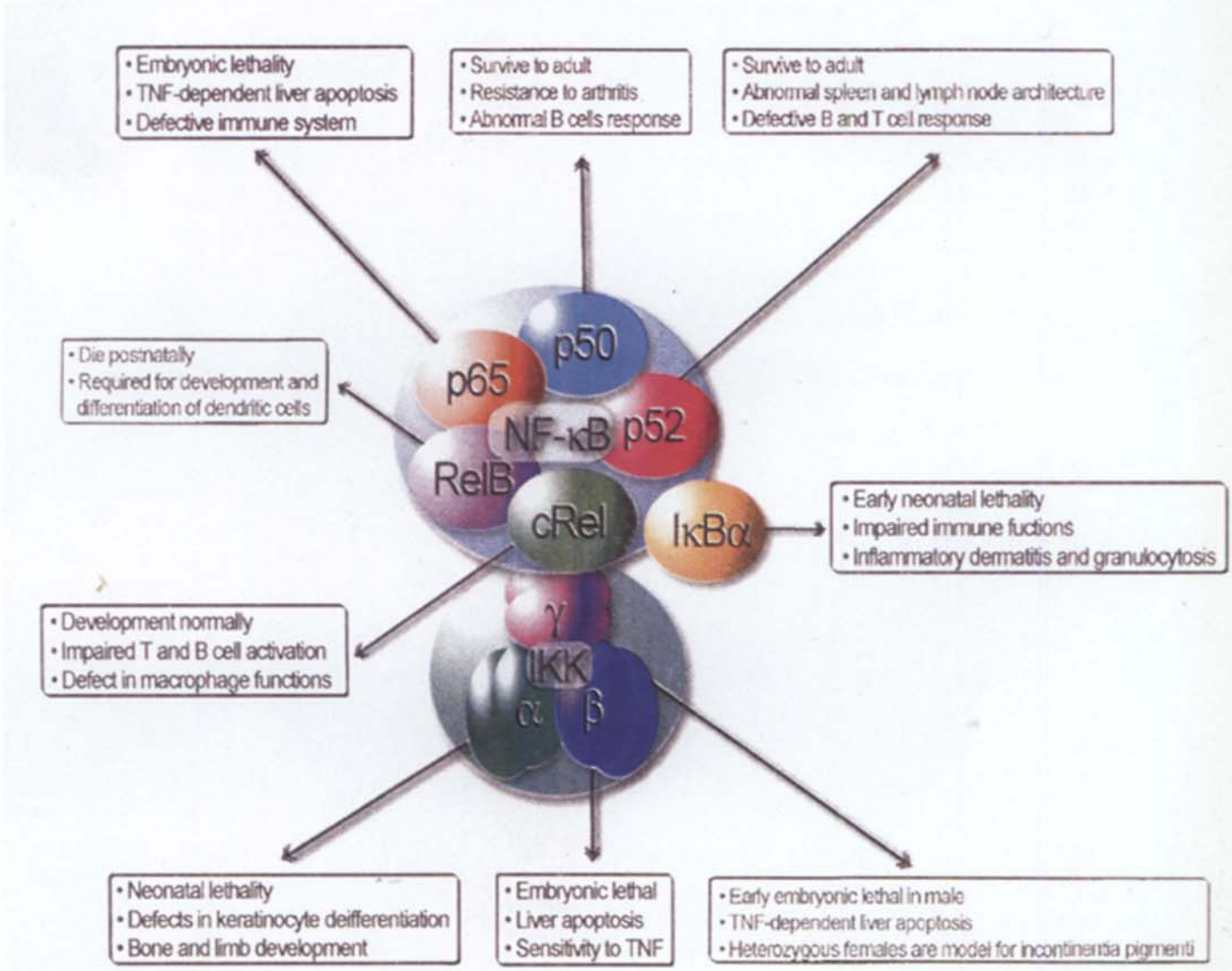
Figure - 2
NF-kB Activation Pathway



Mackichan and Israel,1992; Mercurio et al, 1993). So far well defined IκB members include IκB α, IκB β, IκB ε, p105 and p100 subunits (Baldwin,1996). These inhibitory proteins function by blocking the nuclear translocation and DNA binding activities of NF-κB. Another ARD-containing the protein, Bcl-3, which is structurally homologous to IκB and binds to certain NF-κB members (p50 and p52) was originally classified to IκB Family (Franzoso,1992; Wulczyn and Scheidereit,1992). However it is now clear that Bcl-3 is not an inhibitor of NF-κB, but rather functions as a co activator of the p50 and p52 homodimers (Bours et al,1993; Fujita et al, 1993). The presence of multiple IκB proteins may be important for controlling the specificity of NF-κB function, since different IκB, proteins have preferences for targeting distinct NF-κB and may also respond to different cellular stimuli (Whiteside 1997).

NF-κB can be activated by a large variety of stimuli, such as inflammatory cytokines, growth factors, DNA damaging agents, bacterial components and viral proteins (Pahl,1997). A key step in NF-κB activation is degradation of its associated IκB, which enables the released NF-κB dimers to move to the nucleus and transactivates the target genes. The nuclear NF-κB transactivates a large body of genes involved in diverse cellular functions such as cell proliferation, apoptosis and cell adhesion and cell migration (Pahl,1997). Under normal conditions, the signals mediating NF-κB activation are transient, which is instrumental for programmed cell proliferation and survival. However deregulated NF-κB activation has been detected in a large variety of human malignancies (Rayet,1999; Baldwin,2001) such as Hodgkin's disease (Bargou et al,1996), acute lymphoplastic leukemia (Kordes et al,2000), breast cancer (Sovak et al,1997;

Figure-.3
Functions of NF-kB subunits



Nakshatri et al, 1997), colon cancer (Lind et al,2001), ovarian cancer (Huang et al,2000), prostate cancer (Palayoor,1999), liver cancer (Tai et al, 2000), and melanoma(Meyeskens et al, 1999; Yang and Richmond, 2001). An essential role of NF-κB in the growth and survival of the malignant cells has been demonstrated by a number of studies. In addition to its direct contribution to oncogenesis, NF-κB also mediates resistance of cancer cells to anti-cancer therapies (Wang, Mayo and Baldwin, 1996, Iwanaga et al, 1998; Barinaga, 1996). Thus a fundamental understanding of how NF-κB is activated will provide important insights into the molecular mechanisms of NF-κB deregulation associated with various malignancies.

Transcriptional Regulation by NF-κB Subunits

Consistent with their roles as transcription factors c-Rel, Rel B and Rel A contain transcriptional activation domains (Dobrazanski, Ryseck and Bravo, 1993; Blair et al,1994). Additionally, both c-Rel and Rel A interact with the TATA-binding protein (TBP) and the c-terminus of Rel-A interacts with the basal factor TFIIB (transcription factor IIB) (Schmitz et al, 1995). Studies using *in vivo* and *in vitro* indicate that different NF-κB dimers have different transcriptional activation properties (Schmidt et al,1991). Strong evidence indicates that interactions between NF-κB subunit and other transcription factors influence the ability of NF-κB to regulate gene expression in a selective manner. Interactions between NF-κB proteins and bZIP proteins are implicated in the inducible regulation of the genes encoding IL-8, E-selectin and G-CSF. A complex that binds to enhancer-A in the class-I MHC promoter and that is modulated by interferon-γ or glucocorticoids reportedly contains the p50 NF-κB subunit in association with the bZIP protein

Table-2

Genes regulated by NF-κB (Neurath, Becker and Barbulescu,1998)

Cytokines and growth factors	Interleukin-1 Interleukin-6 Interleukin-8 Interleukin-12p40 TNF-α β-interferon Granulocytes/ macrophage colony stimulating factor (GM-CSF)
Adhesion molecule	Endothelial leukocyte adhesion molecule (ELAM-1) Vascular cell adhesion molecule-1 (VCAM-1) Intercellular adhesion molecule-1 (ICAM-1) E-Selectin Mucosal vascular addressin cell adhesion molecule
Cell surface receptors	T cell receptor β- chain T cell receptor γ-chain β2-microglobulin Interleukin-2Rα chain
Transcription factors	c-myc Interferon regulatory factor-1 (IRF-1)
Others	iNOS TAP-1 peptide transporter LMP2 proteasome subunit

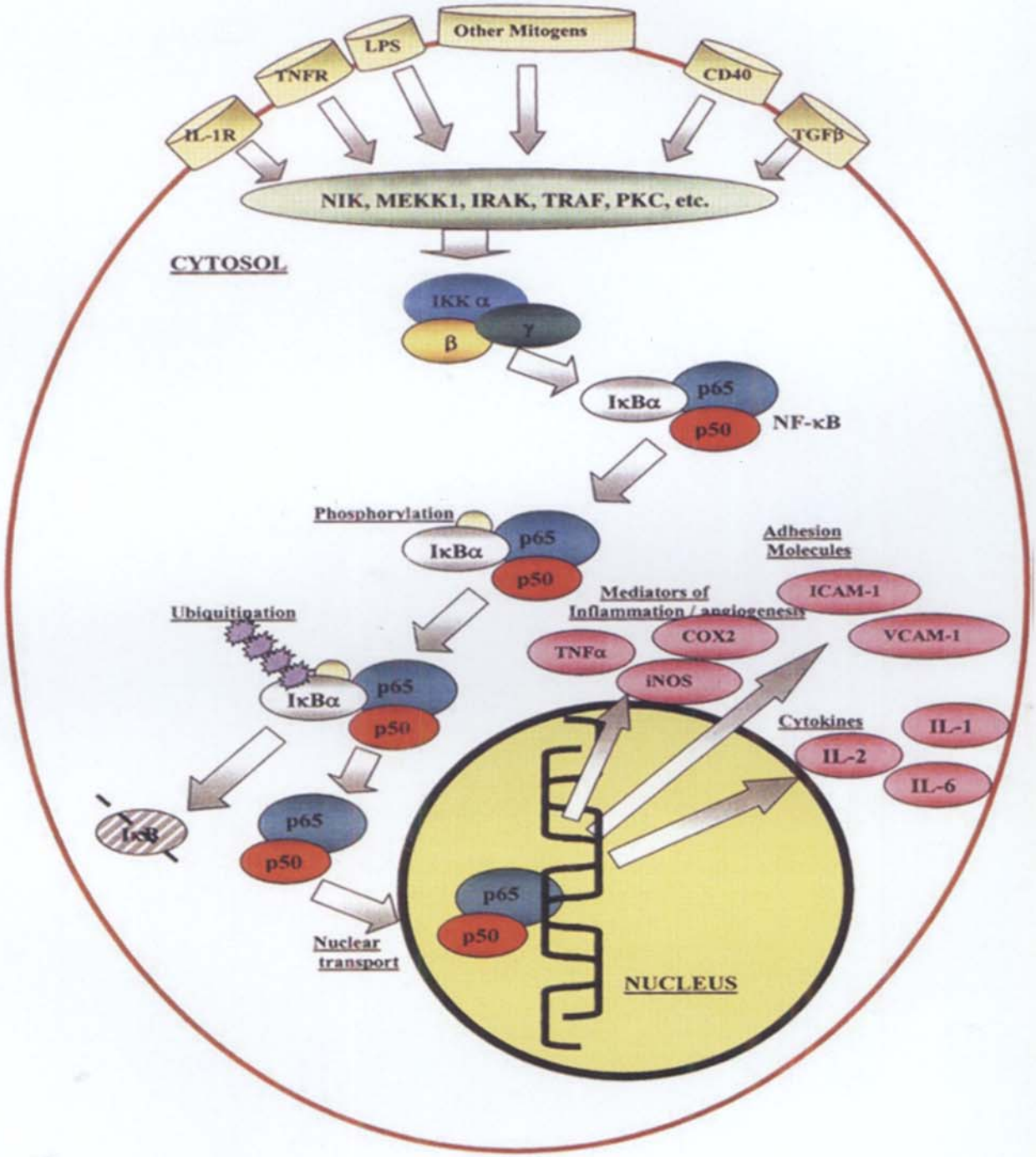
fra-2 (Giuliani et al, 1995). An interaction between the NF-κB subunit Rel-A and the zinc finger protein Sp1 has been identified and appears to regulate transcription directed by HIV-1 (Perkins et al, 1994). The functional outcome of transcriptional induction based on some of these interactions likely involves co-operative DNA binding as well as transcriptional synergy.

Genes regulated by NF-KB

NF-κB is a key regulator of the inducible expression of many genes associated with immune function. For instance, NF-κB plays an essential role in the transcriptional regulation of many cytokine genes such as IL-1, interferon (IFN)-γ, IL – 6, IL-8, IL-12p 40 in epithelial cells, lymphocytes, monocytes and tumour cells (Ghosh, Tan and Rice, 1993). In fact, NF-κB has been shown to have an important function on the regulation of a variety of genes encoding transcription factors such as interferon regulatory factor -1 (IRF-1) c-myc and c-Rel as well as cell adhesion molecules such as endothelial leucocytes adhesion molecule-1(ELAM-1), vascular cell adhesion molecule–1 (VCAM-1), intercellular cell adhesion molecule (ICAM-1) and E-selectin (Neurath, Becker and Barbulescu,1998). Furthermore NF-κB regulates the expression of genes for the transporter associated with antigen processing (TAP-1), proteasome submit latent membrane protein-1 (LMP-1) and the MHC Class II invariant chain, proteins with essential functions for antigen presentation (Xie, Kashiwabhara and Nathan, 1994).

NF-κB itself is extensively up-and down regulated presentation by a wide variety of exogenous stimuli that modulate immune function, thus providing a positive or negative feed back mechanism. For instance, NF-κB transactivates the

Fig-4
Signaling cascade of NF-kB



inducible nitric oxide (NO) synthase promoter in response to LPS giving rise to increased production of NO, a substance that is strongly up-regulated in the inflamed conditions (Xie, Kashiwabhara and Nathan, 1994).

Role of NF-κB in Oncogenesis

Issues involving oncogenesis and resistance to chemotherapy, has been shown that NF-κB activation can suppress cell death pathways (Beg et al, 1995). Thus consistent with the role of NF-κB as an anti apoptotic factor, NF-κB activation is required to protect cells from the apoptotic cascade induced by TNF and other stimuli (Beg et al, 1995, Wang, Mayo and Baldwin, 1996). NF-κB has been shown to activate TRAF1 and 2 and c-IAP1 and 2 to block caspase-8 activation (Wang et al, 1998). Other anti-apoptotic genes have been shown to be activated by NF-κB which include the Bcl-2 homologue A1/Bfl-1, IEX – 1 and XIAP (Wu et al, 1998). The observations that NF-κB can antagonizes p53 function, possibly through the cross-competition for transcriptional co-activators are significant interests (Webster,1999).

Inhibition of NF-κB with the super repressor IκBα led to the induction of apoptosis when an oncogenic allele of H-Ras was expressed (Mayo et al,1997). Since NF-κB has potent effects on cell survival and growth, NF-κB most likely will also be found to control other aspects of cell cycle progression. It is speculated that NF-κB can control metastasis. This idea is supported by the fact that Cdc42 and Rac induce integrin-mediated invasiveness through PI3K pathway, and it was shown that Rac and PI 3K are involved in the NF-κB activation (Keely et al,1997). Recently it was shown that animals null for TNF are inhibited in their ability to undergo skin carcinogenesis (Moore et al, 1999).

Since NF-κB positively upregulates TNF gene expression and TNF activates NF-κB these results may correlate with a role for NF-κB in skin carcinogenesis (Moore et al, 1999). Consistent with this it has been suggested that the up regulation of NF-κB in cancer promotes proinflammatory cytokine production and possibly metastasis (Moore et al, 1999). These gene regulations by NF-κB are presumed to control its major oncogenic functions during the tumorigenesis and the progression of secondary tumour.

Apoptosis

Apoptosis or programmed cell death is a highly regulated process during the development to eliminate cells that are no longer needed after a certain stage, or that had been generated in excess of the final demand. Apoptotic cell death is found through out the animal kingdom and it culminates in the execution, packaging and disposal of the dying cells. It allows the organism to tightly control cell numbers and tissue size and to protect itself from damaged cells that threaten homeostasis. Apoptosis is characterized by distinct morphological and biochemical changes mediated by a family of cysteine proteases called caspases, which are expressed as inactive zymogens and are proteolytically processed to an active state following an apoptotic stimulus.

Two distinct pathways (extrinsic and intrinsic) leading to caspase activation have been characterized (Wang,2001). The extrinsic pathway is initiated by ligation of transmembrane death receptors (CD95, TNF receptor and TRAIL) to activate membrane-proximal (activator) caspases (caspase-8 and caspase-10), which in turn cleave and activate effector caspases such as caspase-3 and caspase-7 (Wang,2001). The intrinsic pathway requires disruption of the

mitochondrial membrane and the release of mitochondrial proteins including Smac/DIABLO, $HtrA_2$ (Omi) and cytochrome-c. Cytochrome-c functions with Apaf-1 to induce activation of caspase-9, thereby initiating apoptotic caspase cascade while Smac/DIABLO and Htr A_2 bind to the antagonists IAPs (Suzuki, Nakabayashi and Takahashi, 2001).

Disruption of the intrinsic apoptotic pathway is extremely common in several cancers. Besides over expression of antiapoptotic genes, tumours can acquire apoptosis resistance by down regulating or mutating proapoptotic molecules.

Bcl - 2

The members of the bcl-2 family, which regulate apoptosis at the mitochondrial level are an important class of regulatory proteins. They can be divided into anti apoptotic (bcl-2, bcl-x_L, bcl_W, mcl-1 etc) and proapoptotic (bax, bak, bid, bad, bok, mtd etc) proteins according to their function. Although the impact of bcl-2 family members on apoptosis is well known, the bio chemical mechanism of their function is not entirely clear. It is believed that the mitochondrial membrane permeabilization is regulated by the opposing pro and anti apoptotic bcl-2 family members (Reed,1999).

Many oncogenic mutations probably impair apoptosis indirectly by affecting signal transduction pathways that promote or repress expression of Bcl-2 family members. Bcl-2 is over expressed in a variety of cancers (Tyagi et al, 2002) and its over expression can accelerate tumorigenesis in transgenic mice (Adams et al,1999). Over expression of bcl-2 occurs in 70% of breast cancer, 30-60% of prostate cancer, 80% of B-cell lymphomas, 90% colorectal adenocarcinomas and many other forms of cancer. Over expression of Bcl-2 in

patients receiving synchronous chemo or radiotherapy has been reported as an independent indicator of poor survival (Hussain et al, 2003). Mutations or altered expression of bcl-2 related proteins could drastically alter drug sensitivity in experimental models (Reed,1999). Infusion of a bcl-2 antisense oligonucleotide into tumour bearing mice has been shown to reduce tumour growth and the preliminary results of clinical studies seen promising (Jansen et al, 1998). In addition, mutations of altered expression of regulators of Bcl-2 proteins are associated with cancer.

Akt

Akt is also known as proteins kinase II and the consequences of Akt activation that are relevant to cancer cell growth can be catalogued loosely into three categories survival, proliferation (increase in cell number) and growth. Several components of the PI3K-Akt pathway are deregulated in a wide spectrum of human cancers. The mechanism by which Akt protect cells from death is likely to be multifactorial because Akt directly phosphorylates several components of the cell death machinery. For example, BAD a pro-apoptotic member of the bcl-2 family promotes cell death by forming a non-functional heterodimer with the survival factor bcl-$_{XL}$ (Datta et al, 1997). Similarly, Akt inhibits the catalytic activity of a prodeath proteases caspase-9, through phosphorylation (Cardone et al, 1998). Finally phosphorylation of FKHR a member of the fork head family of transcription factors by Akt prevents its nuclear translocation and activation of FKHR gene targets, which include several pro apoptotic proteins such as BIM and Fas ligand.

Akt can also influence cell survival by means of indirect effects of cell death NF-κB and p53 (Romashkova and Makarov, 1999, Mayo and Donner, 2001). Akt can exert a positive effect on NF-κB which, functioning by promoting phosphorylation and activation of IκB kinase (IKK) a kinase that induces degradation of the NF-κB inhibitor, IκB (Romashkova and Makarov, 1999). Akt influences the activity of p53 through phosphorylation of p53 binding protein MDM2, which is a negative regulator of p53 (Mayo and Donner, 2001).

Inhibitor of apoptosis (IAP)

The inhibitor of apoptosis (IAP) gene family, which was discovered less than a decade ago, encodes a group of structurally related proteins that, in addition to their ability to suppress apoptotic cell death are involved in an increase in number of seemingly unrelated cellular functions. As the name implies, the IAP proteins protect cancer cells from death inducing stimuli (Deveraux and Reed, 1999). So far eight human IAPs have been identified, namely IAP-1, IAP-2, XIAP (X-linked IAP), BIR (baculovirus IAP repeat), CARD (caspase recruit domain), ILP (IAP like protein) MIHA (mammalian IAP homologue-A), NAIP (neuronal apoptosis inhibitory factor) and survivin (Rothe et al, 1995). Expression levels of certain IAPs are subjected to tight transcriptional control.

c-IAP2 and XIAP are regulated by the stress responsive transcrition factor, NF-κB (Ambrosini, Adida and Altieri, 1997; Ghosh, May and Kopp,1998). It remains to be tested whether the induction of IAPs such as x-IAP and C-IAP2 by NF-κB directly correlates with the situations in which NF-κB activation exerts an antiapoptotic effect.

Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) functions collectively to protect cells from the potentially fatal consequences of adverse environmental, physical or chemical stresses by their ability to prevent protein aggregation and to promote the refolding of denatured proteins (Parsell and Lindquist, 1993). The protective functions of the HSPs may be extended to include an antiapoptotic role for several members of the HSP family including HSP90, HSP70 and Hsp27 and these proteins are over expressed in cancer tissue samples compared with normal tissues (Kanazawa et al, 2003). HSP90 was recently reported to sustain Akt activity by directly associating with Akt to prevent its dephosphorylation by protein phosphatase 2A (PP2A) (Sato, Fujita and Tsuruo,2000); a mechanism proposed for the modulation of JNK activity by HSP70 (Meriin et al, 1999). However, HSP is not a cancer specific protein, even though elevated levels of Hsp27 are observed in many tumour types and are often correlated with a drug resistant phenotype.

Caspases

Caspases have been called the executioners of apoptosis because once activated, they cleave of various cellular substrates, including other caspases, results in the morphologic features of apoptotic cell death. There are 14 caspases in mammals. All appear to be involved in apoptosis except human caspases-1 (ICE) or interleukin-1β converting enzyme and caspase-4, which serve to proteolytically process the precursors of cytokines (Earnshaw et al, 1999). Because of the thermodynamic irreversibility of proteolysis, caspase activation is a commitment to death that cannot be undone. Hence caspases are tightly regulated; this regulation is achieved by three principal means (a). Caspases are zymogens that require proteolytic processing (b) certain caspases have long

"prodomains" that allow them to enter the complexes with adaptor molecules that promote auto-processing; and (c) specific inhibitors exist (Raff,1998). Caspases have an NH2 - terminal "prodomain" that would removed in the active enzyme. The COOH terminal protease domain comprises two catalytic subunits of the mature enzyme that are denoted by their processed molecular weights. The processing sites between these parts occur at short specific tetrapeptide sequences ending in aspartate residues that dictate that the major processing enzymes are caspases themselves (Earnshaw,1999). For caspases with short prodomains, namely caspase-3, 6 and 7 regarding apoptosis is believed to be the primary regulators. Those with long prodomains harbor protein-interaction domains that allow them to enter activating complexes for specific death pathways. Caspase-8 and caspases-10 have DED (death effector domains) and caspases-5, 9, 11, 12 and 13 have CARD (caspase recruitment domains).

The death inducing effect of caspases is highly specific in that most proteins in the dying cell remain uncleaved. Lethality is therefore due to cleavage of a limited set of target substrates. Proteins known to be cleaved by caspases in the dying cells have been grouped according to apparent functional importance: (a) cytoskeletal proteins such as actin, gelsolin, α-fodrin (b) nuclear structure proteins, especially laminins A and B. (c) DNA metabolism and repair proteins such as PARP (d) protein kinases such as various isoforms of PKC and (c) signal transduction proteins such as STAT1, SREBP-1 and phospholipase C -γ1 (Earnshaw,1999).

Chemoprevention and Natural Products

The term "Chemoprevention" was coined in the 1970's by Sporn et al to distinguish between the process of preventing cancer and the practice of chemotherapy a process of inhibiting the progression of advanced diseases. Major emphasis was placed on natural or synthetic chemicals at nontoxic concentrations, and several organ specific retinoids were identified (Sporn et al, 1976; Kelloff, Sigman and Greenwald, 1999) Chemopreventive agents were further classified as "blocking agents" and "suppressing agents" by Wattenberg (Wattenberg,1985). There are three main avenues by which blocking agents might exist an effect: The agent could prevent activation of carcinogens, enhance detoxification of carcinogens, or trap reactive carcinogens prior to target organ damage. The majority of antioxidants can be grouped into this category and thereby function as antiinitiators (Weisburger,1991; Park and Pezzuto, 2001). Suppressing agents are chemopreventive chemicals that inhibit the progression of overall neoplastic process and prevent cells from becoming malignant. This class of agents has the most prominent transactional value. These anti-promotional agents include retinoids (Decensi,2000), monoterpenes (Crowell,1999), inhibitors of prostaglandin synthesis (Lupulescu,1996; Cuendet and Pezzuto,2000) and inhibitors of polyamine biosynthesis (Verma,1990), flavanoids (Yang,2000; Lu, Anderson and Grady,2000), phytoestrogens such as genistein (Lu, Anderson and Grady,2000) and protease inhibitors (Kennedy,1998).

Traditionally, the anti-promotional activity of any given chemopreventive agent has been equated with its ability to retard the growth of transformed cells. This effect is best displayed in experimental animal models, where treatment with

chemopreventive agents following carcinogen treatment reduces the incidence or multiplicity and severity of the cancer. This chemopreventive effect is brought about by modulating signal transduction pathways in carcinogen-transformed cells, because carcinogenesis can be characterized by a series of events leading to altered signal transduction. Appropriate protection or correction of a specific disorder can lead to molecular prevention or delay of the event. Thus, the molecules responsible for cellular communication, including hormone or growth factor receptors, enzymes associated with hormone receptors and protein kinase-c are among the likely targets for chemopreventive agents (Weinstein,1996). Similarly with altered signaling, second messengers such as cyclic adenosine-5 triphosphate, prostaglandins, NF-κB and MAP kinases have been shown to serve as molecular targets for chemopreventive agents.

Early identification and experimental evaluations did not focus on the mechanism of action of these agents. However, recent progress in the field of chemopreventive agents has resulted in the identification of molecular targets for new chemopreventive agents (Aggarwal, 2000, Burger, Zhang and Seth, 1998). Now it is clear that future chemoprevention strategies must include interaction with molecular targets. To this end chemopreventive agents will be developed based on the potency of their interaction with tissue-specific signal transduction molecules (Primiano, Yu and Kong, 2002). One major advance with chemopreventive agents will come from the selection of yet unknown natural products containing novel compounds that can interact with known or new molecular targets.

Discovery of Chemopreventive Agents 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 chemopreventive 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, Kuttan and Kuttan, 1996) and anticarcinogenic activity (Kuttan, Menon and Kuttan,1996). 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 chemopreventive agents that target different cellular signaling molecules which regulate proliferation and differentiation (Surh,2002; Mehta and Pezzuto,2002; Sporn and Suh, 2002). The past 10 years studies of signal transduction pathways which has resulted in the development of

Figure - 5 Curcumin

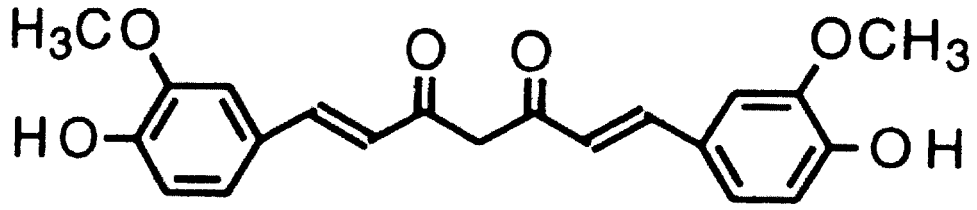


Figure - 6 Tetrahydrocurcumin

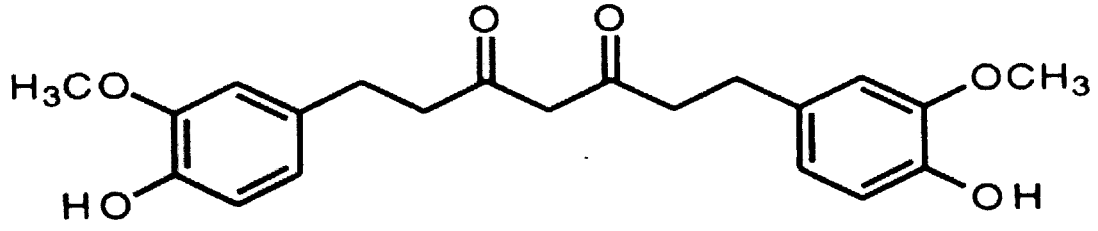
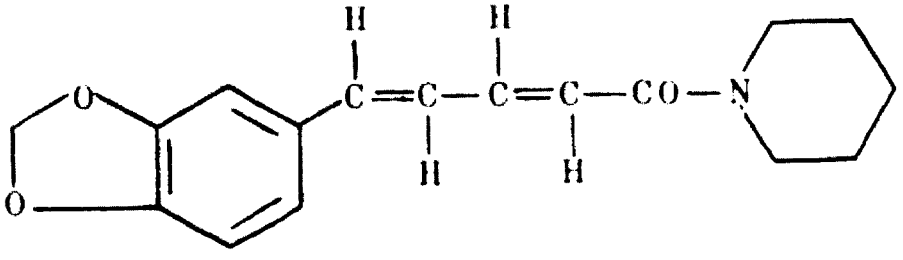


Figure - 7 Piperine



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.

Compounds Included in this Study

Curcumin (Diferuloyl methane)

Curcumin a yellow colouring principle from the rhizome of turmeric (*Curcuma longa* Linn), exhibits profound anticarcinogenic activities in many animal tumour model (Soudamini and Kuttan,1989). One of the most plausible mechanisms underlying the chemopreventive effects of curcumin involves suppression of tumour promotion. Thus, topical application of curcumin strongly inhibited TPA-induced inflammation, hyperplasia, proliferation, generation of ROIs oxidized DNA base modification and pappiloma formation in mouse skin (Lu et al, 1993; Huang et al, 1997). Curcumin inhibits COX-2 and lipoxygenase activities in TPA-treated mouse epidermis (Huang et al, 1991). Curcumin is reported to inhibit production of inflammatory cytokines by peripheral blood monocytes and alveolar macrophages (Lin,1997). Curcumin inhibits cycloxygenase and lipooxygenase expression (Huang et al; 1997). Curcumin easily penetrates into the cytoplasm of cells, accumulating in membranous structures such as plasma membrane, endoplasmic reticulum and nuclear envelope (Jaruga et al, 1998). Previously we have reported the antimetastatic activity of Curcumin *in vitro* as well as *in vivo* (Menon, Kuttan and Kuttan, 1999).

Tetrahydrocurcumin

This is a reduced, a colourless variety of Curcumin. Tetrahydrocurcumin (THC) derived from Curcumin by hydrogenation is supposed to be an effective antioxidant. Inhibitory effect of Tetrahydrocurcumin on the tetradecanoyl phorbol-13 acetate induced tumour promotion has been reported (Huang et al, 1995). Effect of Tetrahydrocurcumin on the inhibition of tumour promoter induced reactive oxygen species generation in leukocytes also reported previously (Nakamura et al,1998).

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; Guido et al,1998). Piperine is a potent inhibitor of mixed function oxygenase and it is a nonspecific inhibitor of p450 isoenzymes (Atal, Duby and Singh, 1985). We have reported its antimetastatic activity and its inhibitory potential of nitric oxide (NO) and tumour necrosis factor-α (TNF-α) production. Piperine was selected due to its structural similarity with Capsaicin, a known NF-κB inhibitor.

Catechin

Catechin is a flavanol, which is widely distributed in plants and is a major component in tea leaves. It is a major component of *Areca catechu*. Catechin has been shown to inhibit lung metastasis induced with B16F-10 melanoma cells in mice (Menon, Kuttan and Kuttan,1995). Recently it was reported that tea catechin inhibit angiogenesis *in vitro* measured by tumour endothelial cell growth migration and tube formation through the inhibition of VEGF receptor binding

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Figure - 8 (+) Catechin

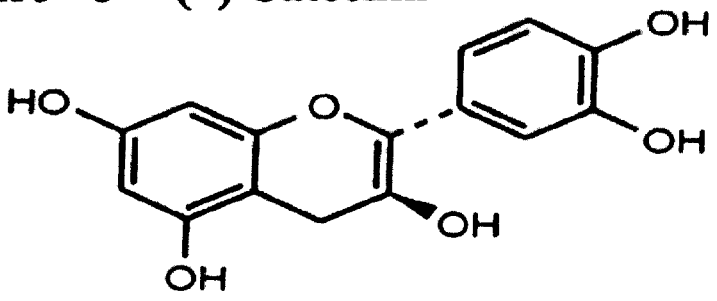


Figure - 9 β-Carotene

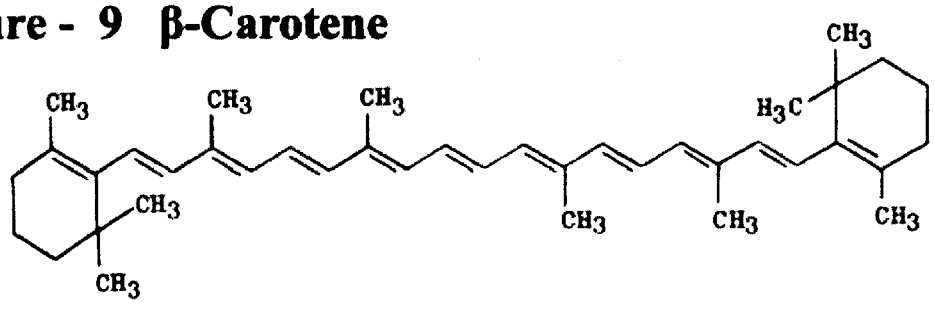
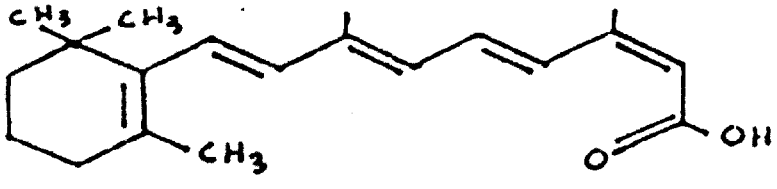


Figure - 10 13cis-Retinoic acid



(Kondo et al). Catechin also have been reported to restrain mutation, tumour initiation, tumour promotion and tumour cell growth (Kuroda and Itara, 1999)

β-Carotene

The selection of the β-carotene for these trials was based largely on data indicating that consumption of large amounts of fruits and vegetables was associated with a reduced risk of developing cancer (Ommen,1996). Two intriguing properties of beta-carotene is its potential for chemoprevention and its ability to trap certain organic free radicals (Krinsky and Deneke, 1982; Yu and Dotler, 1997).

13cis- Retinoic acid

Retinoids forms an essential class of nutrients needed in the vertebrates for the maintenance of different epithelial structures, vision, reproductive functions and health. Retinoid has multiple effects on cell growth and proliferation. Retinoic acid has antiinflammatory and antitumour action, it is emerging as an effective natural compound for chemoprevention for certain types of bladder carcinomas. The action of Retinoic acid mediated through RAR-β and RAR-α receptors (Thacher, Vasudev and Chandraratna, 2000). Retinoic acid attenuates iNOS expression and activity in cytokine stimulated murine mesanglial cells (Waliszewski,1999; Datta and Lianos,1999).

Chapter-2

Materials and Methods

A. Materials

1.Test Compounds

Curcumin obtained by the alcoholic extraction of *Curcuma longa* was purchased from Kancore Pvt. Ltd, Angamali, India. Tetrahydrocurcumin and Piperine was obtained as a gift from Dr. Majeed, Sami Chemicals, Banglore, India. (+)Catechin, β-carotene and 13cis-Retinoic acid were purchased from Sigma Chemicals, St. Louis, USA.

2. Cells Used

B16F-10 metastatic mouse melanoma cells and L929 lung fibroblast were obtained from National Centre for Cell sciences, Pune, India. Dalton's Lymphoma Ascites Cells were initially procured from Adayar Cancer Institute, Chennai, India.

3. Animals Used

BALB/C mice, C57BL/6 mice and Swiss albino mice were purchased from National Institute of Nutrition, Hyderabad, India.

4. Chemicals

Minimum Essential Medium
Dulbecco's Modified Eagles Medium
Rosewell Park Memorial Institute medium
Trypsin
Casein
Fluid Thioglycollate Medium

— HiMedia Laboratories, Mumbai,

L-Glutamine		
TBA (Thiobarbituric acid)		HiMedia Laboratories, Mumbai,
Sodium Caseinate		
Foetal Calf Serum	:	Biological Industries, Kibbutz, Israel.
Collagen solution type I (from calf skin)		
Gelatin Type A (from Porcine skin)		
Guanidinium thiocyanate		
2-mercaptoethanol		
Sodium acetate		
Bromophenol blue		Sigma chemicals, St. Louis, USA
EGTA		
DEPC (Diethylpyrocarbonate)		
Aprotinin		
Leupeptin		
PMSF		
DTT (Dithiotheritrol)		
Benzamidine		
Triton X-100		
MTT (Methyl thiazol tetrazolium bromide)		

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

Concanavalin – A

Hydroxyproline

4, 6 Glucuronic acid lactone

— Sigma Chemicals, St. Louis, USA.

Acrylamide

N,N-Methylene Bisacrylamide

1, 4- Bis(phenyloxazol-2yl) Benzene (POPOP)

2.5 Diphenyl oxazole (PPO)

Papain (Extracted from Papaya latex)

HEPES buffer

Folins reagent

Acetyl acetone

Glucosamine hydrochloride

NNED (1-Naphthyl ethylene diamino dihydrochloride)

— Sisco Research Laboratories, Mumbai, India

Lipopolysacchride : Difco Laboratories, USA

Polycarbonate filters: : Nucleopone, Cambridge, USA.

Crystal Violet — Romali, Mumbai, India.

Trypan blue

DNTP mixture

Taq DNA polymerase — Genei, Bangalore, India

DNase, RNase free water

5. Reagent Kits

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

Gelcode Blue Stain Reagent : Pierce Inc, USA

Cells to cDNA Kit : Ambion Inc, USA

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

BD Mercury Transfactor Kit : BD Biosciences, USA

6. 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)

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

96 well flat bottom culture plate

24-well tissue culture plate : Laxbro, India.

7. Instruments used

Minicycler – Thermocycler : MJ Research, USA

Gel Documentation system : Biorad, USA

Transilluminator : Vilber Lourmat, USA

CO_2 Incubator : Napco, Canada

Lyophilizer : Labconco Inc, USA

High speed cooling centrifuge : Remi, Chennai, India

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

Disc electrophoresis unit : Balaji Scientific Service Chennai, India

Submerged electrophoresis unit : Bangalore, Genei, India

ELISA-Reader : Awareness Technology, Gujarat, India

Rack Beta Liquid Scintillation Counter : Wallac, Finland

Spectrophotometer : Elico, India

Inverted Microscope : Leica, Germany

Spinwin Microcentrifuge : Tarson, India

Electronic Balance : Schimadzu, Japan

Microtome : LabAgencies, Cochin, India

Deep Freezer : Remi, Chennai, India

8. 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, 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. Griess Reagent

Reagent 1 - 0.1% N(1-Naphthyl ethylene diamino dihydrochloride).

Reagent 2 - 1% sulfanilic acid.

Mix reagent 1 and 2 in 1:1 proportion before use.

F. Crystal Violet

Crystal violet	-	50.0 mg.
20% methanol	-	100 ml.

Solution filtered and used

G. Trypan blue

Trypan blue stain	-	100 mg
Normal saline (0.85% NaCl)	-	100.0 ml

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

H. 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.

B. METHODS

I. Tissue Culture

1. Sterilization of Glass-wares

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

2. Preparation of Culture Media and Sterility Checking

DMEM (9.98g/l), MEM (10.3g/l) and RPMI (10.3g/l) were prepared in autoclaved double distilled water, pH adjusted to 7.2 using sodium bicarbonate. Additional supply of L-glutamine (2mM) added and filtered under negative pressure using a 0.22 μm cellulose filler. 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 6 days and

checked for visible contamination. Broad spectrum antibiotics such as penicillin (100 units/ml) streptomycin (100 μg/ml) and foetal calf serum (10%) were added to the medium prior to use.

3. Maintenance of L929 cells in Tissue Culture

To start with tissue culture techniques, mouse filoroblast cells were maintained for a long time in culture. The spent medium was removed from the confluent bottles and the cells were washed thrice with 2 ml of PBS EDTA. 0.1 ml of trypsin solution containing 0.02% EDTA was added and incubated for 3-4 minutes at 37°C and the bottles were tapped to dislodge the cells and MEM 10 ml 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 10 ml. of complete medium and incubated at 37°C. The spent medium was changed every 3rd day and was subcultured every week.

4. Maintenance of B16F-10 Cell lines in Tissue Culture

To maintain the cells in culture, spent medium from confluent bottles was removed and the cells were washed three times with PBS. 0.1 ml trypsin solution free of EDTA was added and incubated for 2-3 minutes at 37°C. The bottles were then gently tapped and 10 ml. of 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 subcultured every week.

5. Preparation of Cells for *In Vitro* Studies

To perform an experiment, cells of sub-confluent cultures (70-80% confluence) were used. Monolayers were washed three times with PBS and the cells were harvested by mechanical dislocation using a cell scraper. Cell concentration was adjusted to the required number cell viability checked by trypan blue exclusion method and cell suspension with more than 90% viability were used for experiments. (Kuttan et al, 1985).

6. Determination of Cell Viability

Cell viability was determined by trypan blue dye exclusion method (Kuttan et al, 1985). 0.1 ml of cell suspension was mixed with 0.1 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 were counted separately.

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

7. *In vitro* Cytotoxicity Studies

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

8. Long term *in vitro* cytotoxicity studies by tissue culture

B16F-10 melanoma cells or DLA cells growing in log-phase was used for this experiment. Cells were collected by trypsinization and 5000 cells/well were seeded into 96 well flat bottomed tissue culture plate containing 200 µl complete medium and incubated at 37°C. After 24 hours of incubation, various concentrations of the test compounds were added to the wells and then incubation was continued for 48h. Four hours before termination 20 µl of MTT solution (5 mg/ml) was added to each well. After the incubation period the plates were centrifuged, supernatant was removed and 100 µl DMSO was added to each well. The plates were then incubated at room temperature for 15 minutes and the optical density was measured at 570 ηm with reference of 690 ηm.

The percentage of dead cells was determined using the formula

% dead cells = [O.D of drug treated/ O.D of Control] X 100

9. Maintenance of Experimental Animals

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

10. Maintenance of tumours 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. Tumour cells were

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

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

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

For *in vivo* studies, tumour mass from animal was dissected and processed to get a single cell suspension. Tumour was mashed and the pieces were forced through a fine steel mesh using PBS. The cells were separated from RBCs by centrifugation and then suspended in PBS to required cell number and used for *in vivo* experiments after determining the cell viability. $1x10^6$ viable cells were injected in the same manner to another set of animals to maintain the tumour line.

12. Preparation of Peritoneal Macrophages

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

13. Bioassay of Tumour Neurosis Factor - α (TNF-α)

Bioassay of TNF-α is designed on the basis of direct cytotoxicity of tumour necrosis factor - α (TNF-α) on lung fibroblast cell line (L929) which was used for the identification of murine TNF-α activity in tissue culture supernatants (Sugarman et al, 1985; Tomkins et al, 1992). Single cell suspension of target L929 cells suspended in MEM containing 10% goat serum. L929 cells were plated in 96 well titre plate at a concentration of 5000 cells/well. Macrophages were cultured with or without LPS (5 μg/ml) for 24h at 37°C with 5% CO_2. After 24 hours the plates were centrifuged and the supernatant were added to the wells containing target L929 cells in triplicate. The plates were incubated at 37°C with 5% CO_2 atmosphere. After incubation cells were fixed and stained with crystal violet and cellular cytotoxicity was assessed morphologically.

14. Estimation of Nitrite

Nitrite in the serum and culture supernatant was determined by the Griess reaction (Green et al, 1982). Briefly, 100 μl sample was incubated with equal amount of Griess reagent and incubated for 10 min at room temperature. Absorbance were estimated at 540 ηm. The amount of nitrite was calculated from the $NaNO_2$ standard curve.

In Vivo Antimetastatic Studies

1. Determination of the Metastatic Potential of B16F-10 Cells in Animal model

Studies on the metastatic ability of tumour cells (*in vivo*) were done in C57BL/6 mice. Pulmonary colony forming ability of B16F-10 cells was carried out as described by Fidler (Fidler,1978). C57BL/6 mice were injected with B16F-10 cells ($1x10^6$) through the lateral tail vein. Animals were sacrificed on 21st day. Metastasis of the lungs were determined by (a) counting the metastatic tumour nodules on the surface of the lungs (b) measuring biochemical parameters such as lung collagen hydroxyproline, uronic acid, hexosamine, serum sialic acid and serum γ-glutamyl transpeptidase (c) histopathological analysis of lungs and (f) determining the rate of survival.

Biochemical parameters

2. Estimation of Protein by Lowry Method

The assay relies on the formation of protein copper complex and reduction of phosphomolybdate - phosphotungstate reagent (Folin Ciocaltau reagent) by tyrosine and tryphtophan residues of protein (Lowry et al, 1951).

Reagents

Solution A

Sodium potassium tartarate	-	1 ml (2%)
$CuSO_4$	-	1 ml (1%)

Na_2CO_3 - 98 ml (2% in 0.1 N NaOH)

Solution B

Folin's Phenol reagent (2N) - Diluted 1:1 with distilled water.

Procedure

Samples were made up to 1.2 ml with distilled water. To this 6 ml of solution A was added and then incubated at room temperature for 10 min. 300 µl of Solution B was then added to the vortex mixed reaction mixture and incubated at room temperature for 30 min. Absorbance was taken at 660 ηm.

2. Estimation of Hydroxyproline (Bergman and Loxely, 1970)

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

Reagents

1. Oxidant solution

Sodium acetate	-	5.7g.
Trisodium citrate	-	3.75g.
Citric acid	-	0.55 g.
Isopropanol	-	38.5 ml
Distilled Water	-	61.5 ml

2. Ehrlich's Reagent

ρ-dimethyl amino benzaldehyde	-	4.4 g
Perchloric acid (60%)	-	10.2 g
Isopropanol	-	25 ml (final volume)

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

Procedure

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

3. Extraction and Estimation of Uronic acid (Bitter and Mair, 1962)

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 (10 mg/g dry weight of tissue) in 5 ml of 0.5M acetate butter pH 5.5 containing 0.005 M cysteine and 0.005M disodium salt of EDTA at 65°C for 24h. An aliquot of the sample containing uronic acid was estimated by the method of Bitter and Mair (Bitter and Mair, 1962).

Reagents

0.025 M sodium tetraborate in concentrated H_2SO_4

Acetate Buffer (0.1M)

Procedure

Aliquote (5 ml) of sulphuric acid reagent (prepared with 0.025 M sodium tetrabonate in conc. H_2SO_4) was taken in tubes and cooled at 4°C for some time. 1ml of sample on standard glucuronolactone solution containing 5-20 mg was layered on the acid. Tubes were closed with glass stoppers and the rack was shaken first gently and then vigorously. Tubes were kept in a boiling water bath for 10 minutes and cooled at room temperature. 0.2 ml of carbazole reagent (0.125 % carbazole in absolute alcohol) was added and the tubes were shaken heated in a boiling water bath for 15 minutes and cooled. The pink colour thus developed was read at 530 ηm. Uronic acid content of the tissues were expressed as mg/100 mg wet weight.

4. Estimation of Hexosamine (Elson and Morgan, 1993)

Lyophilized tissue samples (5 mg) 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.

Preparation of 2% acetyl acetone in 0.5M Na_2CO_3

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

Preparation of Ehrlischs reagent

Dissolve 1.33 g of ρ-dimethyl amino benzaldehyde (PDAB) in 100 ml of 1:1 ethanol and conc.HCl.

Procedure

Aliquots containing 10-15 μg hexosamine were treated with 1 ml 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, 5 ml of 95% ethanol and 1 ml of Ehrlich's reagent (equal proportion of 1.33% ρ-dimethylamino benzaldehyde in 1:1 ethanol : conc. HCl mixture) were added and mixed thoroughly. The purple red colour developed was read after 30 minutes at 530 ηm. 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.

5. Estimation of Protein Bound Serum Sialic acid (Skoza and Mohos, 1976)

Hydrolysed serum liberate sialic acid forms a coloured compound with thiobarbituric acid.

Reagents

1. 0.2N H_2SO_4
2. Periodic acid – 25 μM in 62.5 mM H_2SO_4
3. Sodium arsenite – 0.2% in 0.5M HCl
4. Thiobarbituric acid – 6% (pH 9.0)

5. Dimethyl sulphoxide

Procedure

200 µl of sample was mixed with equal volume of 0.2 N H_2SO_4 and hydrolyzed for 1h at 80°C. To this hydrolysate 50 µl periodic acid of (25 µM) was added and incubated for 30 minutes at 37°C. To this reaction mixture 50 µl of sodium arsenite was added, followed by 100 µl of thiobarbituric acid and was heated in a boiling water bath for 7.5 minutes. After heating, 400 µl of DMSO was added to intensify the colour and read at 549 ηm with reference to 532 ηm.

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

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

Reagents

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

Procedure

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

7. Histopathological Analysis

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

8. Determination of the Rate of Survival

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

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

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

In vitro Antimetastatic Studies

1. Collagen Matrix Invasion Assay (Albini, Reed and Mc Nutt, 1997).

Invasion of collagen matrix by tumour cells was carried out using modified boyden chambers (Blind well chambers) as described by Albini et al. The lower compartment of the chamber was filled with DMEM with 10% conditioned medium and polycarbonate filters coated with 25 μg.of Type I collagen were placed in between the upper and lower compartment of blind well chamber. B16F-10 cells ($1x10^5$/0.15 ml) prepared and suspended in DMEM with

out FCS. The cell suspension was added to the upper chamber and incubated at 37°C for 10h in a 5% CO_2 atmosphere. After the incubation period medium and cells from the upper chamber was removed using a cotton swab. The filter was then fixed in methanol for 5-8 minutes and stained for 3 min with crystal violet. Cells that penetrated the polycarbonate filters were counted in 10 random fields under a microscope. Results were calculated as % inhibition of invasion using the formula.

$$\% \text{ Inhibition of Invasion} = 100 - \frac{\text{Mean no: of invaded cells in test}}{\text{Mean no: of invaded cells in control}} \times 100$$

2. Tumour Cell Motility Assay

B16F-10 cells ($1x10^5$/0.15 ml) were seeded on the upper compartment of blind well chamber, containing polycarbonate filter in between the upper and lower compartments with out collagen coating. Chambers were incubated at 37°C in 5% CO_2 atmosphere for 24h. Migrated cells were collected from the lower chamber and counted using a haemocytometer. Results were calculated as % inhibition using the formula.

$$\% \text{ Inhibition of Motility} = 100 - \frac{\text{Mean no: of migratory cells in test}}{\text{Mean no: of migratory cells in control}} \times 100$$

3. Tumour Cell Adhesion Assay (Inokuchi et al, 1991)

B16F-10 melanoma cells ($5x10^3$ cells/well) were added to 96 well flat bottom titre plates, pre coated with type I collagen (25 µg/ml) 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 min and stained using crystal violet for 20 minutes each. The cells were counted under an inverted microscope. Results were calculated as % inhibition using the formula.

$$\% \text{ Inhibition of adhesion} = 100 - \frac{\text{Mean no: of adherent cells in test}}{\text{Mean no: of adherent cells in control}} \times 100$$

4. Gelatin Zymography

Proteases of tumour cell supernatant were initially resolved on SDS – polyacrylamide gels, which were incorporated with gelatin. Following the incubation of the gel in the activation buffer, protease separated on the gel will break down the gelatin and appears as transparent zones.

Procedure

Gelatin zymography was performed according to the modified procedure of Billings et al. The conditioned medium from subconfluent (70% confluent) of B16F-10 melanoma cells were used for zymographic analysis. Conditioned medium was collected, centrifuged, and the protein concentrations were estimated. Conditioned medium containing the proteases were activated with 2.5 µl of trypsin (75 µg/ml)for 30 minutes at room temperature. Trypsin treated and

untreated samples (equivalent to 50 μg protein were mixed with an equal volume of sample buffer (2X), and loaded on 11% polyacrylamide gels containing 0.8% gelatin and carry out electrophoresis at 10°C for 3h with a constant current of 2 mA 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 times of 30 min to remove SDS which could interfere with the proteolytic activity. This was followed by 2 h washing with activation buffer and the gels were finally incubated in the same butter at 37°C for 18h.. Gels were then fixed and stained with Coomassie blue stain and clear digested zone were visualized against dark back ground.

Preparation of Gels

Resolving gel

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

29.2% acrylamide + 0.5% bisacrylamide	-	11 ml
0.1 M Tris-HCl, pH 6.8	-	1.2 ml
20% SDS	-	0.15 ml
Gelatin (240 mg/2 ml H_2O, heated to dissolve)	-	2 ml
Distilled water	-	6. ml
20% Ammonium per sulfate	-	0.10 ml
TEMED	-	0.045 ml

5% stacking gel

29.2% acrylamide + 0.5% bisacrylamide	-	1.67 ml
0.1 M Tris HCl pH 8.8	-	1.75 ml
20% SDS	-	0.10 ml
20% Ammonium per sulfate	-	0.10 ml
Distilled water	-	6.36 ml
TEMED	-	0.02 ml

2% Triton X – 100

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

10 mM EDTA solution

EDTA	-	372.24 mg
0.1 M Tris – HCl, pH7.8	-	1000 ml

0.25 M Sucrose – 0.01 M Tris-HCl buffer, pH 7.4

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

Running Buffer pH 7.8

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

Made up to 1 litre with distilled water.

Trypsin Solution

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

Activation Buffer pH 7.8

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

Sample Buffer (2x)

Glycerol	-	1 ml
1M Tris-HCl, pH 6.8	-	0.25 ml
20% SDS	-	1 ml
Bromophenol Blue	-	1.65 mg

Made up to 5ml with distilled water.

In Vitro Apoptotic Studies

1. Morphological Analysis

To detect the morphological changes, 5 million Dalton's Lymphoma Ascites 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, centrifuged and the cell pellets were suspended in PBS and cell smear was prepared 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.

2. 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, 5 million Dalton's Lymphoma Ascites 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, centrifuged and the cell pellets were 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 (1000 g, 4^0C). The supernatant obtained was incubated over night with RNase (2 μg/ml) at room temperature and then with proteinase K (50 μg/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 50 μl of Tris-EDTA buffer. The extracted DNA were resolved on 1.5% agarose gels.

Gene Expression Studies

1. cDNA Synthesis

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

Materials provided in the kit

1 x PBS	-	40 ml
Cell Lysis Buffer	-	10 ml
DNase I (2 U/µl)	-	200 µl
10 X RT Buffer	-	500 µl
M-MLV Reverse Transcriptase	-	100 µl
RNase inhibitor (10 U/µl)	-	100 µl
dNTP (2.5 mM each d NTP)	-	400 µl
Oligo $(dT)_{18}$ Primers	-	50 µM
Nuclease Free water	-	3.5 ml.

Principle

In the cells to cDNA II kit, a crude cell lysate is subjected to RT-PCR without purifying the RNA. Cells from tissue culture were washed in PBS and then heated in cell lysis buffer. This treatment has two important effects. First it raptures the cells, releasing the RNA into the cells lysis buffer. The heating step also inactivates endogenous RNases, protecting 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. At this point the cell lysate is ready for reverse transcription and PCR were carried out using two step RT- PCR strategy.

Procedure

B16F-10 melanoma cells ($1x10^4$ cells/well) were seeded in the 96-well flat bottom titre plate using DMEM supplemented with 10% FCS and antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml) and incubated for 12h at 37°C in 5% CO2 atmosphere. After incubation medium was removed and the cells were washed with ice cold PBS. Ice cold cell lysis buffer 100 µl were added to the cells and immediately transferred to a water bath, incubated for 15 min at 75°C cells then transferred to 200 µl nuclease free microcentrifuge tubes. To this 2 µl DNase-1 per 100 µl cell lysis buffer were added and incubated for 15 min at 37°C. DNase were inactivated by heating at 75°C for 5 min.

Following reagents were assembled in nuclease free microcentrifuge tubes

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

Reagents were mixed by vortexing, centrifuged briefly and placed on crushed ice. The remaining Reverse transcription reagents to the same microcentrifuge tubes as follows.

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

The reagents were mixed by vortexing and centrifuged briefly.

Reverse Transcription Reaction

The reaction mixture was incubated at the following temperatures and time by using a thermocycler.

Temperature	Time
42^0C	60 minutes
92^0C	5 minutes
4^0C	5 minutes

2. Polymerase Chain Reaction (PCR) Analysis of Proinflammatory Cytokine gene expression.

cDNA synthesized above were directly used for PCR amplification (5µl for each reaction). PCR was performed with Message Screen Mouse Inflammatory Cytokine Set Multiplex PCR kit from Biosource International, USA.

Biosource's message Screen Mouse Inflammatory Cytokine Multiplex PCR kit have been designed to detect the expression of Mouse GAPDH, IL-6, IL-1β,

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 IL-6, IL-1β, GM-CSF, TNF-α and IL-12p40 gene expression and to normalize the expression of these genes against GAPDH.

Materials provided in the kit

10X dNTPs	-	250 µl
2 X PCR Buffer	-	1250 µl
Dnase free Water	-	1500 µl
10 X Positive Control	-	50 µl
10 X PCR Primers	-	250 µl
DNA. M.W . Marker	-	100 µl

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-1β	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 them thoroughly and centrifuged briefly. All the reagents were assembled in nuclease free microcentrifuge tubes as follows.

Components	Amount
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 +ve Control	5 µl

Reaction mixture were vortex and centrifuged briefly before and after adding the cDNA sample. PCR thermal cycling was performed according to the protocol

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	

Polymerase Chain Reaction (PCR) analysis of Bcl –2 and Caspase-3 gene expression

cDNA synthesized from Dalton,s Lymphoma Ascites (DLA) cells ($1X10^4$ /well) through the above method was directly used for PCR amplification. PCR was performed with primers obtained from Maxim Biotech, Inc, USA.

Materials provided in the kit

Pre Mixed forward and reverse primers	-	1000 µl
Optimized PCR buffer (Chemicals, Enhancer, Stabilizer, dNTPs)	-	750 µl X 4 tubes
Positive Control	-	100 µl
Nuclease free water	-	1000 µl

Procedure

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

Master mixture preparation

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

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

Reaction mixture were 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

10 µl of each PCR product resolved on 2% agarose gel electrophoresis.

Reagents

10 X TEB

Tris-HCl, pH 8.3	-	21.6 g
EDTA	-	0.372 g
Boric acid	-	11 g

Made upto 200ml using double distilled water.

10X Loading dye

Bromophenol blue	- 0.05 %
TEB 10X pH 8.3	- 1 ml
Glycerol	- 100 µl

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 the clean, dry, plastic gel tray was sealed with sealing tape and the comb was placed.

iii. Agarose powder (2%) added to the electrophoresis buffer and heated the slurry in a boiling water bath or gas flame until the agarose dissolved and the slurry became visible.

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 30-45 minutes and the gel tray placed in the electrophoresis tank after removing the sealing tape.

v. 10µl of PCR products mixed with 2 µl 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.

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

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). This method is faster, easier and more sensitive than electrophoretic mobility shift assays (EMSA) and does not require the use of radioactivity.

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 bind 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.

Materials provided in the kit

Transfactor plate

Transfactor Rack

Primary antibody

Secondary antibody

Wild-Type competitor Oligos

TMB substrate

10 × Transfactor Buffer

Blocking Reagent

Stop solution (Na Azide)

Transfactor ELISA procedure

Cytosolic and Nuclear Extract Procedure

The following procedure was used to prepare cytosolic or nuclear extracts from cell line culture (Dignam, Lebovitz and Roeder, 1983; Dyer and Herzog, 1995; Lee et al, 1998). This procedure is designed for extraction from 1×10^7 cells, which is roughly equivalent to three 15 cm tissue culture plates at 80% confluency. 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)

15 mM $MgCl_2$

100 mM KCl

Pre -Extraction Buffer

20 mM HEPES (pH 7.9)

1.5 mM $MgCl_2$

0.42 M NaCl

0.2 mM EDTA

25%(v/v) glycerol

Protease Inhibitor Cocktail

Aprotinin (1mg/ml)	–	5 µl
Leupeptin (1mg/ml)	–	5 µl
PMSF (100 mM in Isopropanol)	–	5 µl
DTT (100 mM in D.W.)	–	10 µl
Benzamidine (1mg/ml)	–	5 µl
Triton × 100 (10% in D.W)	–	10 µl
Tris pH 7.4 (1M)	–	20 µl
NaCl (5M)	–	50 µl
EDTA (0.5 M)	–	5 µl
EGTA (10 mg/ml)	–	5 µl

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

1 X Lysis Buffer

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

a.Cell Lysis

1. Cells were collected after incubation using a cell scraper, and transferred to a clean centrifuge tube , centrifuged for 5 min at 450 x g. Supernatant was decanted.

2. The cell pellet were rinsed with ice cold PBS and centrifuged for 5 min at 450 x g 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 15 min on ice.
6. The cell suspension was centrifuged for 5 min 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.

b.Cell Disruption

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

c.Nuclear Extraction

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

   Pre- extraction Buffer – 147 µl

   0.1 M DTT – 1.5 µl

   Protease inhibitor cock-tail – 1.5 µl

2. The crude nuclear pellet obtained in Step b.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 as step b.1 - b.3.
4. Nuclear suspension were allowed to shake gently for 30 min at $4^{O}C$
5. The nuclear suspension was centrifuged at 20000-21000 × g for 5 min.
6. The supernatant were transferred to a clean, chilled test tube and this fraction is the nuclear extract.
7. The protein concentration of the nuclear extract was measured by BradFord method.
8. 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 colour development is virtually complete in 2 min and the colour 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

Brad Ford 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.

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 150 µl of 1X Transfactor buffer for 15 min. at room temperature. Nuclear extracts (6µl) were made up to 50 µl with 1X Transfactor buffer and added to the assay wells and incubated 60 min at room temperature. Wells were washed with 1X Transfactor buffer and incubated with 100 µl primary antibody at room temperature for 60 min, then washed with 1X Transfactor buffer and incubated with 100 µl of secondary antibody at room temperature for 30 min. Wells are washed with 1X Transfactor buffer and incubated with 100 µl of TMB substrate to each well and incubated at room temperature for 10 min. After seeing the blue colour development the reaction

were stopped by adding 100 ml of sodium azide stop solution. Absorbance was measured at 655 ηm.

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

Chapter-3

Effect of some natural products on the NO and TNF-α production in the in vitro as well as in vivo models

Introduction

Nitric oxide (NO), since its discovery, has been shown to play a significant role in several physiological and pathological processes in different tissues and organism. It has been demonstrated to widen blood vessels, thus regulating blood pressure and diverting blood flow to several areas of the body. It has also been shown to battle against bacterial infection (Moncada, Palmer and Higgs, 1991; Beutler et al, 1992). NO is enzymatically formed from a terminal guanidine-nitrogen of L-arginine by the enzyme Nitric Oxide Synthase (NOS), yielding L-citrulline as a co-product . There are two principal systems by which NO is produced in the body, the inducible nitric oxide synthase (iNOS) system and the consecutive nitric oxide synthase (cNOS) system (Schmidt and Walter, 1994). The iNOS system is Ca^{2+} independent but cNOS system is Ca^{2+} or calmodulin dependently. iNOS is present in macrophages, cardiomyocytes, vascular smooth muscle, hepatocytes, intestinal epithelium, megakaryocytes, keratinocytes and chondrocytes (Schmidt and Walter, 1994; Steage et al, 1998). The cNOS system is the constitutive (basal production) form. It is Ca^{2+} and calmodulin dependent and is rapidly activated (10-15 min) by elevated intracellular free Ca^{2+} in different types from several organism (Fimiani et al, 1999; Balligand et al, 1995). NO acts as an important mediator of tissue damage in inflammatory diseases, elevated NO levels are thought to play a central role in tissue damage observed during septic shock. iNOS mediated NO production have a critical role in carcinogenesis and excessive amount of NO has been implicated in pathogenesis of may diseases like cardiomyopathy, chronic degenerative disease and rheumatoid arthritis (Moncanda, Palmer and Higgs,1991). So it is necessary that the production of NO should be tightly regulated.

Proinflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) released during endotoxaemia are likely to be involved in the regulation of NO production and these cytokines play important role in the up regulation of NO synthesis (Jorens et al, 1991; Feinstair, 1994). TNF-α is mainly produced by activated macrophages and monocytes. LPS is presumably the most efficient and certainly the most studied inducer of TNF-α (Beutler, Han, and Krays, 1992). Concanavalin-A (Con-A) possesses mitogenic properties. It also activates T-lymphocytes and induces the release of IFN-γ and other cytokines (Gunther et al, 1973; Reeke et al, 1975). In the present study we investigated the effect of some natural products on the inhibition of NO and TNF-α production which may have therapeutical importance.

Materials and Methods

Animals

Four to six week old Balb/c mice were used in the present study. The animals were fed with normal mouse chow (Sai feeds, India) and water *ad libitum.*

Cells used

L929 cell line was used for Bioassay studies.

Compounds studied

Curcumin, Tetrahydrocurcumin, Catechin, Piperine, β-Carotene and 13cis-Retinoic acid

Effect of natural products on the production of NO and TNF- α by macrophages (*in vitro*)

Macrophages were isolated as mentioned in Chapter-2. Macrophages were washed with PBS and resuspended in RPMI-1640 with 10% FCS. The cells were plated in 96 well culture plate and incubated for 2 hours at 37^0 C, in a 5% CO_2 . After

incubation non adherent cells were removed and the adherent macrophages were incubated (2 X 10^5 cells/ well) in complete culture medium (RPMI-1640, 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin, 2 mM glutamine). Macrophages were cultured with or with out LPS (5 μg/ml) in the presence and absence of natural products (0.5 μg/ml - 10 μg/ml) for 24 hours at 37^0C with 5% CO_2 . After 24 hours the plates were centrifuged and the supernatant was used for the estimation of NO and TNF- α.

Estimation of nitrite *(In vitro)*

Nitrite in the culture supernatant was determined by the Griess reaction (Green et al, 1982). Briefly 100 μl sample was incubated with equal amount of Griess reagent (1 part of 0.1% of N (1-naphthyl)-diamine dihydrochloride in distilled water and 1 part 1% sulfanilamide in 5% concentrated H_3PO_4) and incubated for 10 min at room temperature. Absorbance was estimated at 540 nm. The amount of nitrite was calculated from the $NaNO_2$ standard curve.

Bioassay of TNF- α production

Tumor necrosis factor- α (TNF- α) can cause direct cytotoxicity to the lung fibroblast cell line (L929). Bioassay of TNF- α is designed on the basis of this cytotoxicity of TNF- α , which can be used for the identification of murine TNF- α activity in tissue culture supernatants (Sugarman, 1985; Tomkins et al, 1992). Single cell suspension of target L929 cells suspended in MEM containing 10% goat serum, were plated in 96 well titre plate at a concentration of 5000 cells/ well. After 24 hours the supernatant from the macrophage culture were added to the wells containing target L929 cells in triplicate. The plates were incubated at 37^0C in 5% CO_2 atmosphere for 48 hours. After incubation cells were fixed and stained with crystal violet and cellular cytotoxicity was assessed morphologically.

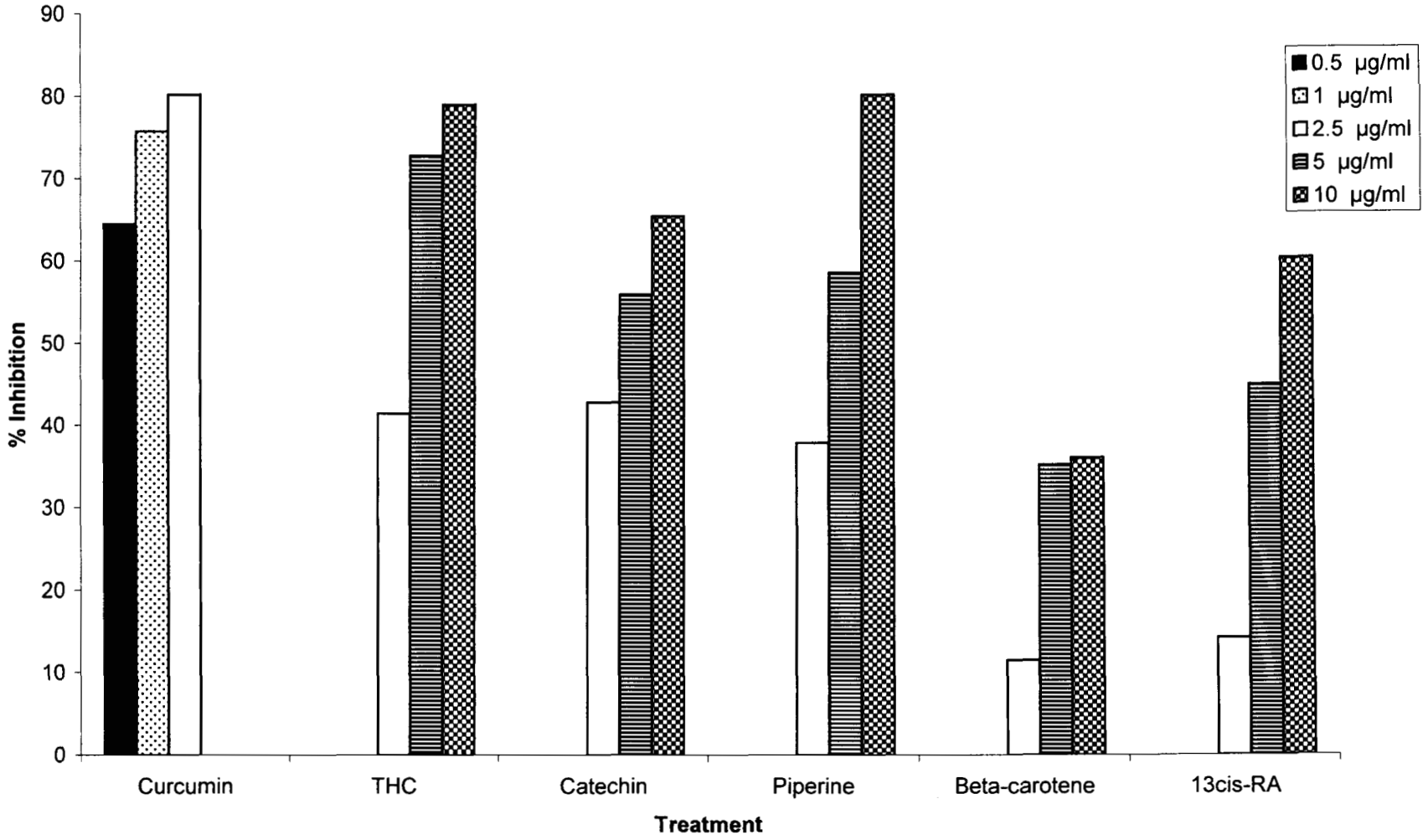
Determination of the effect of natural products on the production of NO and TNF- α by the stimulated macrophages (*In vivo*)

Balb/c mice weighing 20-25g were separated into 3 major groups.. Each major group was divided in to VII subgroups of 3 animals. Group-I animals were treated with respective natural products (200 μ moles/ Kg body weight/ animal/ dose) intraperitoneally for five consecutive days and on the 5th day stimulated with a high dose LPS (250 μg in 0.5 ml PBS) intraperitoneally , Group-II animals were treated with respective natural products (200 μ moles/ Kg body weight/ animal/ dose) intraperitoneally for 5 consecutive days and on the 5th day stimulated with Con-A (100 μg in 0.5 ml PBS) intraperitoneally and Group-III animals were treated with respective natural products (200 μmoles/ Kg body weight/ animal/ dose) intraperitoneally for five consecutive days and on the 5th day stimulated with LPS (250 μg in 0.5 ml PBS)+Con-A (100 μg in 0.5 ml PBS) intraperitoneally . SubgroupVII in each major group was kept as untreated normal animals. Six hours after the challenging treatment of LPS and Con-A, blood was collected from the tail vein. Serum was separated and used for the estimation of nitrite and TNF-α.

Effect of natural products on serum nitrite levels of *In vivo* stimulated animals

Nitrite level which is an indicator of NO synthesis was measured in serum after reducing nitrate to nitrite using bacterial nitrate reductase by microplate assay method based on Griess reaction (Phizackery and Dabagh, 1983). To the serum, equal amount of Griess reagent was added and incubated for 10 min at room

Fig-I. Effect of natural products on the production of nitrite of the activated macrophages (*in vitro*)



temperature. The absorbance was determined at 540 nm . Amount of nitrite was calculated from the $NaNO_2$ standard curve.

Estimation of serum TNF-α by ELISA

Serum TNF-α was estimated using the ELISA kit according to the manufacture's procedure .

Statistical analysis

Results are expressed as Mean±SD. The student's t- test was used to make a statistical comparison between the groups. Results with $P< 0.001$ were considered as statistically significant

Results

Effect of natural products on the production of nitrite of the activated macrophages *in vitro*

Treatment of different natural products could inhibit the production of nitrite by the LPS activated macrophages (Fig-I). Natural products such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, β-Carotene and 13cis-Retinoic acid could inhibit the level of nitrite production dose dependently. Curcumin at concentrations of 0.5 μg/ml, 1μg/ml and 2.5 μg/ml could inhibit the nitrite level by 64.49%, 75.74% and 80.17% respectively. Tetrahydrocurcumin at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml could inhibit the production of nitrite 41.42%, 72.78% and 78.98% respectively. Catechin at concentration of 2.5 μg/ml, 5 μg/ml and 10 μg/ml could reduce the nitrite concentration 42.77%, 55.91% and 65.38% respectively. Piperine at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml could inhibit the production of nitrite 37.86%, 58.57% and 80.17% respectively. β-carotene could not produce a high inhibition of the nitrite production. β-carotene at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml could

Fig-II Effect of natural products on TNF-α induced toxicity on L929 cells

a) L929 cells incubated with normal macrophage culture supernatant.

b) L929 cells incubated with culture supernatant collected from LPS activated macrophages.

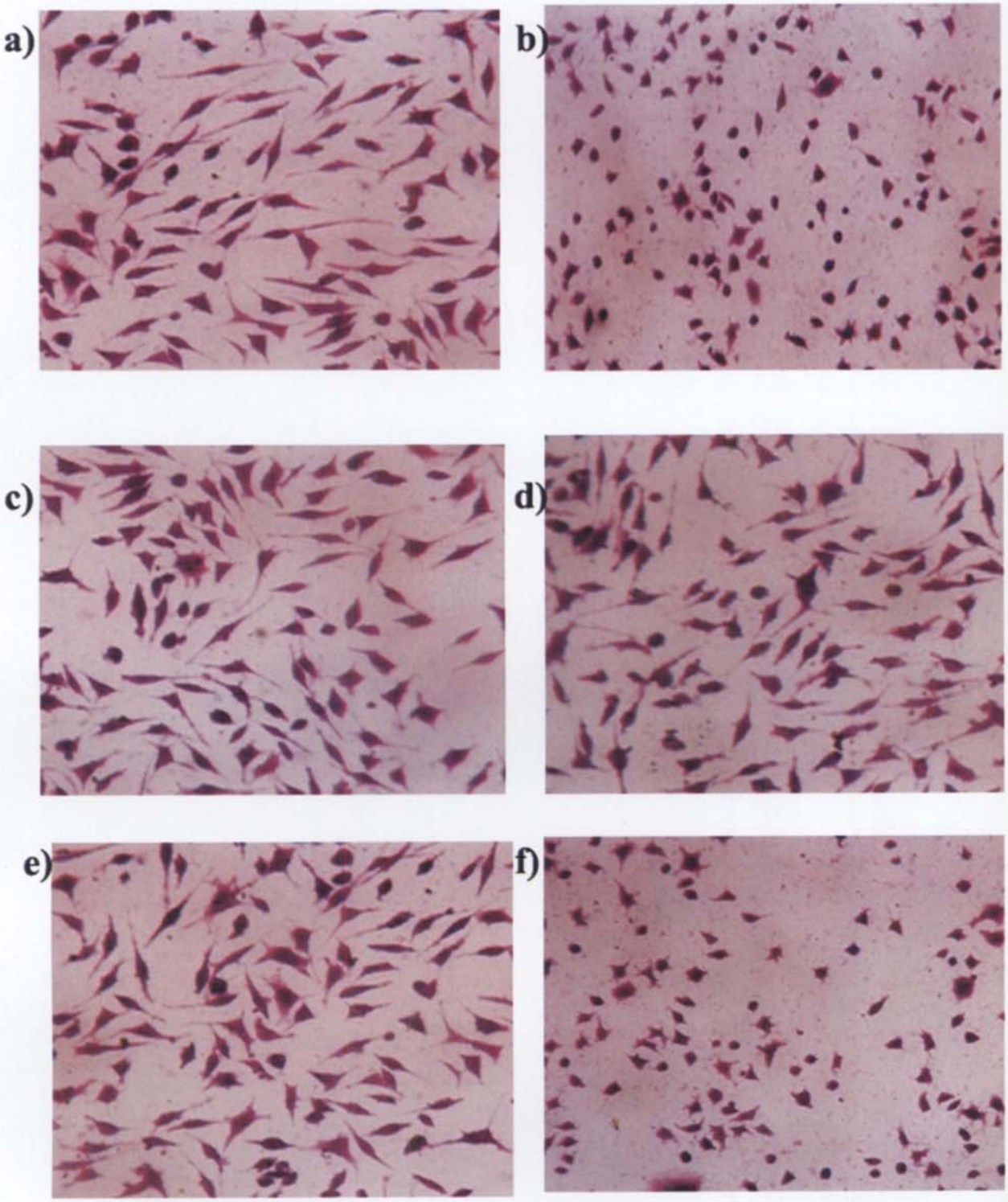
c) L929 cells incubated with culture supernatant collected from LPS and Curcumin (0.5 μg/ml) treated macrophages .

d) L929 cells incubated with culture supernatant collected from LPS and Curcumin (1 μg/ml) treated macrophages.

e) L929 cells incubated with culture supernatant collected from LPS and Curcumin (2.5 μg/ml) treated macrophages.

f) L929 cells incubated with culture supernatant collected from LPS and Tetrahydrocurcumin (2.5 μg/ml) treated macrophages.

Fig-II Effect of natural products on TNF-α induced toxicity on L929 cells



g) L929 cells incubated with culture supernatant collected from LPS and Tetrahydrocurcumin (5 µg/ml) treated macrophages.

h) L929 cells incubated with culture supernatant collected from LPS and Tetrahydrocurcumin (10 µg/ml) treated macrophages.

i) L929 cells incubated with culture supernatant collected from LPS and Catechin (2.5 µg/ml) treated macrophages.

j) L929 cells incubated with culture supernatant collected from LPS and Catechin (5 µg/ml) treated macrophages.

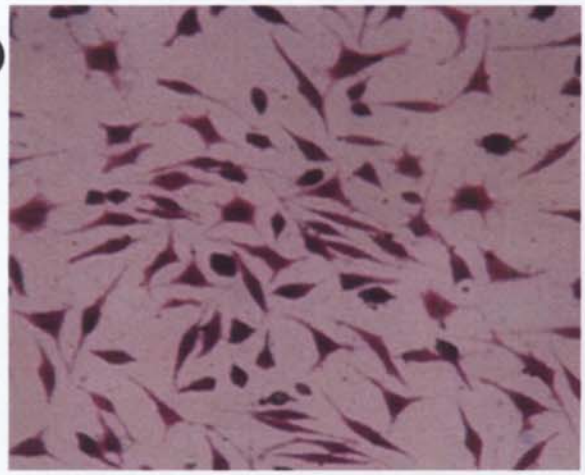
k) L929 cells incubated with culture supernatant collected from LPS and Catechin (10 µg/ml) treated macrophages.

l) L929 cells incubated with culture supernatant collected from LPS and Piperin (2.5 µg/ml) treated macrophages.

m) L929 cells incubated with culture supernatant collected from LPS and Piperin (5 µg/ml) treated macrophages.

n) L929 cells incubated with culture supernatant collected from LPS and Piperin (10 µg/ml) treated macrophages.

g)



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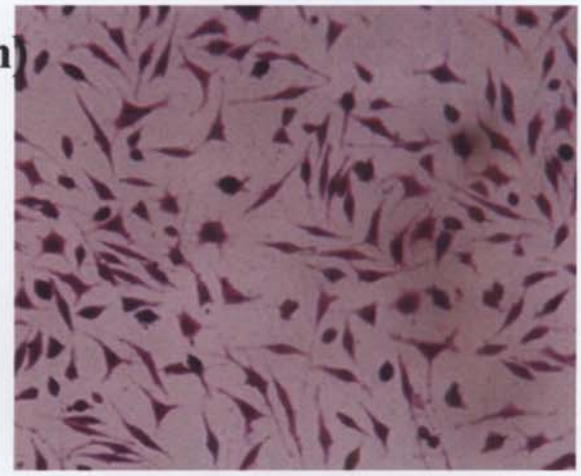

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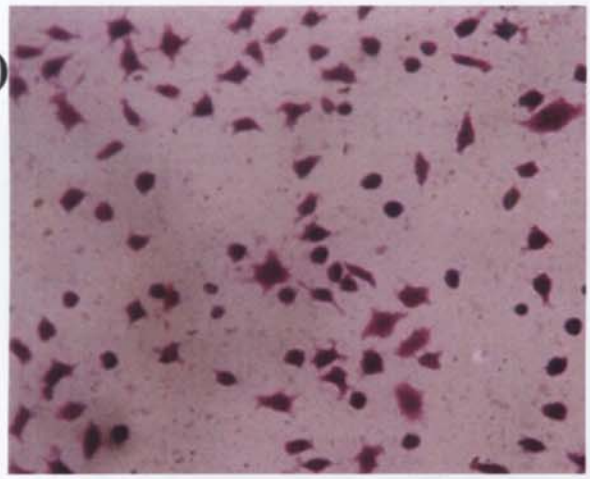

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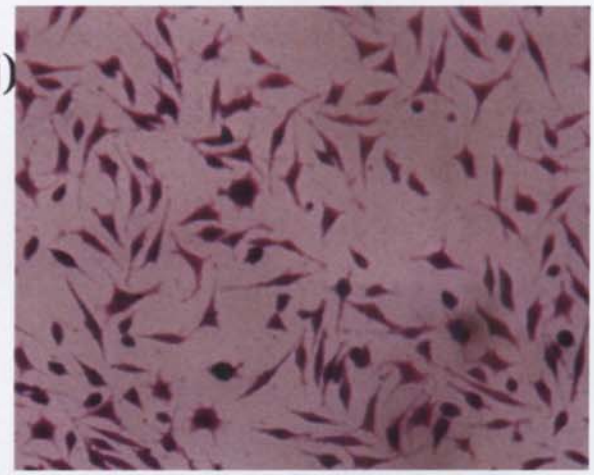

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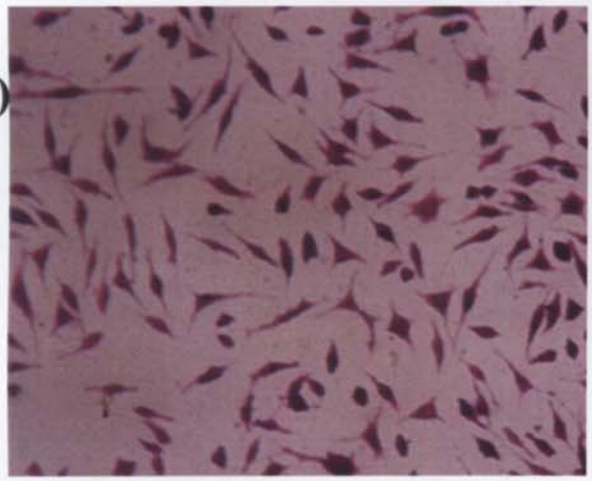

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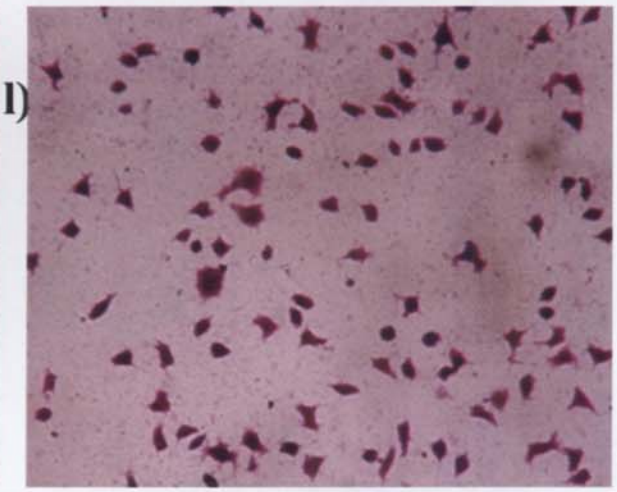

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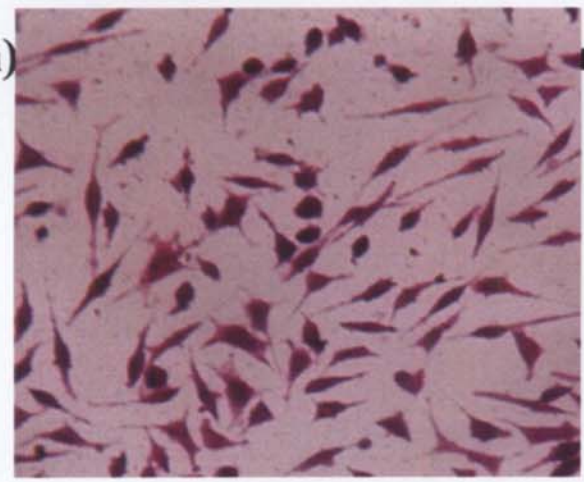

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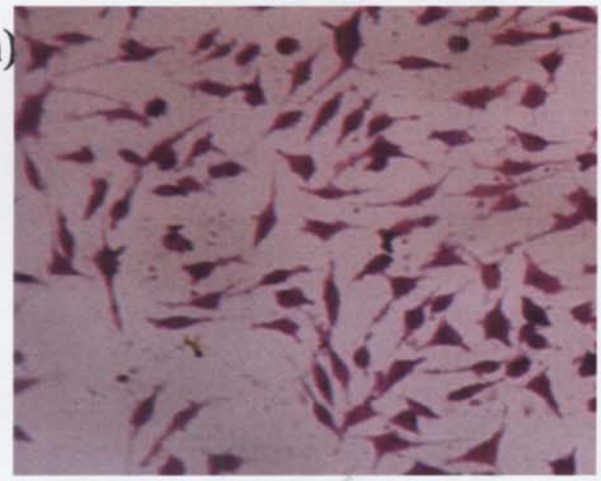


o) L929 cells incubated with culture supernatant collected from LPS and β-Carotene (2.5 μg/ml) treated macrophages.

p) L929 cells incubated with culture supernatant collected from LPS and β-Carotene (5 μg/ml) treated macrophages.

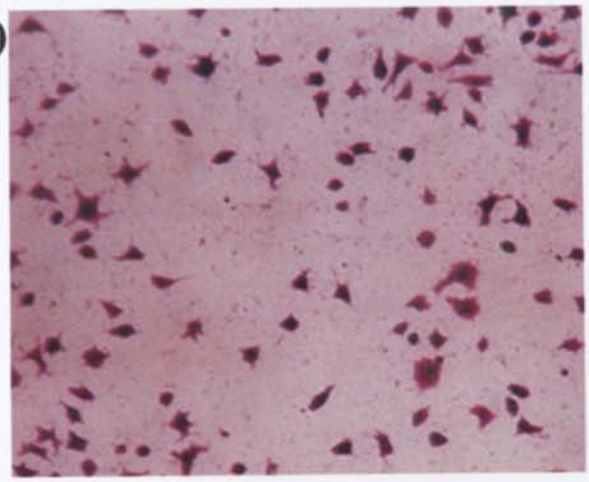
q) L929 cells incubated with culture supernatant collected from LPS and β-Carotene (10 μg/ml) treated macrophages.

r) L929 cells incubated with culture supernatant collected from LPS and 13cis-Retinoic acid (2.5 μg/ml) treated macrophages.

s) L929 cells incubated with culture supernatant collected from LPS and 13cis-Retinoic acid (5 μg/ml) treated macrophages.

t) L929 cells incubated with culture supernatant collected from LPS and 13cis-Retinoic acid (10 μg/ml) treated macrophages.

u) Normal L929 cells with out any treatment.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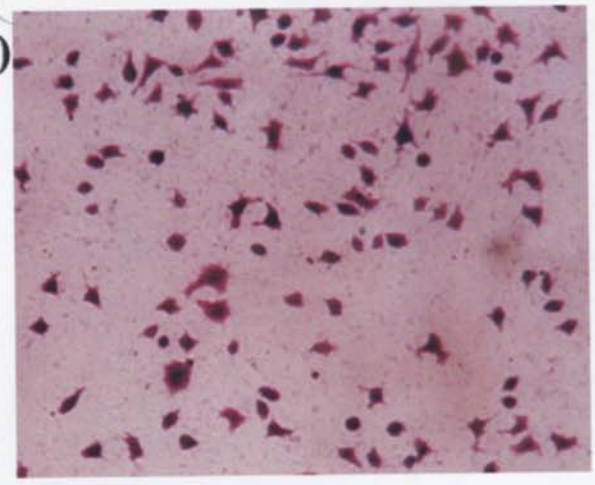
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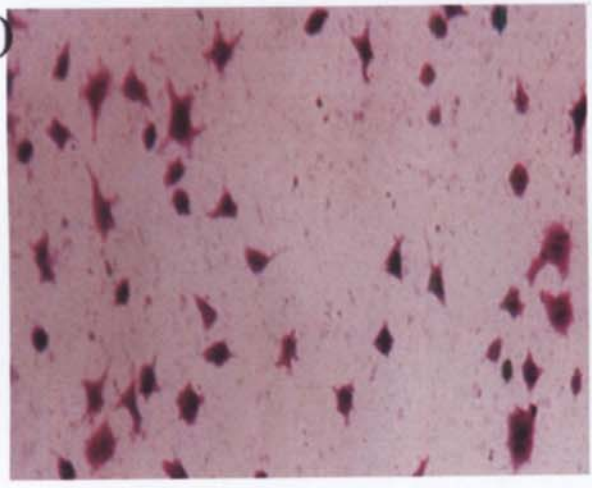
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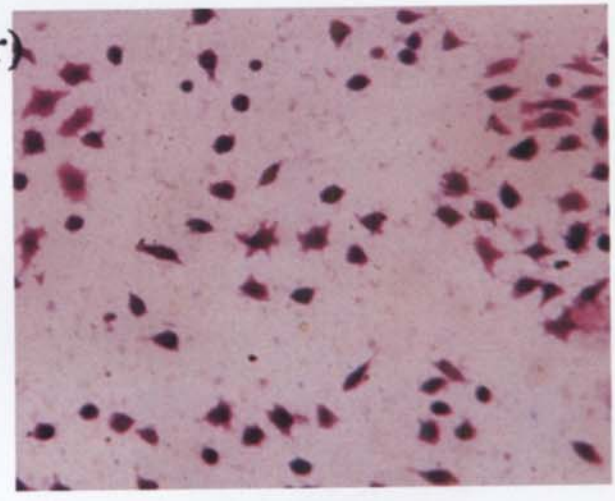
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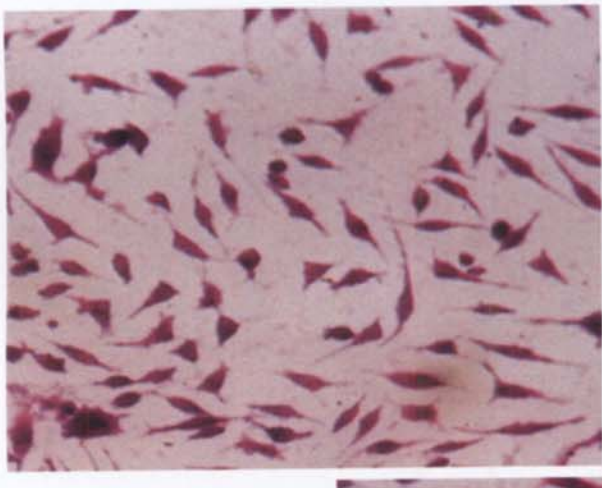
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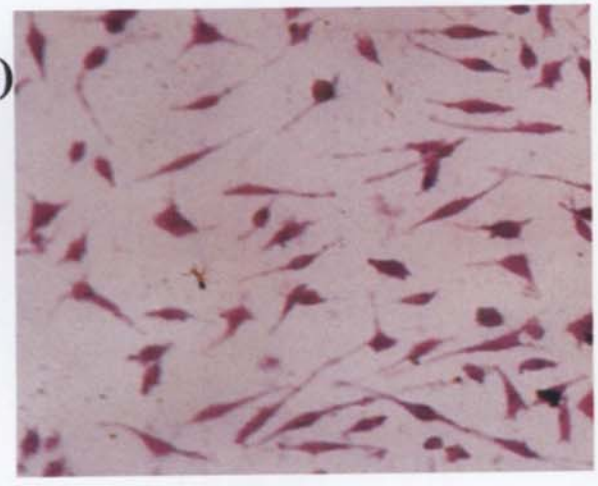
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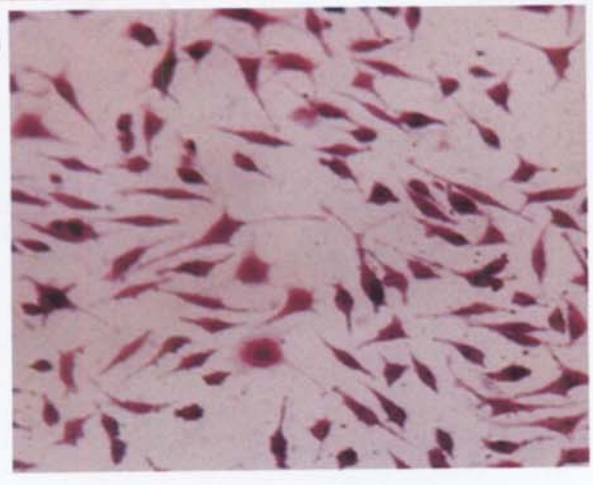
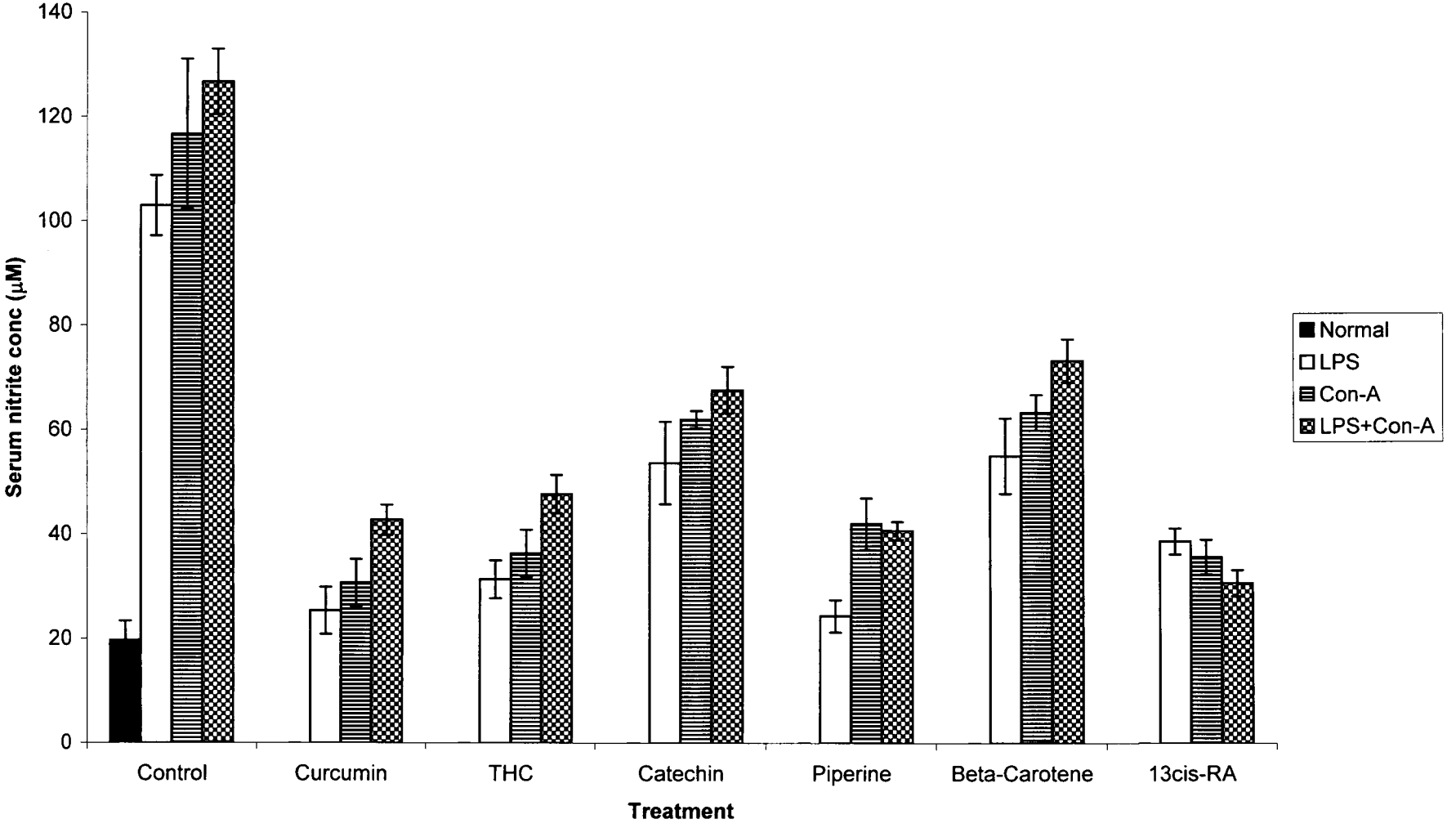

u)

produce only 11.59%, 35.20% and 36.09% respectively but 13cis-Retinoic acid at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml could inhibit the production of nitrite 14.2%, 44.96% and 60.35% respectively.

Effect of natural products on the production of TNF-α by the activated macrophages (*in vitro* Bioassay)

Effect of natural products on the production of TNF-α of the activated macrophages is shown in Fig-II. Fig-IIa. shows L929 cells incubated with the culture supernatant from the untreated normal macrophages. Culture supernatant collected from LPS activated macrophages produced 100% cytotoxicity to the TNF-α sensitive L929 cells (Fig-IIb). Culture supernatant from LPS activated macrophages in the presence curcumin (0.5 μg/ml, 1 μg/ml and 2.5 μg/ml) inhibited the production of TNF-α and the cell growth pattern was normal (Fig-IIc, IId and IIe). Treatment with lower concentrations of tetrahydrocurcumin (2.5μg/ml) treated could not inhibit the production of TNF-α (Fig-IIf) but higher concentrations of tetrahydrocurcumin (5 μg/ml, 10 μg/ml) could inhibit the production of TNF-α and L929 growth pattern was normalized (Fig-IIg and IIh). Similarly catechin also at lower concentration (2.5 μg/ml) could not inhibit the production of TNF-α (Fig-IIi) while higher concentrations of 5 μg/ml and 10 μg/ml could inhibit the production of TNF-α production (Fig-IIj and Fig-IIk).Piperine also inhibited the production of TNF-α dose dependently (Fig III, FigIIm and FigIIn). β-carotene upto 10 μg/ml could not inhibit the production of TNF-α production (Fig-IIo, IIp, IIq) but 13cis-Retinoic acid could inhibit the TNF-α production dose dependently. ((Fig-IIr, Fig-IIs and IIt).

Fig-III- Effect of natural products on the serum nitrite levels in the *in vivo* stimulated animals



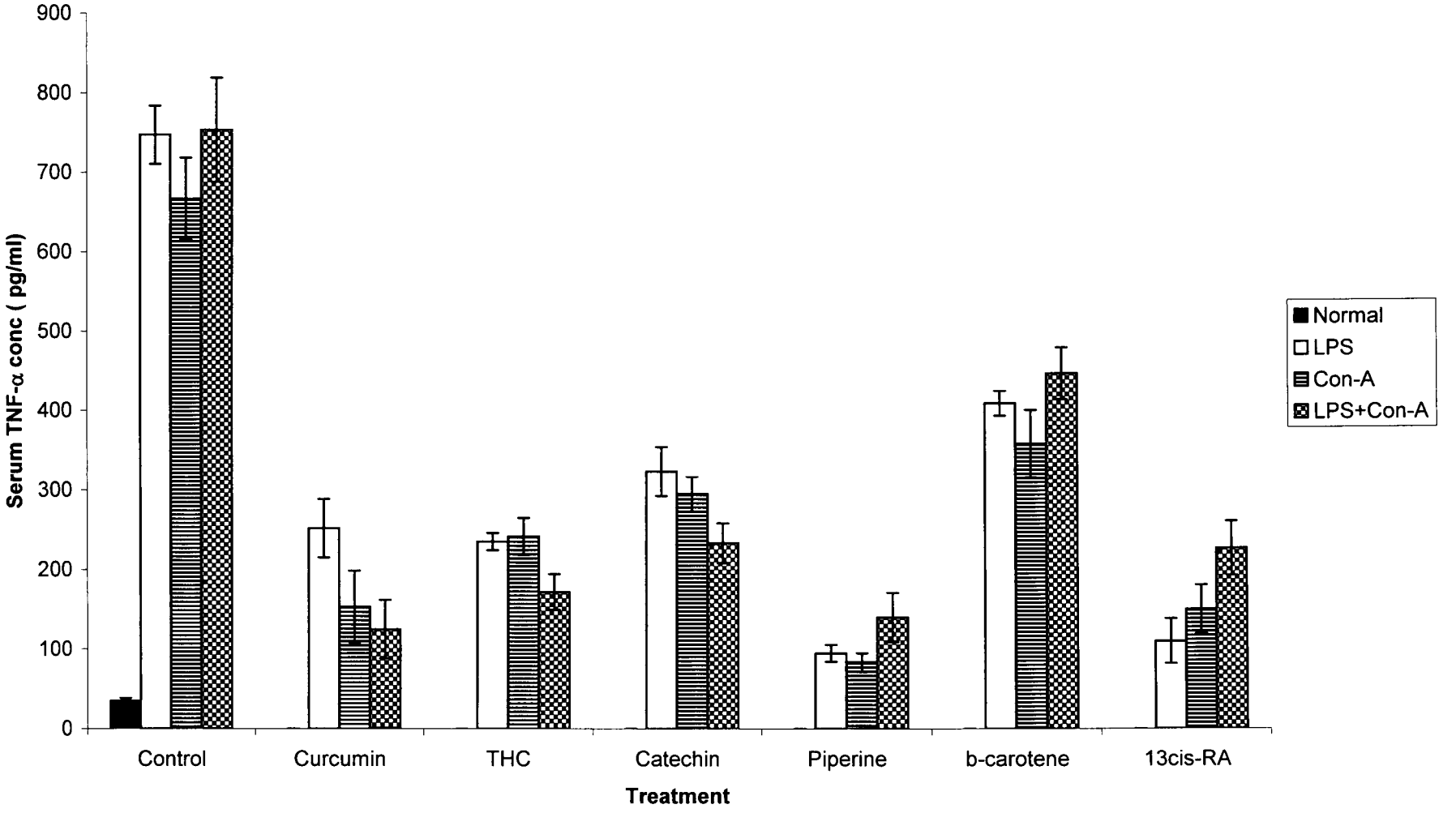
Effect of natural products on the serum nitrite of in vivo stimulated animals

Effect of natural products on the serum nitrite level of the LPS, Con-A and LPS+Con-A treated animals are shown in Fig-III. LPS (103.66±5.79 μM of nitrite), Con-A (116.67±14.34 μM of nitrite) and LPS+Con-A (126.67±6.24 μM of nitrite) treated animals were produced very high concentrations of serum nitrite levels positively. Curcumin could inhibit the production of serum nitrite of LPS, Con-A and LPS+Con-A stimulated animals to 25.33±4.50 μM of nitrite, 30.67±4.5 μM of nitrite and 42.67μM of nitrite respectively. Tetrahydrocurcumin treatment could inhibit the production of the serum nitrite of LPS, Con-A and LPS+Con-A stimulated animals to 31.33±3.63 μM of nitrite, 36.33±4.50 μM of nitrite and 47.67±3.68 μM of nitrite respectively. Similarly catechin also inhibited the production of serum nitrite of the stimulated animals to 53.67±7.93 μM of nitrite, 62±1.63μM of nitrite and 67.67±4.5 μM of nitrite respectively. β-carotene could not inhibit the serum nitrite level in an effective manner, but 13cis-Retinoic acid could inhibit the serum nitrite of the LPS, Con-A and LPS+Con-A treated animals in a dose dependent manner.

Effect of natural products on the serum TNF-α production of *in vivo* stimulated animals

Effect of natural products on the serum TNF-α level in the LPS, Con-A and LPS+Con-A stimulated animals is shown in Fig-IV. LPS, Con-A and LPS+Con-A treated animals produced very high amount of TNF-α such as 747±36.74ρg/ml, 666.67±51.37ρg/ml and 753±65.36 ρg/ml, respectively. Curcumin treatment could inhibit the TNF-α level of LPS, Con-A and LPS+Con-A stimulated animals to 251.67±36.59 ρg/ml,, 153.33±44.97 ρg/ml, and 125±36.74 ρg/ml respectively

Fig-IV. Effect of natural produts on the serum TNF-α levels of the *in vivo* stimulated animals



by the same mode of treatment. Tetrahydrocurcumin (THC) also inhibited the serum TNF-α production. Catechin could inhibit the serum TNF-α in LPS, Con-A and LPS+Con-A stimulated animals to 323.33±30.41 ρg/ml,, 295±21.60 ρg/ml and 233.33±24.97 ρg/ml respectively. Piperine treatment also inhibited the production of TNF-α to 95±10.80 ρg/ml, 83.33±11.79 ρg/ml and 140±30.82 ρg/ml respectively. β-carotene could not inhibit the production of TNF-α effectively compared to other compounds but 13cis-Retinoic acid could inhibit the TNF-α production of LPS, Con-A and LPS+Con-A stimulated animals to 110±28.14 ρg/ml, 150±30.33 ρg/ml and 226.67±33.79 ρg/ml respectively.

Discussion

NO is a highly reactive molecule and participate in the non-specific defence mechanism of the immune system even though elevated level of NO are thought to play a role in the tissue damage in inflammatory diseases (Gupta, 1998; Nussler and Billiar, 1993). NO has the potential to interact with oxygen, metals and other free radicals. NO can also form peroxynitrite ($ONOO^-$) and dinitrogen trioxide (N_2O_3) following an interaction with the superoxide radicals and oxygen respectively (Moncanda, Palmer and Higgs, 1991). NO overproduction plays a major role in cancer metastasis because its toxic nature may rupture the cancerous membrane surrounding certain types of cancer, especially those of the gastrointestinal tract. In addition NO plays a major role in tumour angiogenesis, tumour progression and liver sepsis (Rialis, 2002).

TNF-α is an important cytokine because it is fairly unique among cytokines in causing severe toxicity, at higher concentrations leading to death. Homologous TNF causes hypotension, acidosis, oligourea, transient hyperglycemia, acute renal tubular necrosis etc. Similar pathology has been seen

in various mammals from mice to humans (Vasali, 1992). TNF-α can produce various immune responses such as the release of soluble mediators like prostaglandin E_2 (PGE_2), leukotrienes, platelet activating factor, NO and reactive oxygen intermediates (Foote, Chang and Denny,1970). All these characters may participate in the TNF-toxicity.

Pharmacological modulation or natural products that could inhibit NO and TNF-α will have great medical interest. In this study we found that natural products such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, β-carotene and 13cis-Retinoic acid could inhibit the nitrite and TNF-α production *in vitro* as well as *in vivo* models which may be useful for the treatment of inflammatory diseases including cancer.

Chapter-4

Effect of some natural products on the inhibition of metastasis of B16F-10 melanoma cells - *in vivo* as well as *in vitro* analysis

Introduction

The major determinant of morbidity and mortality for numerous patients already diagnosed with primary, solid, malignant tumours is related to failure to effectively eliminate or control the emergence, expansion and progression of established metastatic tumour colonies. Therapeutic treatment modalities of chemotherapy, radiotherapy and surgery while effective for some patients with solid malignancies can not be successfully employed for many other patients who expire from the direct or indirect effects of the progressive outgrowth of metastatic foci or from the adverse and toxic effects associated with some standard therapeutic modalities (Goldfarb & Brunson, 1992). There is a cascade of events leading to the metastasis of tumours. These include the separation of tumour cells from the primary site, circulation through the blood or lymph, adhesion to the basement membrane, invasion and proliferation to distant sites (Poste, 1986; Albino et al., 1997).

It is necessary for the tumour cells to attach to the extracellular matrix, which is a mechanical barrier in the process of metastasis. Adhesion of tumour cell-extracellular matrix mediated by specific cell surface molecules present on the surface of the tumour cells (Natali et al, 1991) . It is recognized that tumour invasion, the destructive penetration of surrounding tissue by malignant cells, is an important facet of the metastatic spread of tumour cells. Investigation of the biochemical mechanisms involved in tumour invasion and degradation of host extracellular matrices have documented a crucial role for proteolytic enzymes

(Liotta et al ., 1991). Matrix metalloproteinases (MMPs) are a family of secreted or transmembrane proteins that are capable of digesting extracellular matrix and basement membrane components. Several experimental observations suggests the existence of a direct association between MMPs and neoplastic progression that probably is related to both the capacity of breaking down the physical barrier of extracellular matrix and regulating angiogenesis through the modulation of growth factors and cytokines stored in extracellular matrix (Chambers & Matrisian, 1997; Sato et al .,1999). It appears therefore that agents directed towards the inhibition of proteolytic enzymes in inhibition of tumour invasion and inhibition of further proliferation of tumour cells, have some potential for the inhibition of metastatic spread and tumour angiogenesis.

Antitumourigenic activity of several natural products were reported from our lab (Soudamini and Kuttan, 1989). We previously reported the antimetastatic effect of curcumin and catechin on B16F-10 melanoma cells by the inhibition of matrix metalloproteinase invasion (Menon , Kuttan and Kuttan, 1999). The use of natural products against metastasis is less toxic and more effective. Here in this chapter the antimetastatic activity of some of the natural products such as Tetrahydrocurcumin, Piperine, 13cis-Retinoic acid and β-Carotene were studied using *in vitro* as well as *in vivo* models.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6-8 week old) were used for this study.

Cell line

B16F-10 melanoma cells were used to induce metastasis

Compounds studied

Tetrahydrocurcumin, Piperine, 13cis-Retinoic acid and β-Carotene were used for this study.

***IN VIVO* ANTIMETASTATIC STUDIES**

Drug administration

Tetrahydrocurcumin, Piperine, 13cis-Retinoic acid and β-Carotene were administered intraperitoneally at a dosage of 200 μmoles/ kg body weight/ dose/animal simultaneously with metastatic tumour cells from the same day of tumour inoculation. The drugs were continued for 10 consecutive days.

The animals were induced metastasis by injecting B16F10 melanoma cells (10^6 cells/animal) via lateral caudal vein (Liotta, 1986). Animals were divided into 5 groups, which contain 6 animals/group. The Ist, II^{nd}, III^{rd}, IV^{th} and V^{th} groups were treated with Piperine, Tetrahydrocurcumin, 13cis-Retinoic acid and β-Carotene respectively. Another group of animals were kept as control without any drug treatment. Animals were sacrificed on the 21^{st} day after tumour induction, lungs were excised and the number of lung tumour colonies were counted. Lungs were used to estimate lung collagen hydroxylproline content (Bergman and Loxely, 1970), Uronic acid levels (Bitter and Mair, 1962), hexosamine content (Elson and Morgan, 1993) as described in Chapter-2.

Histopathological analysis was carried out by fixing the lung tissues of both control and treated tumour-bearing animals in 10% formaldehyde. After

several treatments in different concentrations of alcohol, the dehydrated tissue was embedded in paraffin wax. Sections were then cut (4μm) and stained with eosin and haematoxylin.

Blood was collected by heart puncture, serum separated and used to estimate serum sialic acid (Skoza and Mohos, 1976) and γ-glutamyl transpeptidase (GGT) levels by ρ-nitroaniline method (Tate and Meister, 1974) (Chapter-2).

Determination of the effect of natural products on the survival rate of metastatic tumour bearing animals.

Three groups of C57BL/6 mice were divided into six sub-groups each (6nos/sub-group) and a similar set of experiments was performed in order to determine the effect of these compounds on the survival rate of metastatic tumour bearing animals. The mortality of the animals was observed and the percentage increase in life span was calculated. (Chapter- 2)

***IN VITRO* ANTIMETASTATIC STUDIES**

Determination of the effect of natural products on the growth of B16F-10 melanoma cells

B16F-10 melanoma cells (5000 cells/ well) plated in a 96-well flat bottom titre plate were incubated at 37^0C in 5% CO_2 atmosphere. After 24h, various concentrations (5μg/ml-100 μg/ml) of natural products were added and the incubation was continued for 48h under the same conditions. Cell viability was determined by MTT assay as described in Chapter-2.

Tumour cell adhesion assay

Tumour 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 natural products and incubated at 37^0c for 5 hrs. Tetrahydrocurcumin and Piperine were used at concentrations of 2.5 μg/ml, 5 μg/ml and10 μg/ml where as 13cis-Retinoic acid and β-Carotene were used at concentrations of 5 μg/ml, 10 μg/ml and 25 μg/ml. 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, 1987). Briefly, the lower compartment of the chamber was filled with DMEM and a type-I collagen-coated poly carbonate filter membrane was placed on it. B16F-10 cells were then seeded on to the upper chamber.

To test the effect of natural products on the invasion of B16F10 melanoma cells, the compounds were added along with the cells to the upper chamber at non-toxic concentrations. Tetrahydrocurcumin and Piperine were used at nontoxic concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml where as 13cis-Retinoic acid and β-Carotene were used at concentrations of 5 μg/ml, 10 μg/ml and 25 μg/ml which were nontoxic. All experiments were performed in triplicate and the results are expressed as percentage inhibition of invasion.

Tumour cell motility assay

Tumour cell motility assay was performed in the same manner as the invasion assay except that polycarbonate filters were collagen free. Natural products (2.5 μg/ml – 25 μg/ml) were added along with B16F-10 melanoma cells to the upper compartment of the Boyden chamber. Tetrahydrocurcumin and Piperine were used at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml where as 13cis-Retinoic acid and β-Carotene were used at concentrations of 5 μg/ml, 10 μg/ml and 25 μg/ml. 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 Billings et al (Billings et al, 1991) with some modifications. B16F-10 cells were treated with these compounds (2.5 μg/ml – 25 μg/ml) for 24h at 37°C in the presence of 5% CO_2. Tetrahydrocurcumin and Piperine were used at concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml where as 13cis-Retinoic acid and β-Carotene were used at concentrations of 5 μg/ml, 10 μg/ml and 25 μg/ml. Cells were washed with PBS and then incubated for 24h at 37°C in serum free DMEM. Supernatants were collected and subjected to zymographic analysis with or with out trypsin activation. For this, 5 μl of trypsin solution (75 μg/ml) in 0.1 M Tris-HCl, 10 mM $CaCl_2$ buffer (p^H 8.0) were added to 100 μl of all cell lysate and incubated for 1h at room temperature. Samples were mixed with an equal volume of 2X sample buffer and loaded onto 11% polyacrylamide gels containing 0.8% gelatin

Table-1

Effect of natural products on the lung tumour colony formation

Treatment	No.of Colonies	% inhibition	% increase in life span (ILS)
Control	250	-	-
Piperine	13 ± 4.53*	95.2	190.32
Tetrahydrocurcumin	97.5 ± 7.08*	61	110.22
β-carotene	71.58±12.32*	71.36	122.58
13cis-Retinoic acid	67.5 ± 4.78*	73	118.55

B16F-10 melanoma cells ($1x10^6$) were injected to each animal via lateral tail vein. Different compounds were administered intraperitoneally for 10 consecutive days starting simultaneously with the tumour induction. Animals were sacrificed after 21 days, lungs excised and lung tumour nodules counted and another group of animals were observed for the percentage increase in life span. Values are expressed as Mean±S.D. Values are statistically significant from the untreated control. *$P<0.001$.

(porcine skin , Sigma chemicals). Electrophoresis was carried out a 10 ° C with a constant current of 2mA/ tube until the bromophenol blue reached the periphery . The gels were then washed (30 min each, three times) on a shaker with 2% Triton X-100 in 0.1M Tris-HCl, 10mM $CaCl_2$ (p^H 7.8) and incubated in 0.1M Tris-HCl, 10mM $CaCl_2$ (p^H 7.8 at 37°C for 18h followed by staining with Coomassie blue stain visualized the clear area against the dark background. The experiments were repeated three times.

RESULT

***IN VIVO* ANTIMETASTATIC STUDIES**

Effect of natural products on the lung tumour nodule formation

Metastatic tumour bearing animals treated with Piperine, Tetrahydrocurcumin, 13cis-Retinoic acid and β-Carotene showed significant reduction in tumour nodule formation. (Table-1). Untreated control animals developed a massive number of tumour nodules on the lungs and were assigned an arbitrary number of 250 (Hill, 1994). Administration of different natural products produced significant inhibition of tumour nodules in Piperine(95.2%) and Tetrahydrocurcumin (61%), 13cis-retinoic acid (73 %) and β-carotene (71.36 %) treated animals.

Table-2

Effect of natural products on lung collagen hydroxyproline, uronic acid and hexosamine contents of metastatic animals

Treatment	Lung collagen hydroxyproline µg/mg protein	Lung uronic acid content µg/100mg wet weight	Lung Hexosamine content (mg/100g dry weight)
Normal	0.95±0.05	32±1.81	0.4±0.02
Control	22.37±1.01	355.83±29.92	4.2±0.4
Piperine	2.59±0.62*	62.5±4.7*	0.98±0.14*
Tetrahydrocurcumin	10.67±1.3*	172.25±4.7*	2.05±0.14*
β-carotene	4.19±1.56*	87.87±14.27*	1.58±0.16*
13cis-Retinoic acid	8.57±0.44*	169.83±1.86*	1.65±0.13*

B16F-10 melanoma cells ($1x10^6$) were injected through lateral tail vein. Different natural products were administered as i.p for 10 consecutive days by simultaneous mode of administration. Animals were sacrificed after 21 days and lungs were excised. Values are expressed as Mean±S.D. Values are statistically significant from the untreated control.*$P<0.001$

Effect of natural products on the increase in the life span of metastatic tumour bearing animals

There was a significant increase in the life span of Piperine (190.32%), β-Carotene (122.58%), Tetrahydrocurcumin (110.22%) and 13cis-Retinoic acid (118.55%) treated animals compared to tumour bearing control animals (Table-1)

Effect of natural products on the lung collagen hydroxyproline content of metastatic tumour bearing animals.

The effect of natural products on the lung collagen hydroxyproline content is shown in Table-2. Untreated tumour bearing animals showed an elevated level of lung collagen hydroxy proline (22.37±1.01 μg/mg protein). Among the four compounds analyzed, the treatment of Piperine(2.59±0.62 μg/mg protein) and β-Carotene (4.19±1.56 μg/mg protein) treated groups had the lowest level of hydroxy proline content compared to Tetrahydrocurcumin (10.67±1.3 μg/mg protein), 13cis-Retinoic acid (8.57±0.44 μg/mg protein)

Effect of natural products on lung uronic acid levels in metastatic tumour bearing animals.

The effect of Tetrahydrocurcumin, Piperine, β-Carotene and 13cis-Retinoic acid on the lung uronic acid level is shown in Table-2. In untreated control animals, the lung uronic acid level was drastically elevated (355.83±29.92 μg/100mg wet wt) which was significantly reduced after the simultaneous administration of Piperine (62.5±4.7 μg/100mg wet wt), β-Carotene (87.87±14.27

Table-3

Effect of natural products on serum sialic acid acid and serum GGT levels of metastatic animals

Treatment	Serum sialic acid μg/ml serum	Serum GGT n mole ρ-nitroaniline/ ml serum
Normal	32±1.81	23.5±1.25
Control	111.68±9.8	103.72±1.9
Piperine	26.95±8.43**	28.8±11.4**
Tetrahydrocurcumin	58.83±8.3*	46.85±5.21*
β-carotene	34.93±4.67*	44.65±12.63**
13cis-retinoic acid	169.83±1.86*	52.21±5.22*

B16F-10 melanoma cells ($1x10^6$) were injected through lateral tail vein. Different natural products were administered as i.p for 10 consecutive days simultaneous by with the tumour cells. Animals were sacrificed after 21 days. Blood was collected by heart puncture and serum was separated. Values are expressed as Mean±S.D. Values are statistically significant from the untreated control.*P<0.001,**P<0.005.

μg/100mg wet wt), Tetrahydrocurcumin (172.25±4.7 μg/100mg wet wt) and 13cis-Retinoic acid (169.83±1.86 μg/100mg wet wt).

Effect of natural products on lung hexosamine contents in metastatic tumour bearing animals.

Lung hexosamine content of the lungs of the natural products treated animals is shown in Table–2. In tumour bearing control animals, hexosamine content was 4.2±0.4 mg/100mg dry weight. Treatment with Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid significantly reduced the hexosamine contents to 0.98±0.14mg/100mg dry weight, 2.05±0.14 mg/100mg dry weight, 1.58±0.16mg/100mg dry weight and 1.65±0.13 mg/100mg dry weight respectively.

Effect of natural products on serum sialic acid levels in metastatic tumour bearing animals

The effect of some natural products such as Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid on serum sialic acid levels is shown in Table-3. The elevated level of serum sialic in the tumour bearing control animals (111.68±9.8μg/ml) compared to normal animals (32±1.81 μg/ml) was significantly reduced by the simultaneous administration of Piperine (26.95±8.43μg/ml), β-Carotene (34.93±4.67 μg/ml), Tetrahydrocurcumin (58.83±8.3 μg/ml) and 13cis-Retinoic acid (169.83±1.86 μg/ml).

Fig-I. Effect of natural products on the Histopathology of metastatic animals after H&E staining.

a) Untreated control

b) Piperine treated

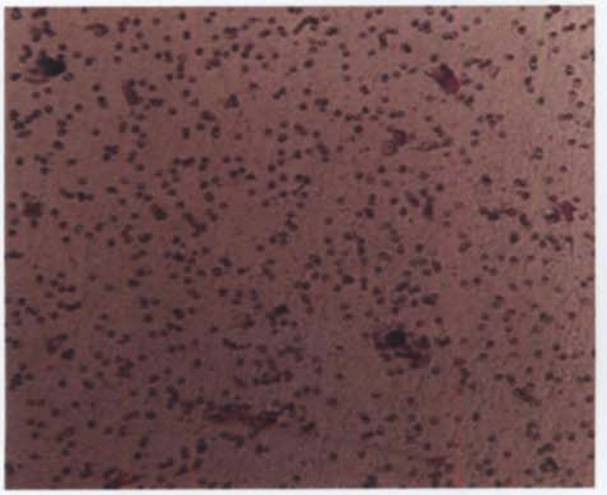
c) B-Carotene

d) Tetrahydrocurcumin

e) 13cis-Retinoic acid

f) Normal

g)



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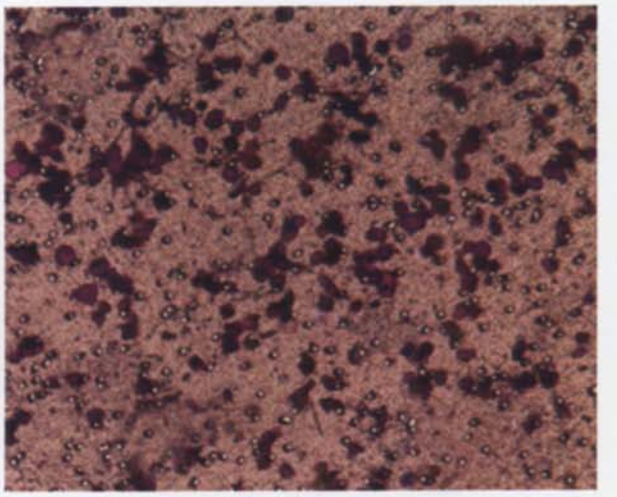

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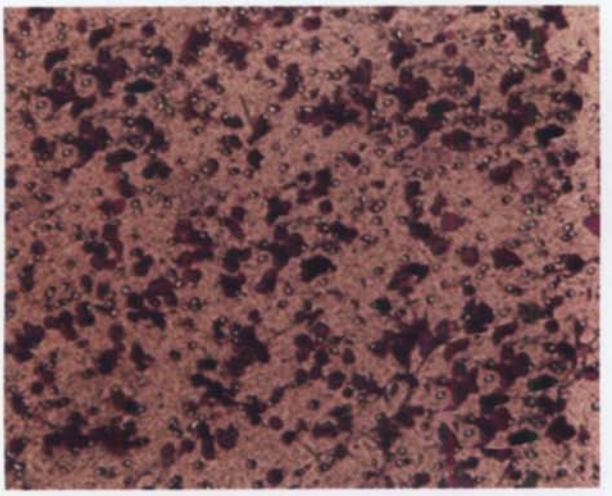

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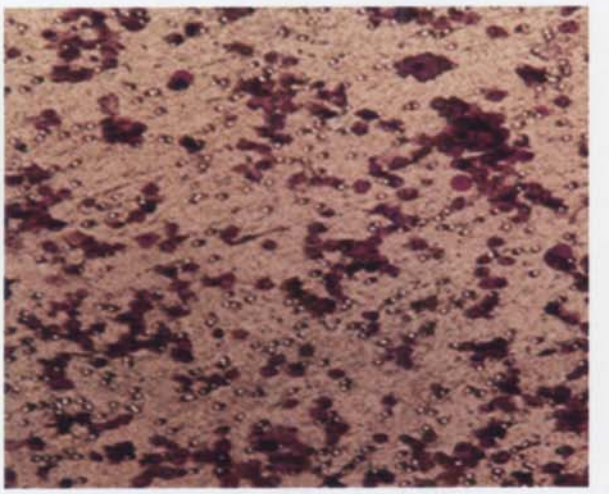

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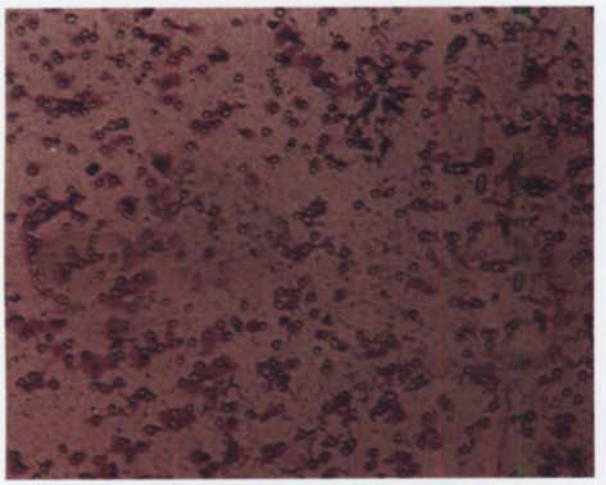

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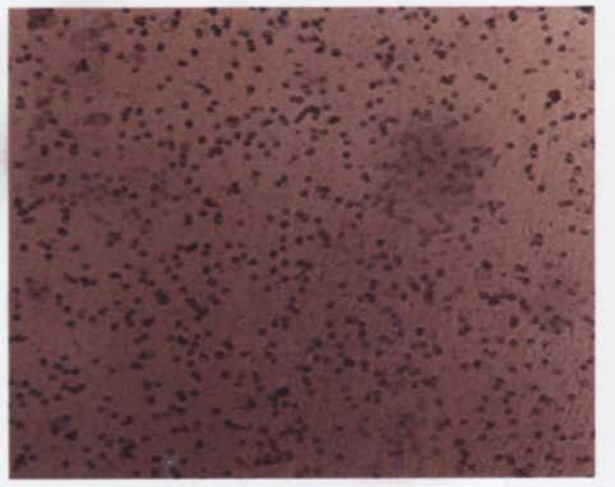


Effect of natural products on serum GGT levels in metastatic tumour bearing animals

Serum GGT levels of the compounds such as Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid is shown in Table-3. In control animals GGT level was highly elevated (103.72±1.9 ηmole/ml) compared to normal animals (23.5±1.25 ηmole/ml). Treatment with Piperine, β-Carotene, Tetrahydrocurcumin and 13cis-Retinoic acid could reduce the GGT levels to 28.8±11.4 ηmoles/ml, 44.65±12.63 ηmoles/ml, 46.85±5.21 ηmoles/ml and 52.21±5.22 ηmoles/ml respectively.

Effect of natural products on the histopathology of the lungs of metastatic tumour bearing animals.

Lungs of both treated and control animals were subjected to histopahological analysis. Control animals (Fig .Ia) showed prominent tumour nodules around terminal bronchiole. These tumour nodules composed of polygonal tumour cells which posses prominent nucleolus. Intracellular melanin deposition and clear area of necrosis were also present. Alveolar passage could not distinguish due to the massive infiltration of neoplastic cells and this infiltration extending up to the pleura. Metastatic tumour bearing animals treated with Piperine (Fig.Ib), β-Carotene (Fig Ic), Tetrahydrocurcumin (Fig.Id) and 13cis-Retinoic acid (Fig.Ie) showed prominent reduction in tumour mass. Alveoli and bronchioles were relatively tumour free, alveolar passage lined with healthy ciliated columnar epithelial cells as similar to normal lung (Fig. If).

Fig-II. Effect of natural products on the growth of B16F-10 cells

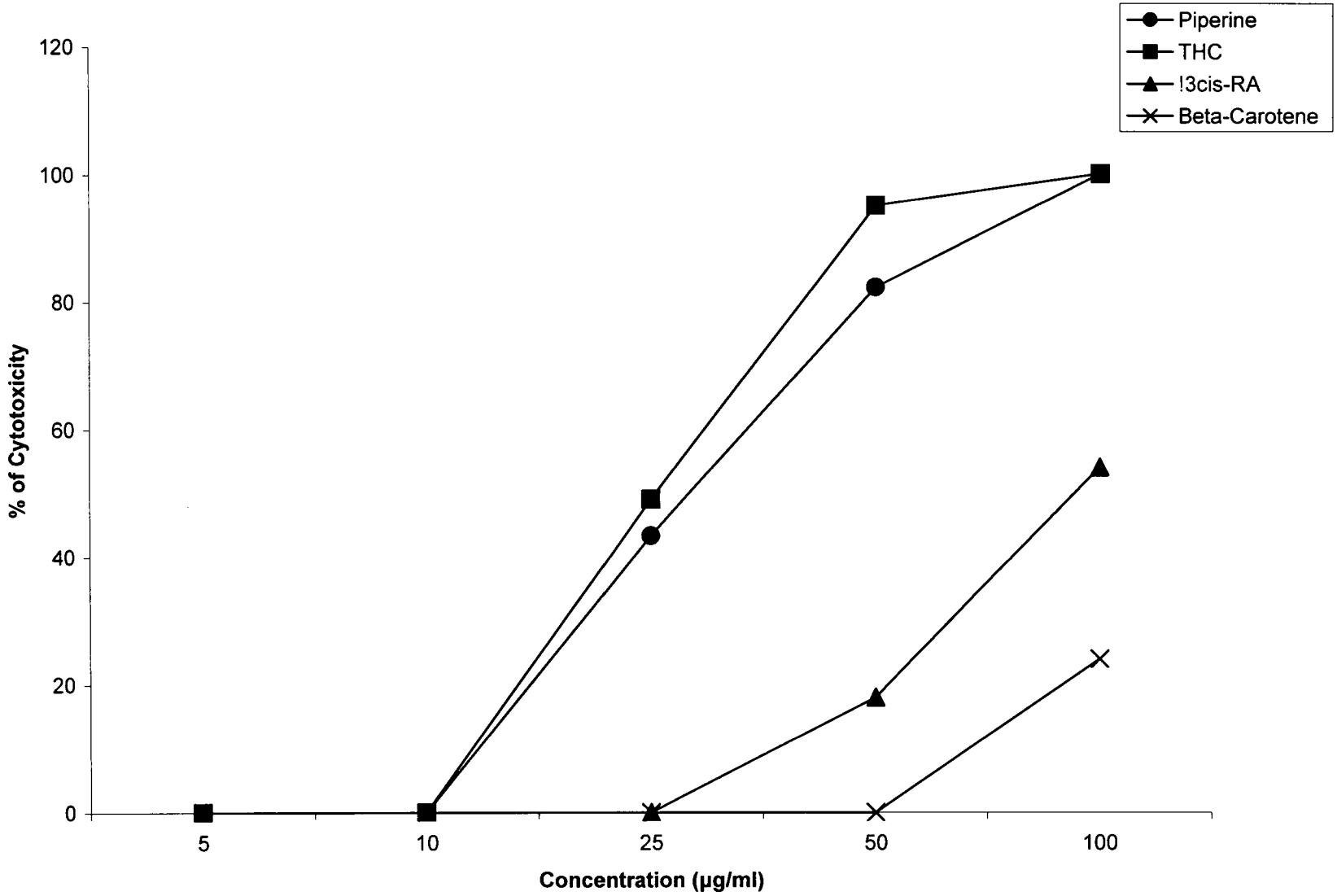


Table-4

Effect of natural products on the Adhesion of B16F-10 melanoma cells to the collagen matrix

Treatment	% inhibition of Adhesion			
	25 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml
Piperine	ΨToxic concentration	26.4%	4.71%	19.52%
Tetrahydrocurcumin	ΨToxic concentration	19.33%	0.93%	No-Inhibition
β-Carotene	78.27%	61.31%	58.76%	Not-Done¶
13cis-retinoic acid	64.61%	18.86%	3.31%	Not-Done¶

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 natural products for 4h at 37^0C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.

¶ Lower concentrations of 13cis-Retinoic acid and β-Carotene did not subject to the experimental performance.

ΨToxic concentrations were also did not subject to experimental performance.

Table-5

Effect of natural products on the Invasion of B16F-10 melanoma cells through the collagen matrix

Treatment	% inhibition of Invasion			
	25 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml
Piperine	ΨToxic concentration	86.5%	82.36%	69.03%
Tetrahydrocurcumin	ΨToxic concentration	70.61%	50.04%	31.99%
β-carotene	39.97%	26.16%	14.56%	Not-Done¶
13cis-retinoic acid	68.91%	51.60%	45.69%	Not-Done¶

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 natural products for 10h at 37^0C. Filters were removed, fixed, stained and the cells that had migrated in the test and control were counted.

¶ Lower concentrations of 13cis-Retinoic acid and β-Carotene did not subject to the experimental performance.

ΨToxic concentrations were also did not subject to experimental performance.

IN VITRO ANTIMETASTATIC STUDIES

In vitro cytotoxicity of natural products towards B16F-10 melanoma cells

Effect of different natural products such as Piperine, Tetrahydrocurcumin, 13cis-Retinoic acid and β-Carotene towards B16F-10 melanoma cells is shown in Fig-II. Piperine and Tetrahydrocurcumin are nontoxic upto 10 μg/ml. Piperine and Tetrahydrocurcumin could produce 100% cytotoxicity at a concentration of 100 μg/ml. 13cis-Retinoic acid and β-Carotene produced very less toxicity even at 100 μg/ml compared to other compounds.

Effect of natural products on the adhesion of B16F10 melanoma cells to the collagen matrix

The effect of natural products on the adhesion of B16F10 cells to collagen matrix is given in Table-4. Piperine and Tetrahydrocurcumin did not inhibit the adhesion of B16F-10 cells very much. But β-Carotene and 13cis-Retinoic acid were inhibited the tumour cell adhesion in a dose dependent manner. β-Carotene at concentrations of 5 μg/ml and 10 μg/ml, 25 μg/ml inhibited the adhesion by 58.76%, 61.31% and 78.27% respectively. 13cis-Retinoic acid at concentrations of 5 μg/ml and 10 μg/ml, 25 μg/ml inhibited the adhesion of B16F-10 melanoma cells 3.31%, 18.86% and 64.61% respectively.

Effect of natural products on the invasion of B16F-10 melanoma cells

Effect of natural products on the invasion of B16F-10 melanoma cells is shown in Table-5 and Fig-III. All the compounds such as Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid could inhibit the invasion of B16F-10 cells in a dose dependant manner. Piperine at a

Fig-III. Effect of natural products on the invasion of B16F-10 melanoma cells

a) Untreated control

b) Piperine treated (2.5 μg/ml)

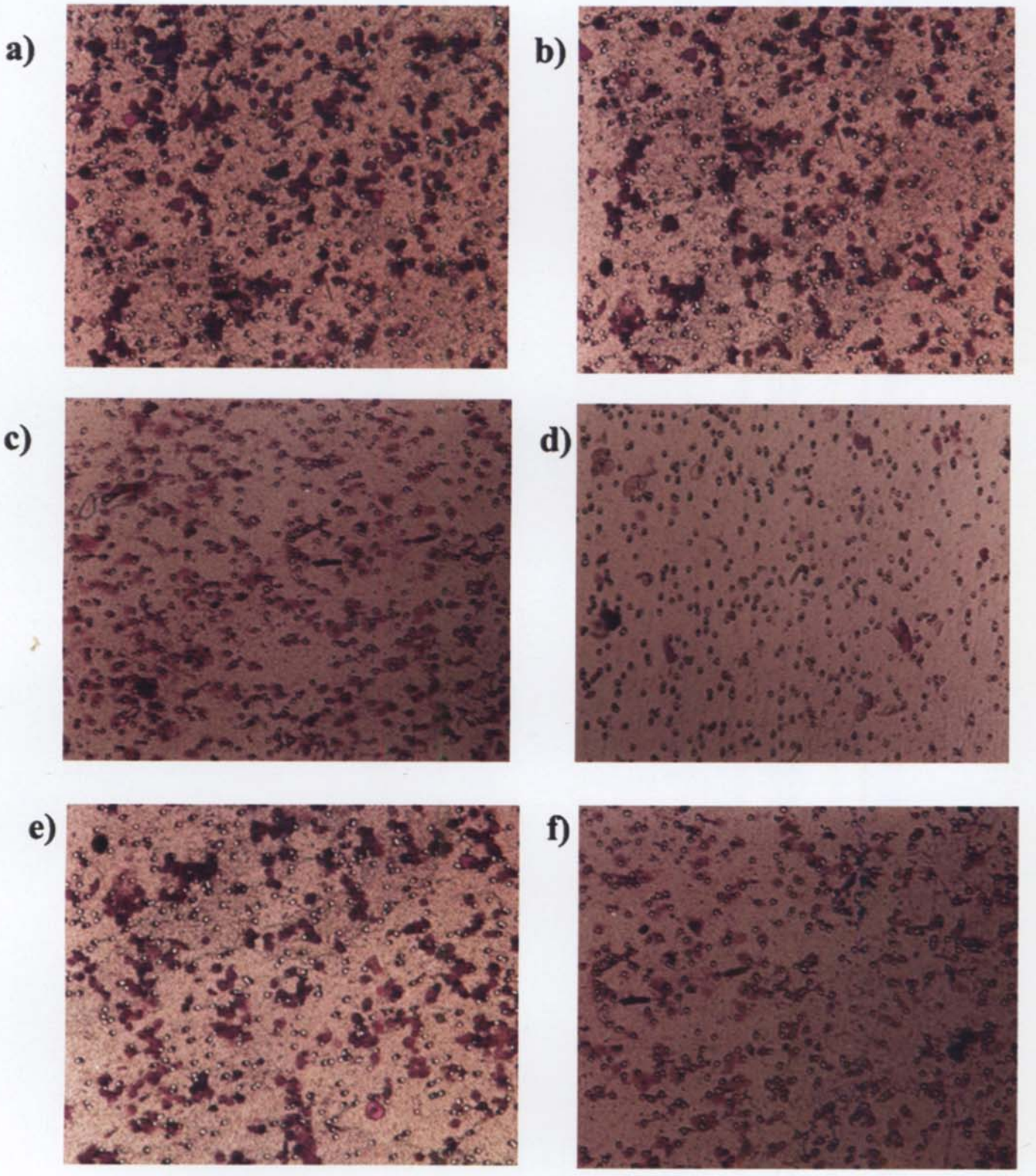
c) Piperine treated (5 μg/ml

d) Piperine treated (10 μg/ml

e) Tetrahydrocurcumin treated (2.5 μg/ml)

f) Tetrahydrocurcumin treated (5 μg/ml

Fig-III. Effect of natural products on the collagen matrix invasion of B16F-10 melanoma cells



g) Tetrahydrocurcumin treated (10 μg/ml)

h) β-Carotene treated (5 μg/ml)

i) β-Carotene treated (10 μg/ml)

j) β-Carotene treated (25 μg/ml)

k) 13cis-Retinoic acid (10 μg/ml)

l) 13cis-Retinoic acid (25 μg/ml)

Fig-I Effect of natural products on the histopathology of the lungs of metastatic animals after H&E staining

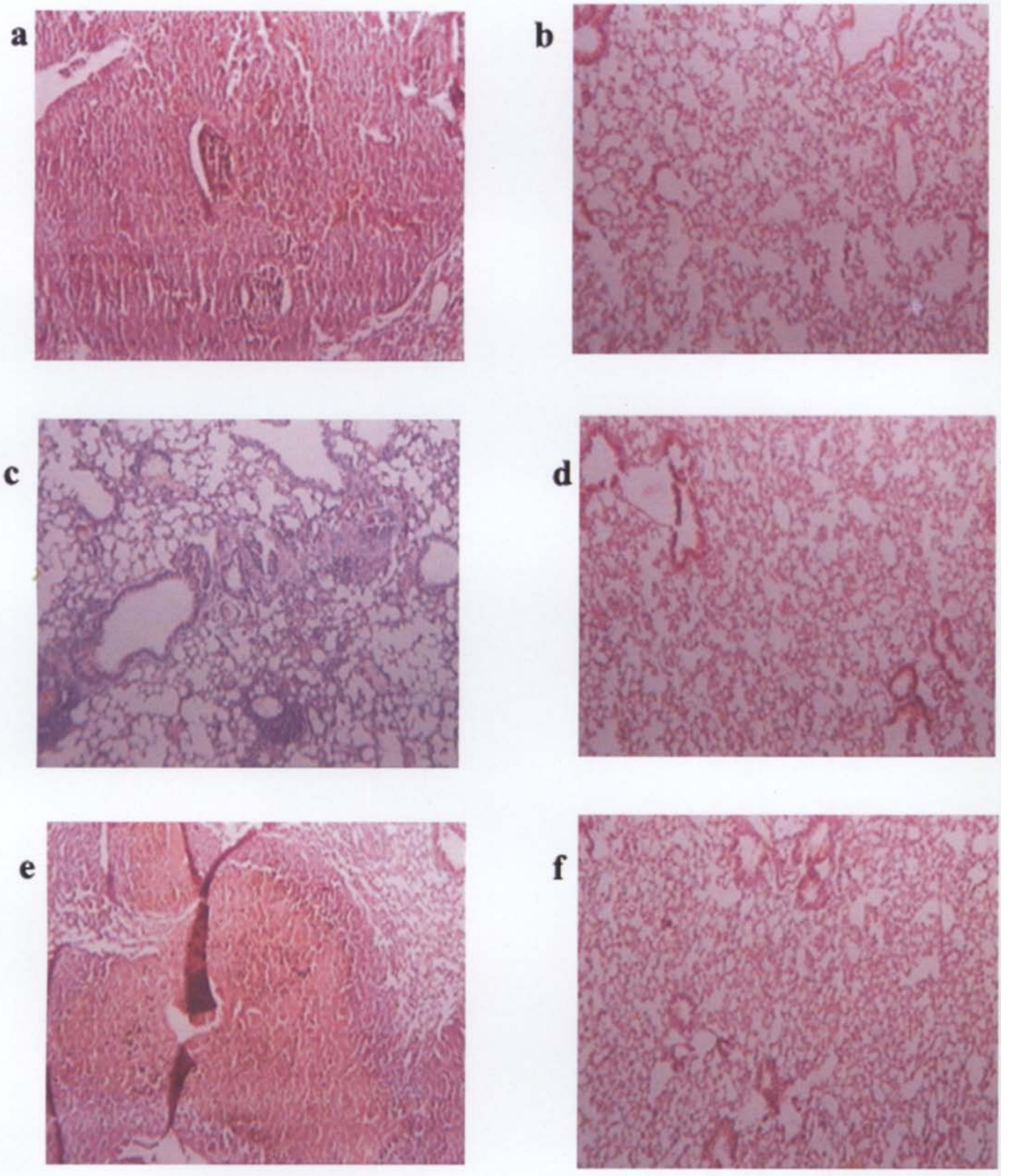


Table-6

Effect of natural products on the migration of B16F-10 melanoma cells through the polycarbonate membrane

Treatment	% inhibition of Motility			
	25 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml
Piperine	[Ψ] Toxic concentration	20%	16%	8%
Tetrahydrocurcumin	[Ψ] Toxic concentration	27.99%	16%	12%
β-carotene	32%	27.99%	24%	Not-Done[¶]
13cis-retinoic acid	44%	36%	27.99%	Not-Done[¶]

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.

[¶] Lower concentrations of 13cis-Retinoic acid and β-Carotene did not subject to the experimental performance

[Ψ]Toxic concentrations were also did not subject to experimental performance.

concentrations of 2.5 μg/ml, 5 μg/ml and 10 μg/ml reduced the invasion 69.03%, 82.36% and 86.5% respectively. At the same concentrations of Tetrahydrocurcumin inhibited the invasion at 31.99%, 50.04% and 70.61% respectively. β-Carotene at a concentrations of 5 μg/ml and 10 μg/ml, 25 μg/ml inhibited the invasion at 14.56%,26.16% and 39.97% respectively. Treatment with 13cis-Retinoic acid at the same concentrations inhibited the invasion at 45.69%, 51.60% and 68.91% respectively.

Effect of natural products on the motility of B16F10 melanoma cells

Inhibition of tumour cell motility by Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid is given in Table-6. There is no profound inhibition in the motility of B16F-10 melanoma cells with the treatment of above natural products. Even though β-Carotene and 13cis-Retinoic acid at the higher concentration of 25 μg/ml could reduce the motility 32% and 44% respectively.

Effect of natural products on the matrix metalloproteinase production

Effect of natural products on the matrix metalloproteinase production by the pretreatment is shown in Fig-IV. As shown in zymographic analysis, conditioned medium from untreated B16F-10 cells produce large amount of matrix metalloproteinase enzyme showed digested clear areas at 92KDa and 72KDa (lane-1) compare to the cells treated with natural products. Trypsin activated tumour cell culture supernatant loaded gels, after incubation with 10mM EDTA did not show clear areas which indicate that enzyme is metalloproteinase (lane-2). Conditioned medium from the piperine (2.5 μg/ml) treated B16F-10 cells gave

Fig-IV. Effect of natural products on the production of matrix metalloproteinases

Lane1, Conditioned medium from untreated control.

Lane2, Gel incubated in the presence of EDTA.

Lane3, Conditioned medium from the cells treated with Piperine (2.5 μg/ml).

Lane4, Conditioned medium from the cells treated with Piperine (5 μg/ml).

Lane5, Conditioned medium from the cells treated with Piperine (10 μg/ml).

Lane6, Conditioned medium from the cells treated with Tetrahydrocurcumin (2.5 μg/ml).

Lane7, Conditioned medium from the cells treated with Tetrahydrocurcumin (5 μg/ml).

Lane8, Conditioned medium from the cells treated with Tetrahydrocurcumin (10 μg/ml).

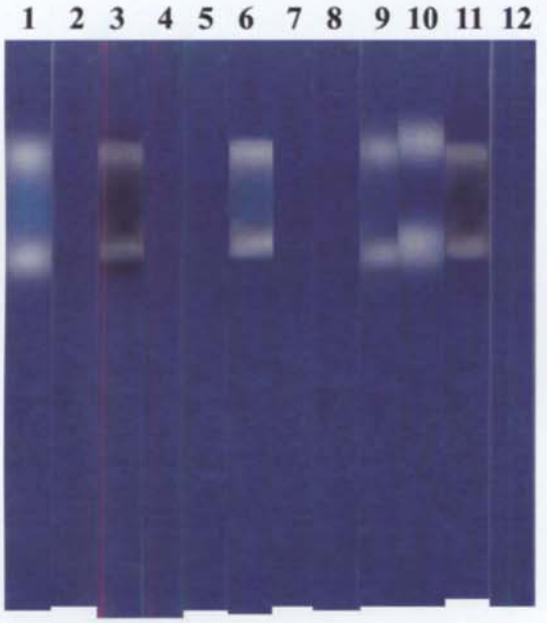
Lane9, Conditioned medium from the cells treated with β-Carotene (25 μg/ml).

Lane 10, Conditioned medium from the cells treated with 13cis-Retinoic acid (5 μg/ml).

Lane11, Conditioned medium from the cells treated with 13cis-Retinoic acid (10 μg/ml).

Lane12, Conditioned medium from the cells treated with 13cis-Retinoic acid (25 μg/ml).

Fig-IV. Effect of natural products on the production of matrix metalloproteinases



partially degraded areas (lane-3) indicating that lower dose of piperine treatment partially inhibit the production of matrix metalloproteinases. Conditioned medium from the piperine (5 μg/ml and 10 μg/ml) treated B16F-10 cells after the trypsin activation did not give any clear degraded areas (lane4and5) indicating that piperine treatment could inhibit the production of matrix metalloproteinases. Conditioned medium from the cells treated with tetrahydrocurcumin at a concentrations of 2.5 μg/ml did not inhibit the production of MMPs but 5 μg/ml and 10 μg/ml concentration's treatment could inhibit the production of MMPs (lane6-lane8). Even the higher concentration of β- Carotene (25 μg/ml) did not inhibit the production of matrix metalloproteinases and clear area of digestion were produced (lane-9). 13cis- Retinoic acid at concentrations of 5 μg/ml, 10 μg/ml could not inhibit the production of metalloproteinases but the pretreatment with 25 μg/ml concentration could inhibit the production of matrix metalloproteinases. (lane10-1ane12)

Discussion

Treatment of cancer metastasis is a major problem to the oncologists. In the majority of patients , metastasis may well have occurred by the time of diagnosis of primary malignant neoplasms (Sugar and Ketchem, 1997; Fidler, Gertsen and Hart, 1978). By the time of diagnosis, primary tumours and metastasis consists of multiple subpopulations of cells exhibiting a wide range of biological heterogeneity in such parameters as cell surface properties, antigenecity, immunogenecity , karyotype , sensitivity to various cytotoxic drugs and the ability to invade and metastasize(Fidler and Hart, 1998).

Chemotherapeutic drugs are not favorable for the treatment of malignancy due to their serious side effects such as neurotoxicity , nephrotoxicity, acute cochlear ototoxicity, peripheral neuropathy and cytopenia (Bruce, 1993). Highly malignant tumour cell subpopulations are often resistant to chemotherapeutic and cytoreductive anticancer drugs. All standard combinations for metastasis therapy has low efficacy and low response rate (Poste, 1986; Pizzo, 1984). For the successful therapy of metastasis will have to include new agents to overcome the development of resistance to therapy. So it is necessary to develop second generation of drug which are equally effective and less toxic. The use of natural products against metastasis is less toxic and more effective. Several reports on novel antimetastatic agents have been published from our lab (Menon, Kuttan and Kuttan, 1999).

In the present study we analyzed the effect of some natural products such as Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid on the inhibition of lung metastasis induced by B16F-10 melanoma cells in C57BL/6 mice as well as *in vitro* models. Administration of these compounds simultaneously with the tumour cells could inhibit the number of tumour nodules after 21 days of induction compared to the untreated control. Tumour nodules are the metastatic colonies of B16F-10 melanoma cells formed in the lungs which initiates the hung fibrosis and collagen deposition. There was also a corresponding increase in the life span of these drug treated animals.

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. 15-30 percentage of collagen is hydroxy proline (Voet and Voet, 1995) which results the reduction in pulmonary function. Administration of Piperine and β-Carotene 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 Tetrahydrocurcumin and 13cis-Retinoic acid reduced the hydroxylproline content of the lung but to a lesser extent

The oxidation of the primary alcohol group of aldoses of sugar derivatives in the tumour cells results the uronic acid production and leads to the formation of glucuronic acid lactone which is an esterified form of glucuronic acid . In the presence of glucuronic acid lactone, prolyl hydroxylase enzyme converts the prohydroxy proline to hydroxyproline thus activates the fibre formation during lung fibrosis (Voet and Voet, 1995). Hexosamine is a significant sugar derivative present in the tumour cells. It has an important role in the synthesis of N- acetyl neuraminic acid (Sialic acid), which is a component of glycolipids present on the surface of tumour cells ((Voet and Voet, 1995). Administration of natural products in simultaneous mode of administration significantly inhibited the lung uronic acid content and lung hexosamine content.

Sialic acid is a family of acylated derivatives of neuraminic acid, occurs as terminal component of carbohydrate chains of glycoproteins and glycolipids. Increased levels of β1-6 branched N - linked oligosaccharides have been observed in many metastatic tumour cells (Dennis and Laferte, 1989). The secretion of glycoproteins and gangliosides has been demonstrated in melanoma

(Sela, Iliopus, Guery, et al., 1989). Metastatic property of tumour cells highly upregulated with the elevated levels of sialic acid content (Fernandes, Sugman, Auger, et al., 1991). Gamma glutamyl transpeptidase (GGT) is a marker of cellular proliferation, was increased in the serum of untreated animals compared with its level in normal animals. Glutathione (GSH) synthesized intracellularly and present in the tumour cell. GGT catalyzes the GSH breakdown by γ-glutamyl cycle (Voet and Voet, 1995). Sialic acid and GGT level in the serum was significantly reduced by the treatment of Piperine, Tetrahydrocurcumin, 13cis-Retinoic acid and β-Carotene. Histopathological analysis also correlated with the above results.

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 . Each step in the cascade involves multiple tumour-host interactions. Interruption of the metastatic cascade at any of these steps can prevent the production of clinically symptomatic metastasis. Our results demonstrate that some of the natural products could inhibit different stages of metastasis in a dose dependent manner *in vitro*. This inhibition helps to prevent the metastasis of tumour cells directly *in vivo*. Metalloproteinase have been implicated in the denaturation of the basement membrane during the metastatic invasion of tumour cells (Tryggvason, 1987; Powel, 1996). As shown in the zymographic analysis, treatment of B16F-10 melanoma cells with Piperine, Tetrahydrocurcumin and 13cis-Retinoic acid could

inhibit the production of matrix metalloproteinases effectively. This could inhibit the invasion of metastatic cells also.

Adhesion of tumour cells on the extracellular matrix play a vital role during trans-endothelial migration of tumour cells (Patarrayo, 1989; Burrow, 1991) and have been implicated in metastasis. In this study we found that β-Carotene and 13cis-Retinoic acid could inhibit the adhesion of the metastatic of B16F10 melanoma cells which in turn inhibit the metastasis *in vivo*. Tumour cell mobility is another accelerating step of metastasis which promote the migration of cells through circulation. In this study we found that none of the above natural products significantly inhibited the motility of B16F-10 cells. In this chapter we found the antimetastatic activity of some natural products such as Piperine, Tetrahydrocurcumin, 13cis-Retinoic acid and β-Carotene *in vivo* and their related activity in the in *vitro* study using the same model of cell lines. We hope that the use of the above natural products will be more effective and less toxic.

Chapter-5

Effect of some natural products on the activation and nuclear translocation of transcription factors and proinflammatory cytokine gene expression.

Introduction

Nuclear Factor-κB (NF-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 (Karin and Delhase, 2000; Silverman and Maniatis, 2001). In mammalian cells NF-κB family is composed of five members, p50, p52, p65 (Rel A), Rel B and c-Rel. The different NF-κB members share strong homology in their N-terminal Rel homology domain (RHD) that is responsible for the DNA binding , dimerization and nuclear translocation functions (Fan and Maniatis, 1991). A typical course of NF-κB activation involves the rapid but often transient nuclear translocation of an early NF-κB species and the subsequent nuclear translocation of the late NF-κB complexes. While the early NF-κB is almost exclusively the Rel A/p50 heterodimer, late NF-κB complexes vary depending on cell types and stimuli. The most commonly detected NF-κB is c-Rel/p50 heterodimer but various other complexes such as c-Rel/p52 have also been reported (Kongten et al, 1995).

Transcription factor AP-1 consists of homodimers and heterodimers of (v-Jun, c-Jun, Jun-B and Jun-D), Fos (v-Fos,c-Fos, FosB, Fra 1and Fra 2) or activating transcription factor (ATF-2,ATF-3,B-ATF) proteins (Karin, Liu and Zandi, 1997). AP-1 and its activation have been shown to play an important role on cell proliferation (Huang, Ma and Dong, 1996). CREB is cyclic AMP response element binding protein which is a transcription factor that down stream in the protein kinase-A (PKA) signaling pathway. When PKA is activated the catalytic subunit of PKA translocates to the nucleus and phosphorylates CREB. CREB then binds to cAMP

response element in the promotors of specific genes and induce their transcription (Zhong, Voll and Ghosh, 1998; Mayo and Baldwin, 2000).

NF-κB and AP-1 have been selectively enhancing the expression of proinflammatory cytokines such as IL-1, IL-6, GM-CSF, TNF-α and IL-12p40 as well as the expression of degradative enzymes such as MMPs (Bondeson et al, 1999; Barnes and Karin, 1997; Andela et al, 2000; Siebenlist, Franzaso and Brown, 1994). There has been substantial body of data that dietary factors and wide variety of phenolic substances from spices have a profound impact on prevention as well as etiology of cancer. Some of these compounds possess antimutagenic and anticarcinogenic activities (Surh, Lee and Lee 1998; Surh, 2002). In the previous chapter we were discussed the antimetastatic effect of some natural products such as Piperine, Tetrahydrocurcumin, β-Carotene and 13cis-Retinoic acid. We have reported the antimetastatic activity of Curcumin and Catechin previously (Menon, Kuttan and Kuttan, 1999). In this chapter we are demonstrating the effect of all these compounds such as Piperine, Terahydrocurcumin, β-Carotene , 13cis-Retinoic acid, Curcumin and Catechin on the activation of transcription factors and the proinflammatory cytokine gene expression in B16F-10 melanoma models *in vitro*.

Materials and Methods

Cells

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

Compounds studied

Curcumin, Tetrahydrocurcumin, Piperine, Catechin, 13cis-Retinoic acid and β-Carotene were used in this study.

Chemicals and Kits used

EGTA, , DEPC, Aprotinin, Leupeptin, PMSF, DTT, Benzamidine ,TritonX-100 , ELISA kits of IL-1β, IL-6, GM-CSF and TNF-α , Cells to cDNA synthesis kit, Message screen mouse inflammatory cytokine multiplex PCR kit and Mercury transfactor kit were used for this study.

ELISA measurement of IL-1β, IL-6, GM-CSF and TNF-α produced by B16F-10 melanoma cells

B16F-10 melanoma cells (5 X 10^4 cells/well) plated in 96 well flat bottom plate was incubated at 37° C in 5% CO_2 atmosphere in DMEM with 10% FCS . After 24h , the cells were washed and incubated further for 24h in serum free medium in the presence of either Curcumin (10μg/ml), Tetrahydrocurcumin (10μg/ml),Piperine (10μg/ml), Catechin (10μg/ml), β-carotene (25 μg/ml) or 13cis-Retinoic acid (25 μg/ml). The culture supernatant was collected, centrifuged and concentrations of IL-1β, IL-6, GM-CSF and TNF-α were determined by quantitative ELISA according to the manufacturer's directions.

RNA isolation and RT-PCR

To determine the effect of piperine on the gene expression level of IL-1β, IL-6, GM-CSF ,TNF-α and IL-12p40, total RNA were subjected to cDNA synthesis using cells to cDNA™ II kit (Ambion Inc,USA). B16F-10 cells (2X 10^4 cells/well) were grown in 96 well titre plate in the presence of Curcumin (10μg/ml), Tetrahydrocurcumin

(10μg/ml), Piperine (10μg/ml), Catechin (10μg/ml), β-carotene (25 μg/ml) or 13cis-Retinoic acid (25 μg/ml) for 4h at 37° in 5% CO_2 in serum free medium. Total RNA was extracted from B16F-10 cells and cDNA was synthesized ,using moloney murine leukaemia 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-1β, IL-6,GM-CSF, TNF-α and IL-12p40 genes. PCR products were analysed by agarose gel electrophoresis and visualized by ethidium bromide.

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 Curcumin (10μg/ml), Tetrahydrocurcumin (10μg/ml),Piperine (10μg/ml), Catechin (10μg/ml), β-carotene (25 μg/ml) and 13cis-retinoic acid (25 μg/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 (10ρg/ml) for 30 min at 37° in 5% CO_2. Cells were washed with PBS, dislodged with a cell scraper and collected by centrifugation. 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

Table-1

Effect of natural products on the inhibition of proinflammatory cytokines

Cytokine	Control (pg/ml)	Curcumin (pg/ml)	Terahydrocurcumin (pg/ml)	Piperine (pg/ml)	Catechin (pg/ml)	Retinoic acid (pg/ml)	β-Carotene (pg/ml)
IL-1β	185±8.16	35±4.08*	34.66±4.92*	51.66±6.23*	42.5±3.94*	38±4.08*	147.33±2.62
IL-6	203±12.47	56±8.28*	73±6.68*	58.33±6.23*	98±16.87**	67±9.41*	166±11.77¶
TNF-α	191.66±13.2	60.66±4.49*	58.83±4.13*	53.66±4.92*	54±3.74*	49±2.44*	142±5.09¶
GM-CSF	96±6.97	22±3.26*	31.66±3.09*	27.33±2.49*	24±3.26*	32±2.16*	80±4.08

B16F-10 melanoma cells (5 X 10^4 cells/well) plated in 96 well flat bottom plate was incubated at 37° C in 5% CO_2 atmosphere in DMEM with 10% FCS . After 24h , the cells were washed incubated for 24h in serum free medium Curcumin (10μg/ml), Tetrahydrocurcumin (10μg/ml),Piperine (10μg/ml), Catechin (10μg/ml), β-carotene (25 μg/ml) and 13cis-retinoic acid (25 μg/ml). The culture supernatant was collected and quantitative ELISA was performed. Values are expressed as Mean ± SD. Values are statistically significant from untreated control. *P<0.001, **P<0.005, ¶ P<0.05.

will bind to their consensus sequences in the well. Bound transcription factors in the DNA were detected by specific primary antibody towards NF-κBp65, NF-κBp50, NF-κB 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).

Statistical analysis

Results were expressed as the mean± standard deviation. Statistical evaluation was done by Student's t-test and the experiments were repeated twice.

Results

Effect of natural products on the production of proinflammatory cytokines

Effect of natural products on the proinflammatory cytokines is shown in table-1. B16F-10 tumour cells produced very high quantity of proinflammatory cytokines such as IL-1β (185±8.16 pg/ml), IL-6 (203±12.47 ρg/ml), GM-CSF (96±6.97 ρg/ml) and TNF-α (196.66±13.12 ρg/ml). When the cells were treated with natural products there was a significant reduction in the cytokine level. Treatment with β-Carotene treatment did not gave much inhibition in the cytokine level compared to other compounds.

When the cells were treated with Curcumin there was an inhibition in the level of cytokines such as level as follows IL-1β (35±4.08 pg/ml), IL-6 (56±8.28 ρg/ml), GM-CSF (22±3.26 ρg/ml) and TNF-α (58.83±4.13 ρg/ml). Tetrahydrocurcumin also produced a significant inhibition in the proinflammatory cytokine level as follows

Fig-I. Effect of natural products on the proinflammatory cytokine gene expression

Lane 1, Catechin (10 μg/ml) treated.

Lane 2, 13cis-Retinoic acid (25 μg/ml) treated.

Lane 3, β-Carotene (25 μg/ml) treated.

Lane 4, Tetrahydrocurcumin (10 μg/ml) treated.

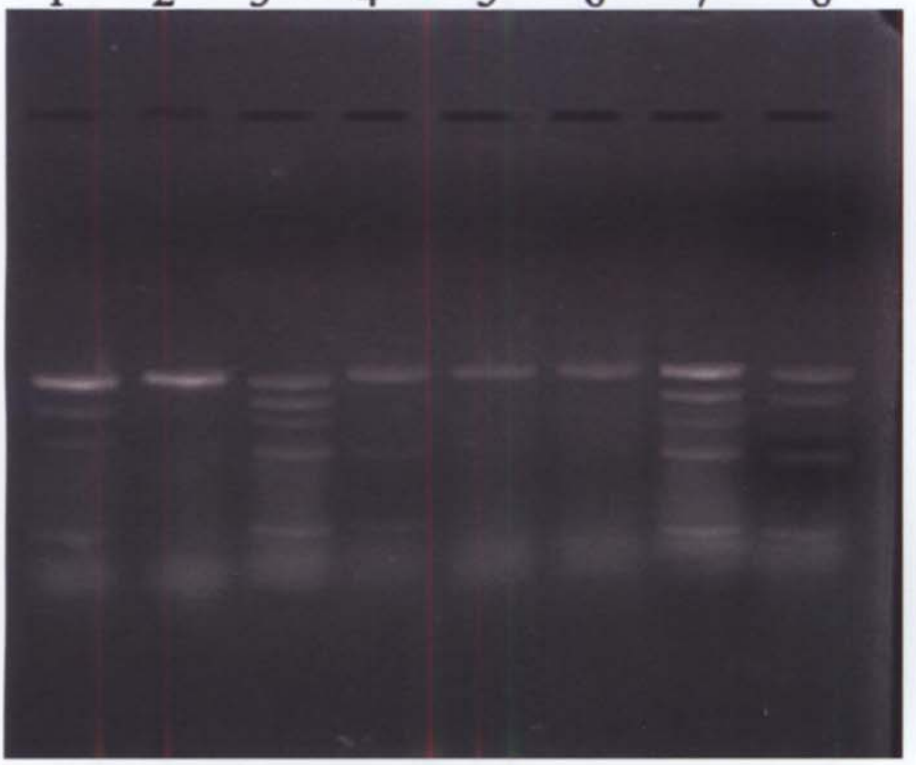
Lane 5, Curcumin (10 μg/ml) treated.

Lane 6, Piperine (10 μg/ml) treated.

Lane 7, Untreated control.

Lane 8, Positive Control.

Fig-I. Effect of natural products on the proinflammatory cytokine gene expression



GAPDH (557 bp)
IL-6 (484 bp)
IL-1β(430 bp)
GM-CSF(375 bp)
TNF-α (290 bp)
IL-12p40 (239 bp)

IL-1β (34.66±4.92 pg/ml), IL-6 (73±6.68 ρg/ml), GM-CSF (31.66±3.09 ρg/ml) and TNF-α (56.66±3.39 ρg/ml). Treatment with Piperine significantly reduced proinflammatory cytokine levels were significantly reduced to IL-1β (53.66±4.92 pg/ml), IL-6 (58.33±6.23 ρg/ml), GM-CSF (27.33±2.49 ρg/ml) and TNF-α (54±3.74 ρg/ml). Catechin also gave significant reduction in the levels of IL-1β (42.5±3.94 pg/ml), IL-6 (98±16.87 ρg/ml), GM-CSF (24±3.26 ρg/ml) and TNF-α (54±3.74 ρg/ml). 13cis-Retinoic acid inhibited the proinflammatory cytokine in a significant manner as follows IL-1β (38±4.08 pg/ml), IL-6 (67±9.41 ρg/ml), GM-CSF (32±2.16 ρg/ml) and TNF-α (49±2.44 ρg/ml). β-Carotene did not gave much inhibition in the cytokine level compared to the other compounds.

Effect of natural products on the expression of proinflammatory cytokine genes

Effect of natural products on the expression of proinflammatory cytokine genes is shown in Figure-1. B16F-10 melanoma cells have highly elevated expression of proinflammatory cytokines such as IL-1β, IL-6, GM-CSF , TNF-α, IL-12p40. In this study we found that Piperine, Curcumin, Tetra hydrocurcumin, Retinoic acid and Catechin inhibited the expression of these proinflammatory cytokines but β-carotene did not inhibit the expression of proinflammatory cytokines.

Effect of natural products on the nuclear translocation of transcription factors

Effect of natural products on the nuclear translocation of transcription factors is shown in Table-2. We analyzed the effect of those natural products those which inhibited the production and expression of proinflammatory cytokines on the nuclear translocation level of transcription factors. We expressed the activation level as optical density at 655nm and % inhibition of each transcription factor by the

Table-2
Effect of natural products on the nuclear translocation of transcription factors

Transcription Factor	Optical Density of Control	Optical Density of Curcumin (% inhibition)	Optical Density of Tetrahydrocurcumin (% inhibition)	Optical Density of Piperine (% inhibition)	Optical Density of Catechin (% inhibition)	Optical Density of Retinoic acid (% inhibition)
NF-κB p65	0.336 ±0.02	0.09 ± 0.002 (69.64%)	0.155±0.004 (53.86%)	0.102±0.01 (69.64%)	0.155±0.006 (53.86%)	0.127±0.01 (62.2%)
NF-κB p50	0.474±0.01	0.139±0.016 (69.83%)	0.091±0.008 (80.81%)	0.143±0.03 (69.83%)	0.121±0.01 (74.47%)	0.132±0.008 (72.15%)
NF-κB C Rel	0.562±0.04	0.168±0.013 (88.25%)	0.040±0.006 (92.88%)	0.066±0.001 (88.25%)	0.179±0.007 (68.14%)	0.127±0.007 (77.4%)
c-Fos	0.247±0.03	0.027±0.005 (89.06%)	0.098±0.004 (60.32%)	0.176±0.01 (28.74%)	0.149±0.006 (39.67%)	0.135±0.006 (45.34%)
ATF-2	0.316±0.01	0.073±0.009 (76.89%)	0.184±0.009 (41.77%)	0.168±0.005 (46.84%)	0.164±0.009 (48.1%)	0.140±0.005 (55.69%)
CREB	0.269±0.01	0.045±0.005 (83.27%)	0.118±0.003 (56.13%)	0.096±0.007 (64.31%)	0.154±0.006 (42.75%)	0.143±0.009 (46.84%)

B16F-10 cells were grown in 25 cm^2 culture flask and treated with natural products for 2h at 37° in 5% CO_2 Cells were washed with PBS and incubated with TNF-α (10ρg/ml) for 30 min at 37° C in 5% CO_2 . Nuclear extracts were prepared in triplicates and subjected to transfactor assay. Optical density expressed as Mean±SD and results were expressed as % inhibition in brackets.

treatment of natural products. Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid inhibited the nuclear translocation of NF-κB p65 by 69.64%, 53.86%, 69.64%, 53.85% and 62.2% respectively . Percentage inhibition in the translocation of NF-κB p50 with the treatment of Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid is 69.83%, 80.81%, 69.83%, 74.47% and 72.15% respectively. Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid inhibited the translocation of NF-κB c-Rel by 88.25%, 92.88%, 88.25%, 68.14% and 77.40% respectively. Translocation of c-Fos and ATF-2 were also inhibited by the treatment of same natural products. Percentage inhibition of c-Fos with the treatment of Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid was 89.06%, 60.32%, 28.74%, 39.67% and 45.34% respectively. Percentage inhibition of ATF-2 was 76.89%, 41.77%, 46.84%, 48.1% and 55.69% in the above order of treatments respectively. These compounds could inhibit the translocation of cyclicAMP response element binding protein (CREB) effectively.

Discussion

NF-κB proteins have been implicated as playing a role in cellular transformation by either providing continued positive growth stimuli such as that mediated by cytokines, or through inhibition of apoptotic pathways (Huang, Ma and Dong, 1996). Activated or nuclear translocated NF-κB proteins are over expressed in malignant cancers such as colorectal cancer, breast cancer, T-cell leukaemia, pancreatic adenocarcinoma (Lind et al, 2001; Nakshatri, 1997; Mori et al, 1999). The prototypical NF-κB is a heterodimer composed of p50 and p65 subunits, which are the most frequent component of active NF-κB. Subunit p65 containing complexes

bind with high affinity to the consensus DNA sequences 5'-GGGPuNNPyPyCC-3' (p65/p50) or 5'-GGGPuNPyPyCC-3' (p65/c-Rel) leading to the activation of transcription (Neurath, Becker and Barbelescu, 1998). Many other heterodimers like c-Rel/p52, RelB/p50, c-Rel/p50 and homodimers such as p50/p50 and p52/p52 have also been found in different cell types, which may vary depending upon the cell types and stimuli (Sun and Xiao, 2003).

Transcription factor AP-1 consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB and JunD); Fos (v-Fos, c-Fos, FosB, Fra1 and Fra2) or activating transcription factor (ATF-2, ATF-3 and B-ATF) proteins (Karin, Liu and Zandi, 1997). 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 tumour promotion (Karin, Liu and Zandi, 1997, Silverman and Maniatis, 2001). Activation of AP-1 protein required for the preneoplastic to neoplastic progression of different cancers such as prostate cancer and epithelial cancer (Huang et al, 2002; Zerbini et al, 2003) and nuclear translocation of c-Fos and ATF-2 has been reported in different cancer cells (Huang et al, 1998; Angel and Karin, 1991). CREB is a cyclic AMP response element binding protein, which gets activated through protein kinase A (PKA) mediated phophorylation in signaling cascade, CREB bind to the cyclic AMP response element sequence and promotes the specific gene transcription (Zhong, Voll and Ghosh, 1998). In the present study we found that the pretreatment of B16F-10 cells with natural products such as Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic 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.

Variety of genes such as IL-1β, IL-6, IL-8, MMP-2, MMP-9, COX-2, TNF-α and IL-12p40 involved in cancer are regulated by NF-κB and AP-1. Deregulated expression of IL-1β, IL-6, IL-8 is reported as an autocrine growth factor for tumour cells and acts as prometastatic or proangiogenic molecules (Andela et al, 2000; Zerbini et al, 2003). The expression level of these IL-1β, IL-6, IL-8 correlates directly with the metastatic potential of several human carcinomas, such as those of ovary, stomach, colon, pancreas, prostrate and brain (Huang et al, 2000; Yoneda et al, 1998).

GM-CSF is also a pleiotropic cytokine produced by a number of different cell types such as macrophages, T-cells, granulocytes, fibroblasts, endothelial cells and various carcinoma cells. GM-CSF has an important role in tissue repair and tumour progression (Bendtezen et al, 2001). TNF-α was originally discovered as a cytokine responsible for endotoxin induced necrosis but it is one of the most important and potent inflammatory mediators (Aggarwal and Natarajan, 1996). Continued over expression of TNF-α has been reported in various cancers, which can induce activation of transcription factors and enhance the expression of adhesion molecules, cytokine receptors and other proinflammatory cytokines (Hensel et al, 1987; Vandernabeele, Declercq and Fiers, 1995).

IL-12 can be thought of as having vital roles in both innate immunity and later immune responses. IL-12 is a covalent dimer of p35 and p40 genes. Interestingly all the cells that produce IL-12 synthesize large amount of IL-12 p40 than p35 . IL-12p40 is homologous to extracellular domains of IL-6R α , CNTFR α and G-CSF

receptor. Thus for this cytokine, part of the receptors has become part of the cytokine (Trinchieri, 1995). IL-12p40 act as a proinflammatory cytokine which can activate the tyrosine kinase 2, STAT proteins (Oppman, Lesley and Blom, 2002). Treating B16F-10 cells for 4 hours with Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic could inhibit the expression of these proinflammatory cytokines but β-Carotene did not give any inhibition on the proinflammatory cytokine expression. We found similar type of results in the ELISA quantitation of proinflammatory cytokines. This inhibition on the expression of proinflammatory cytokines may be due to the inhibition of activation or inhibition of nuclear translocation of transcription factors.

Epidemiological studies suggest a protective effect of natural products on cancer development (Surh, Lee and Lee 1998; Surh, 2002). In the present study we are explaining the mechanism, how Curcumin, Tetrahydrocurcumin, Piperine, Catechin, β-Carotene and 13cis-Retinoic acid could inhibit the metastasis of B16F-10 cells. The mechanism of action of Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid could inhibit the activation of these transcription factors is not certain. This inhibition may be due to the interference with signaling cascade, which may inhibit the expression of several proinflammatory cytokines. These data suggest that these transcription factors may be a good targets for treatments designed to prevent metastasis.

Chapter-6

Effect of some natural products on the induction of apoptosis in Dalton's Lymphoma Ascites cells

Introduction

Multicellular organisms have to delete excess cells both sculpting the form of the organism and its normal function. In addition cells that are infected or sustained genetic damage are dangerous to the organism, which have to be eliminated. To achieve this, cells activate a regulated program of suicide or apoptosis.

Once the decision to die is taken, individual cells activate highly co-ordinated multistep programs that result in the execution of the cell. In recent years there have been considerable advances in our understanding of events that regulate cell survival and a number of pro and antiapoptotic molecular intermediates have been described(Kaufmann and Hengarner,2001). Apoptotic cells are characterized by cell shrinkage, membrane blebbing and chromatin condensation, DNA fragmentation and ultimate fragmentation of cell as membrane enclosed vesicles designated as apoptotic bodies (Natarajan,1998).

The members of bcl-2 family is another family of genes that regulate apoptosis. They can be divided into antiapoptotic (Bcl-2,Bcl-xl, Mcl-1 etc) and proapoptotic (Bax, Bak, Bid, Bad etc) according to their function. Among them bcl-2 is antiapoptotic which promotes the chemoresistance but increases the recurrence rate, enhances radioresistance and tumour malignant potential (Hockenbery et al,1990; Gazzaniga etal,1996).

Caspases are expressed and activated by the proteolytic cleavage after death stimulus. Caspases function in both initiation of apoptosis in response to apoptotic signals and in the subsequent effector pathway to disassemble the cells (Thornbery and Lazebnick, 1998). Natural products which can be used for long period with out or less side effects are appreciable in the cancer therapy. In this study we used the natural products such as Curcumin, Tetrahydrocurcumin, Piperine , (+)Catechin,

and 13cis-Retinoic acid on the induction of apoptosis in Dalton's Lymphoma Ascites cells .

Materials and Methods

Cell line

Dalton's Lymphoma Ascites cells were used for the *in vitro* study

Compounds studied

Curcumin, Tetrahydrocurcumin, Piperine, Catechin, 13cis-Retinoic acid and β-Carotene.

Chemicals and Kits used

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

Determination of the in vitro cytotoxicity of natural products to DLA cells (Short term)

DLA cells ($1x10^6$) were incubated with various concentrations of (2μg/ml-200 μg/ml) of different natural products in a final volume of 1 ml 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 effect o natural products on the growth of DLA cells (MTT assay)

DLA cells (5000 cells/ well) plated in a 96-well flat bottom titre plate were incubated at 37^0C in 5% CO_2 atmosphere. After 24h, various concentrations of natural products were added and the incubation was continued for 48h under the same conditions. Cell viability was determined by MTT assay described in Chapter-2.

Morphological analysis and DNA ladder studies

One million cells (D L A) were grown in serum free DMEM at 37^0C in the humidified atmosphere containing 5% CO_2. Different concentrations of natural products were added to the cells and incubated further for 48h under the same conditions. Curcumin (1 μg/ml,2 μg/ml, 5 μg/ml), Tetrahydrocurcumin (2 μg/ml, 5 μg/ml and 10 μg/ml), Piperine (2 μg/ml, 5 μg/ml and 10 μg/ml), Catechin (5 μg/ml, 10 μg/ml and 25 μg/ml), 13-Retinoic acid (5 μg/ml,10 μg/ml and 25 μg/ml) and β-Carotene (5 μg/ml, 10 μg/ml and 25 μg/ml) concentrations were used in this study. Following these treatments, 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 and smear was prepared and stained with haematoxylin and eosin. Apoptosis was characterized by the morphological changes (chromatin condensation, nuclear condensation and apoptotic bodies).

1 ml of cytoplasm extraction buffer (10mM Tris-HCl buffer, pH 7.5 containing 150mM NaCl, 5mM $MgCl_2$ and 0.5% Triton X-100) was added to the rest of the pellet. DNA was extracted using phenol-chloroform method. DNA was resolved in 1.5% agarose gel containing ethidium bromide. The bands or ladder were visualized and recorded using the gel-documentation system.

RT-PCR and Gene expression studies

DLA cells were grown in serum free DMEM for 4h in the presence and absence of various natural products. Curcumin (2 μg/ml), Tetrahydrocurcumin (10 μg/ml), Catechin (10 μg/ml) , Piperine (10 μg/ml) and 13cis-Retinoic acid (25 μg/ml) were used in this study. Total RNA was extracted from DLA cells and cDNA was synthesized, using moloney murine leukemia virus reverse transcriptase. Gene expression analysis was done by PCR analysis. The mouse bcl-2 and caspase-3

Fig-Ia. Cytotoxicity of natural products towards DLA Cells (Short-term)

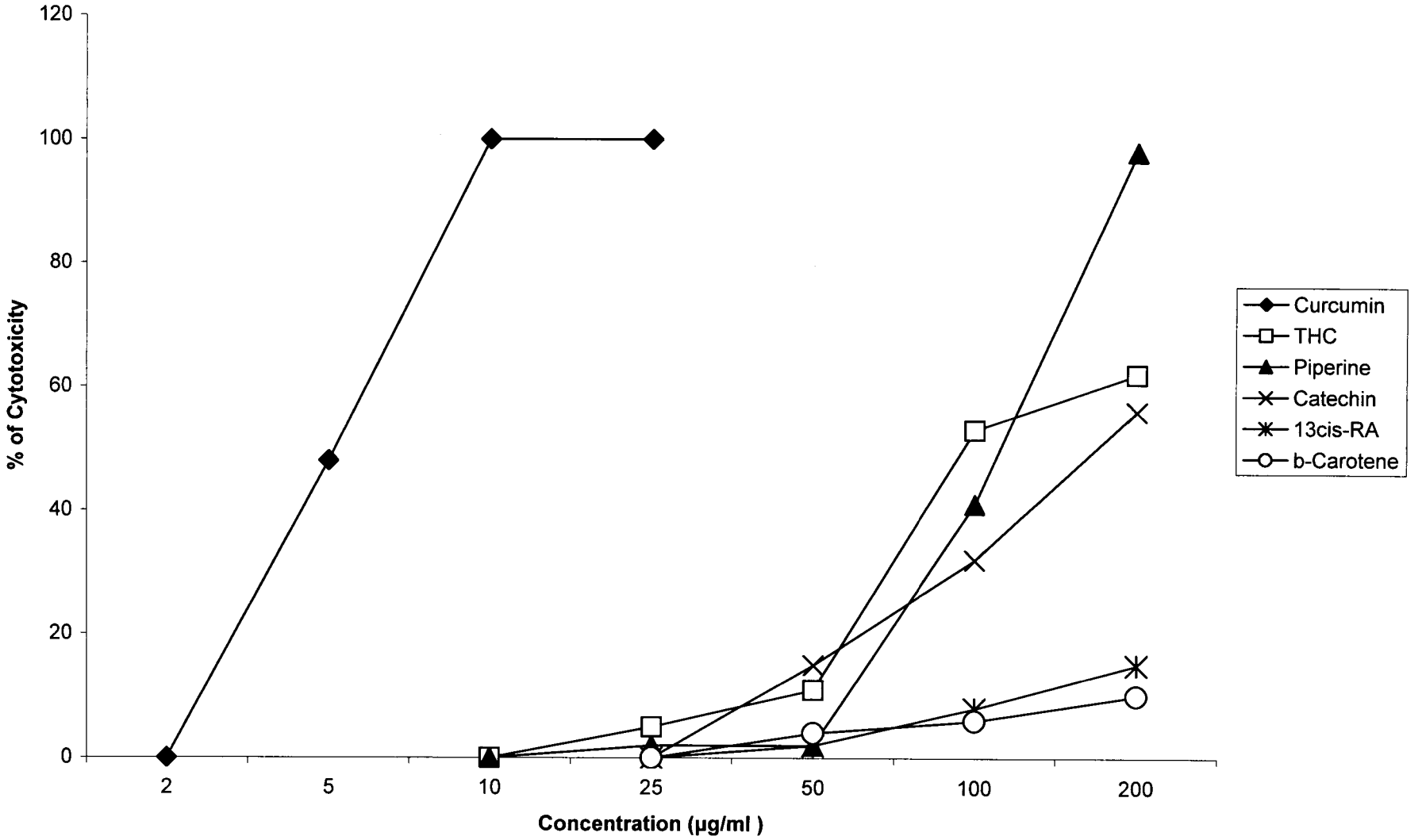
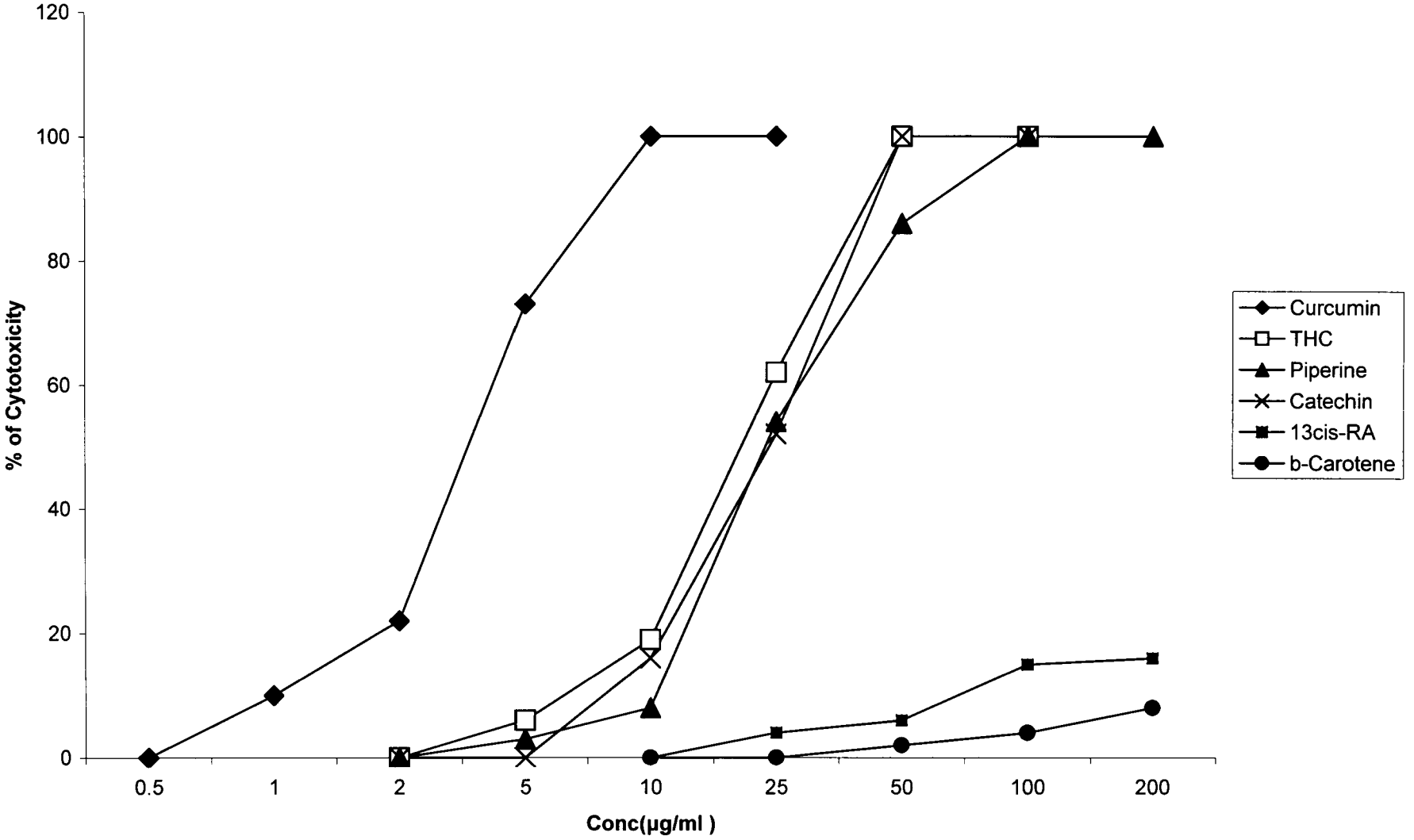


Fig-Ib. Effect of natural products on the growth of DLA cells (MTT assay)



genes were amplified against GAPDH primers obtained from Maxim biotech, USA. The target PCR products generated from a positive control cDNA was also included in this kit. PCR products were analyzed by agarose gel electrophoresis and visualized using gel documentation system.

Statistical analysis

Results were expressed as Mean±SD. Statistical evaluation was done by students t test

Results

Cytotoxicity of natural products towards DLA cells (Short term)

Cytotoxic effects of natural products toward DLA cells is shown in Figure-Ia.. Among these compounds Curcumin was found to be highly cytotoxic at a concentration of 10μg/ml. Tetrahydrocurcumin at concentration of 25 μg/ml gave cytotoxicity of 5%. Piperine at concentrations of 50 μg/ml, 100 μg/ml and 200 μg/ml shows cytotoxicity of 2%, 41% and 98% respectively. Catechin at concentrations of 50 μg/ml, 100 μg/ml and 200 μg/ml shows 15% , 32% and 56% respectively. β-Carotene and 13cis-Retinoic acid showed very less toxicity compared to other compounds even at the higher concentration of 200 μg/ml.

Determination of the effect o natural products on the growth of DLA cells (MTT assay)

Effect of natural products such as Curcumin, Tetrahydrocurcumin, Piperine, Catechin, 13cis-Retinoic acid and β-Carotene on the growth of DLA cells is shown in Figure-Ib. Curcumin was found to be toxic at 1μg/ml concentration and 10 μg/ml concentration of Curcumin gave 100% cytotoxicity. Among these compounds, 13cis-Retinoic acid and β-Carotene were found to be less toxic even at 100 μg/ml

5

Fig-II Effect of natural products on the apoptotic morphology in Dalton's Lymphoma Ascites cells

a) Untreated control with out apoptosis.

b) DLA cells treated with Curcumin (1 μg/ml).

c) DLA cells treated with Curcumin (2 μg/ml).

d) DLA cells treated with Curcumin (5 μg/ml).

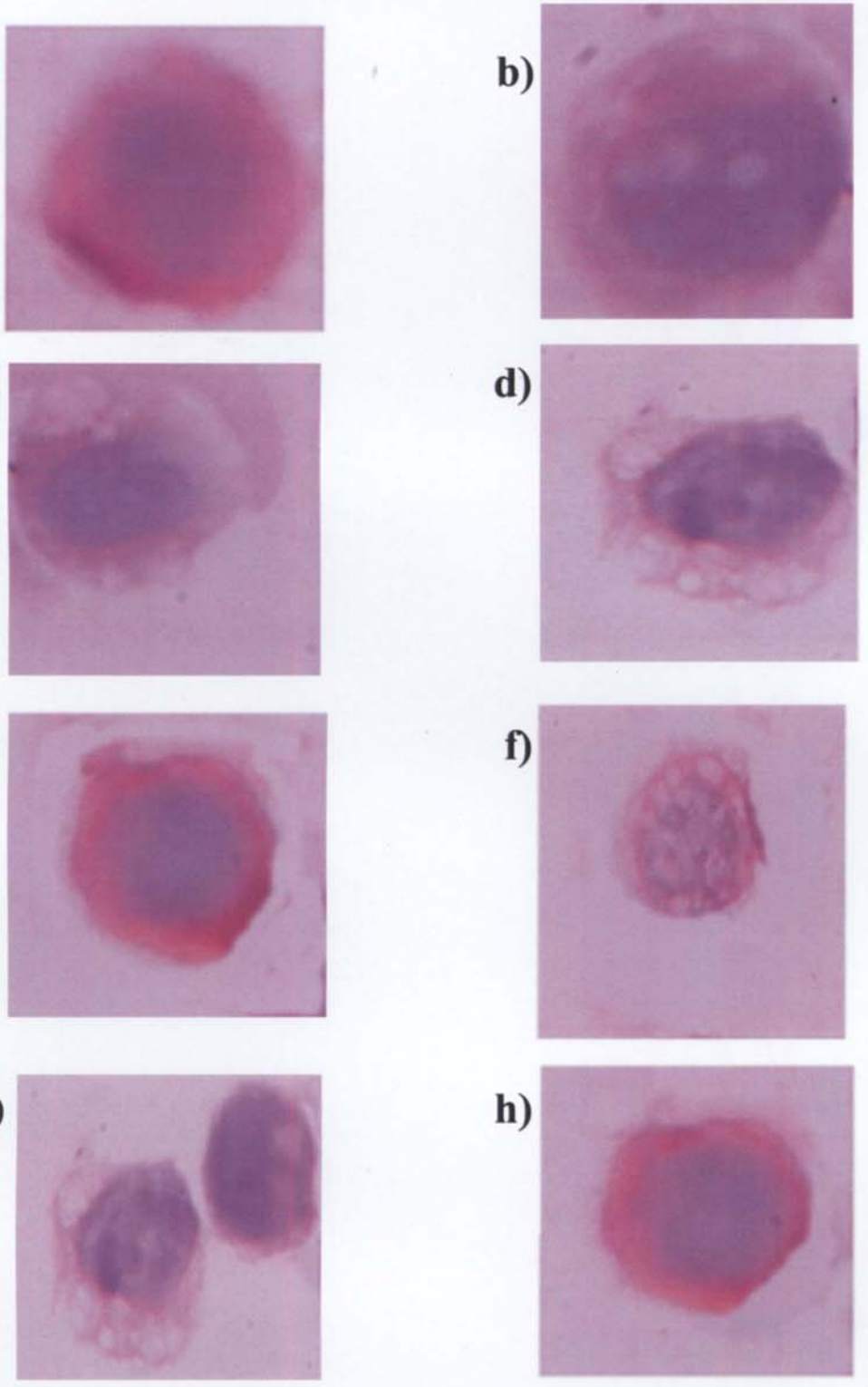
e) DLA cells treated with Tetrahydrocurcumin (2 μg/ml).

f) DLA cells treated with Tetrahydrocurcumin (5 μg/ml).

g) DLA cells treated with Tetrahydrocurcumin (10 μg/ml).

h) DLA cells treated with Piperine (2 μg/ml).

Fig-II. Effect of natural products on the apoptotic morphology in DLA cells.



concentrations. Tetrahydrocurcumin, Piperine and Catechin produced a dose dependent cytotoxicity.

Effect of natural products on the apoptotic morphology in Dalton's Lymphoma Ascites cells

The entire cell is reorganized during apoptosis. The chromatin becomes fragmented and condensed, the cell shrinks and the cell surface blebs leading to budding off the membrane bound apoptotic bodies. Untreated control cells did not show any morphological modifications or reorganizations (FigIIa). Curcumin at concentrations of 1 μg/ml, 2 μg/ml and 5 μg/ml produced the membrane blebs and the budding of membrane bound apoptotic bodies. (Fig-IIb, c, d). Tetrahydrocurcumin did not give such effect at lower concentration of 2 μg/ml (Fig-IIe) but treatment with 5 μg/ml and 10 μg/ml displayed the stages of apoptosis as chromatin condensation or nuclear condensation (Fig-IIf, g). Piperine at a concentration of 2 μg/ml did not give any apoptotic changes (Fig-IIh) but 5 μg/ml and 10 μg/ml produced the formation of apoptotic bodies and chromatin condensation (Fig-IIi, j). Catechin at concentration of 5 μg/ml did not cause any apoptosis (Fig-IIk) but 10 μg/ml and 25 μg/ml concentrations give the cytoplasmic and nuclear changes related to apoptosis (FigII l, m). Lower concentrations of 13cis-Retinoic acid at concentrations of such as 5 μg/ml and 10 μg/ml did not produce such effect (Fig-IIn and IIo) but higher concentrations of retinoic acid at 25 μg/ml displayed apoptosis in the same way (FigIIp). β-Carotene even at higher concentration of 25 μg/ml did not produce any apoptotic morphological reorganization (Fig-IIq).

Effects of natural products on the DNA ladder formation

There are types of DNA fragmentation that occur during apoptosis are (a) internucleosomal DNA cleavage (b) fragmentation into large 50-300 bp lengths and

i) DLA cells treated with Piperine (5 μg/ml).

j) DLA cells treated with Piperine (10 μg/ml).

k) DLA cells treated with Catechin (2 μg/ml).

l) DLA cells treated with Catechin (5 μg/ml).

m) DLA cells treated with Catechin (10 μg/ml).

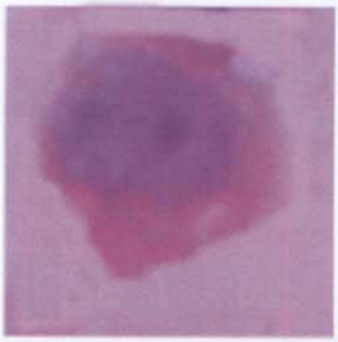
n) DLA cells treated with 13cis-Retinoic acid (5 μg/ml).

o) DLA cells treated with 13cis-Retinoic acid (10 μg/ml).

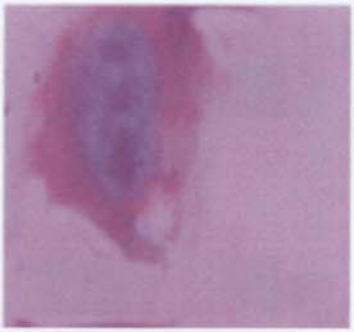
p) DLA cells treated with 13cis-Retinoic acid (25 μg/ml).

q) DLA cells treated with β-Carotene (25 μg/ml)

i)

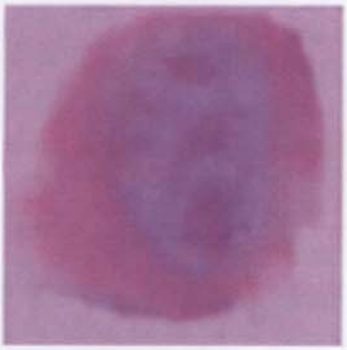


j)

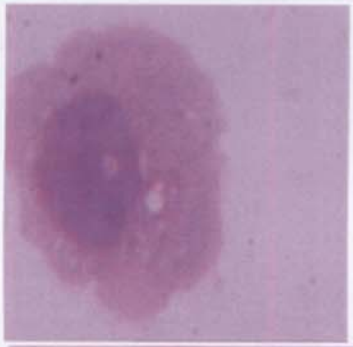


k)

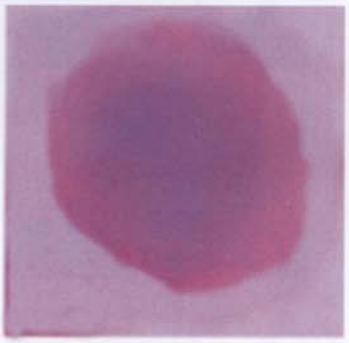
l)



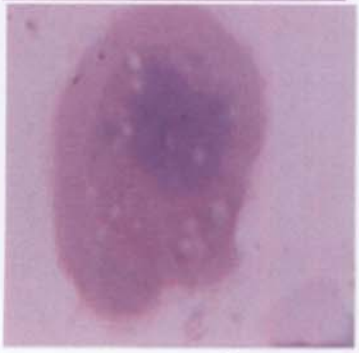
m)



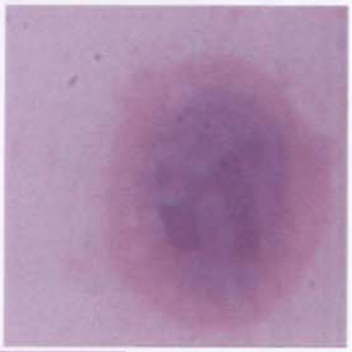
n)



0)



p)



q)

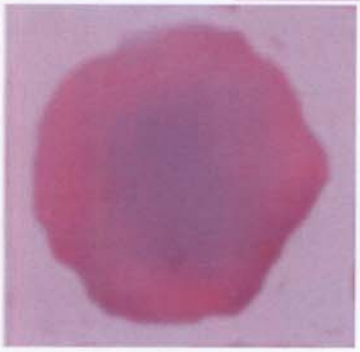


Fig-III. Effects of natural products on the DNA ladder formation

Lane1, Untreated control

Lane2, DLA cells treated with Curcumin (1 μg/ml).

Lane3, DLA cells treated with Curcumin (2 μg/ml).

Lane4, DLA cells treated with Curcumin (5 μg/ml).

Lane5, DLA cells treated with Tetrahydrocurcumin (2 μg/ml).

Lane6, DLA cells treated with Tetrahydrocurcumin (5 μg/ml).

Lane7, DLA cells treated with Tetrahydrocurcumin (10 μg/ml).

Lane8, DLA cells treated with Piperine (2 μg/ml).

Lane9, DLA cells treated with Piperine (5 μg/ml).

Lane10, DLA cells treated with Piperine (10 μg/ml).

Lane11, DLA cells treated with Catechin (2 μg/ml).

Lane12, DLA cells treated with Catechin (5 μg/ml).

Lane13, DLA cells treated with Catechin (10 μg/ml).

Lane14, DLA cells treated with 13cis-Retinoic acid (5 μg/ml).

Lane15, DLA cells treated with 13cis-Retinoic acid (10 μg/ml).

Lane16, DLA cells treated with 13cis-Retinoic acid (25 μg/ml).

Lane17, DLA cells treated with β-Carotene (25 μg/ml)

Fig-III Effect of natural products on the DNA ladder formation

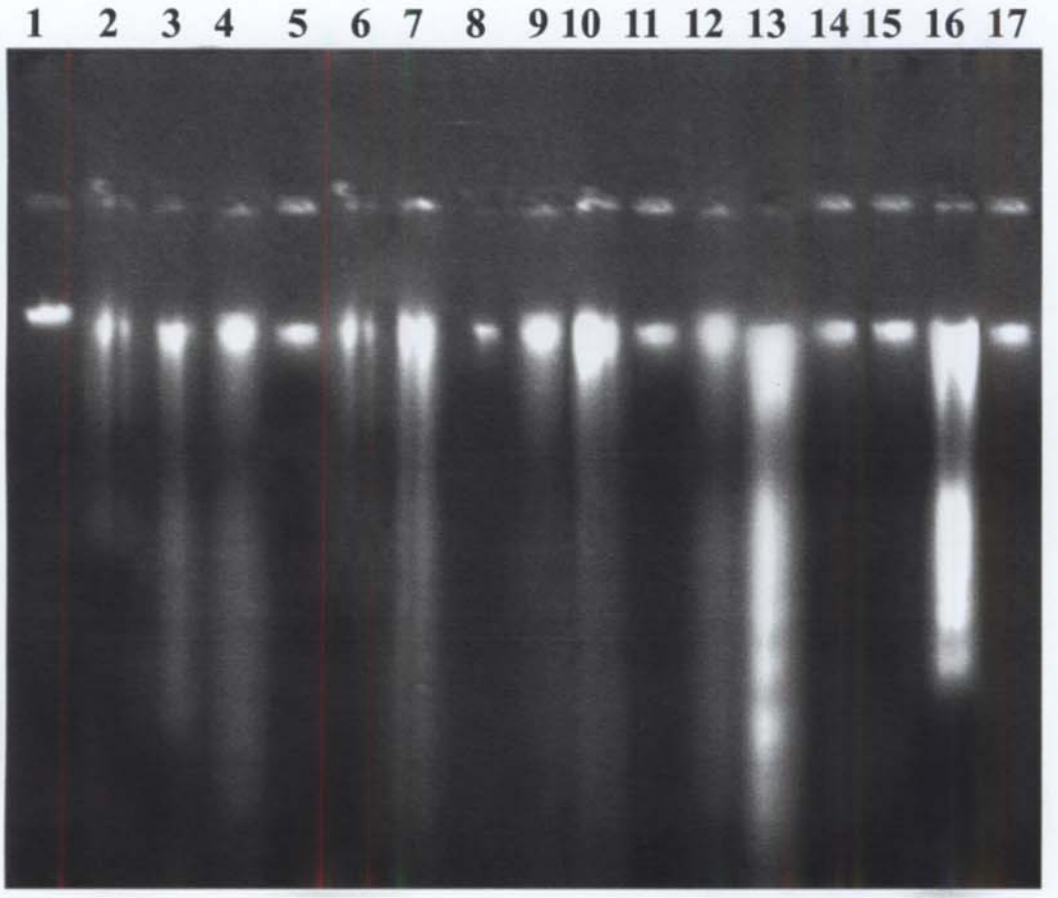


Fig-IV. Effect of natural products on the bcl-2 gene expression in DLA cells

Lane1, bcl-2 Positive control (235 bp)

Lane2, Untreated control

Lane3, Curcumin treated (2 μg/ml)

Lane4, Tetrahydrocurcumin treated (10 μg/ml)

Lane5, Catechin treated (10 μg/ml)

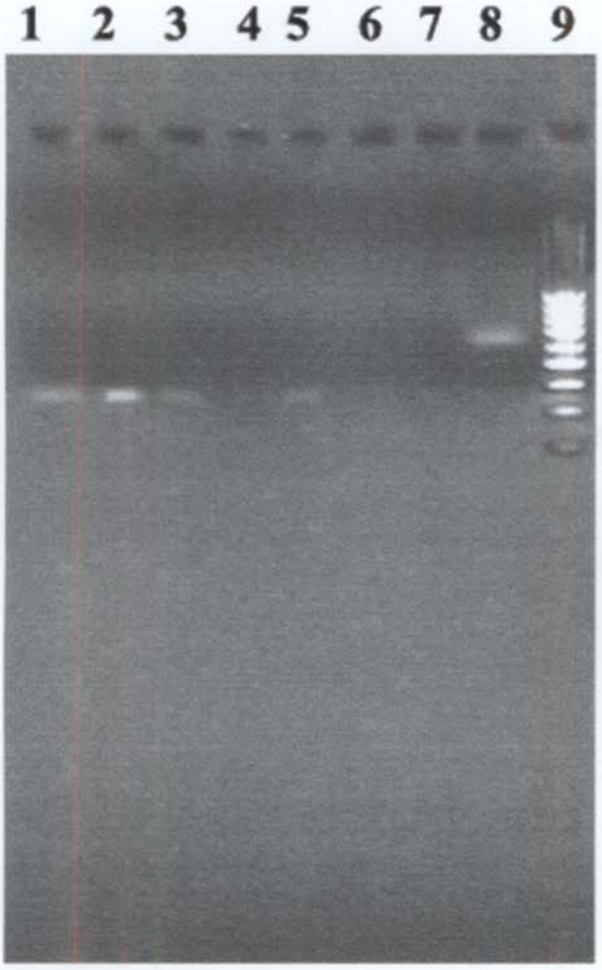
Lane6, Piperine treated (10 μg/ml)

Lane7, 13cis-Retinoic acid (25 μg/ml)

Lane8, GAPDH (527 bp)

Lane9, M.W Markers (100 bp ladder)

Fig-IV. Effect of natural products on the bcl-2 expression in DLA cells



(c) single strand events. The DNA is cleaved to generate 180-200 bp length fragments, giving a ladder like appearance which is a biochemical hallmark of apoptosis in many cells.

Effects of natural products on the DNA ladder pattern is shown in Fig-III. DNA of untreated control cells did not show any ladder pattern (Lane1). Curcumin at concentration of 1 μg/ml, 2 μg/ml and 5 μg/ml gave the DNA ladder formation after 48h incubation (Lane2, 3,4). Natural products such as Tetrahydrocurcumin, Piperine and Catechin produced the ladder pattern dose dependently (Lane5-13). 13cis-Retinoic acid even at the concentrations of 5 μg/ml and 10 μg/ml did not produce any DNA ladder formation (Lane14 and lane15), but 13cis-Retinoic acid at concentration of 25 μg/ml displayed the DNA ladder formation(Lane16). β-Carotene even at higher concentration of 25 μg/ml did not produce any DNA ladder formation.

Effect of natural products on the bcl-2 gene expression in DLA cells

Effect of natural products such as Curcumin, Tetrahyrocurcumin, Piperine, Catechin and 13cis-Retinoic acid is shown in Fig-IV. Lane 1 shows the amplified bcl-2 positive control cDNA (235 bp) included in the kit. Lane2 shows bcl-2 gene in the untreated DLA cells. Bcl-2 level is down regulated or inhibited by the above natural products treatment.

Lane 3- lane7 represents the Curcumin (2 μg/ml), Tetrahydrocurcumin (10 μg/ml), Catechin (25 μg/ml) and 13cis-Retinoic acid (25 μg/ml) treatment respectively. Lane 8 represents the GAPDH (527bp). Lane-9 represents the molecular weight markers.

Fig-V. Effect of natural products on the caspase-3 proapoptotic gene expression in DLA cells

Lane1, Caspase-3 Positive control (414 bp)

Lane2, Untreated control

Lane3, Curcumin treated (2 μg/ml)

Lane4, Tetrahydrocurcumin treated (10 μg/ml)

Lane5, Catechin treated (10 μg/ml)

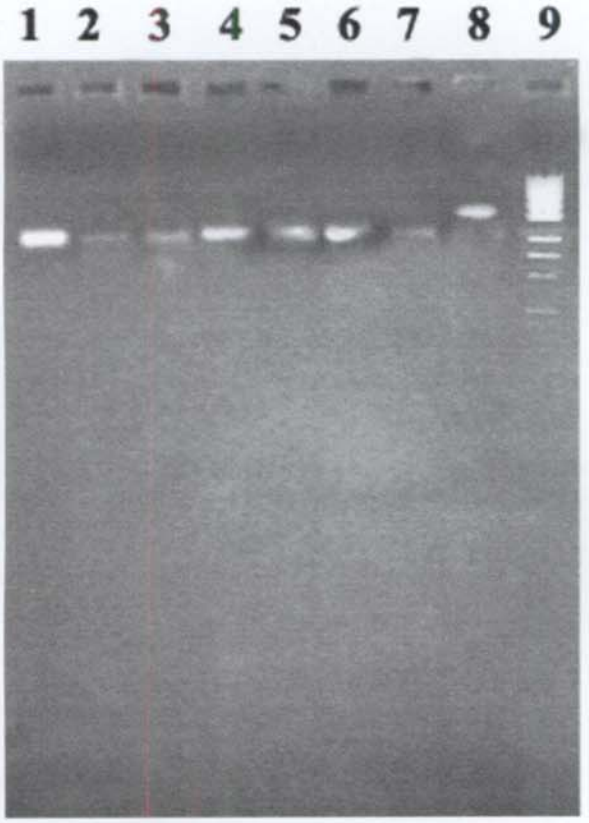
Lane6, Piperine treated (10 μg/ml)

Lane7, 13cis-Retinoic acid (25 μg/ml)

Lane8, GAPDH (527 bp)

Lane9, M.W Markers (100 bp ladder)

Fig-V. Effect of natural products on the caspase-3 expression in DLA cells



Effect of natural products on the caspase-3 proapoptotic gene expression in DLA cells

Effect of natural products on the caspase-3 gene expression in DLA cells is shown in Fig-V. Mouse caspase-3 gene was amplified against mouse GAPDH standard. Lane1 shows the amplified caspase-3 positive control cDNA(414 bp) included in the kit. Lane2 shows the caspase-3 gene in the untreated control DLA cells. Caspase-3 level was very low in the untreated group. Treatment of Curcumin (2 μg/ml), Tetrahydrocurcumin (10 μg/ml), Catechin (25 μg/ml) and 13cis-Retinoic acid (25 μg/ml) could upregulate the Caspase-3 genes (lane3 to lane7) respectively. Lane8 and lane9 shows the GAPDH standard and molecular weight markers respectively.

Discussion

Apoptosis functions effectively in cancer prevention by interfering with the evolution of malignant cells. Same way the apoptotic elimination of drug resistant cells amplifies the chemotherapeutic effects in cancer cells.

Apoptosis is a highly organized physiologic mechanism of destroying injured and abnormal cells. The entire cell morphology is reorganized during apoptosis such as chromatin becomes fragmented and condensed, the cell shrinks and the cell surface blebs leading to budding off the membrane bound apoptotic bodies. (Hale et al,1996). We have selected the lower toxic concentrations of different natural products for the studies of apoptosis such morphological analysis, DNA ladder and gene expression studies. Treatment of DLA cells with natural products such as Curcumin, Tetrahydrocurcumin, Catechin and 13cis-Retinoic acid caused the chromatin condensation and formation of apoptotic bodies. DNA fragmentation is another characteristic feature of DNA, which is the result of an endogenous $Ca2^{+}$ and Mg^{2+} dependant endonuclease activity. During apoptosis DNA is cleaved to 50-

300 kbp length, giving a ladder like appearance, which is a biochemical hallmark of apoptosis in many cells (Natarajan,1998; Walker et al,1991). We found that natural products such as Curcumin, Tetrahydrocurcumin, Catechin and 13cis-Retinoic acid produced the DNA cleavage after 48h incubation.

Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins. During execution phase of apoptosis, caspase-3 is responsible either wholly or in part for the proteolysis of a large number of substrates, each of which contains a common Asp residue. Natural products such as Curcumin, Tetrahydrocurcumin, Catechin and 13cis-Retinoic acid could help to upregulate the expression of caspase-3 after 4h treatment in Dalton's Lymphoma cells which is the primary regulator of DNA fragmentation in apoptosis (Natarajan, 1998; Wolf et al,1999).

The major drawbacks of current cancer treatments, such as chemotherapy and radiation therapy, include neurotoxicity, nephrotoxicity, acute cochlear ototoxicity and peripheral neuropathy (Bruce, 1993). Natural products, which can induce apoptosis could be used for cancer treatment either alone or with any standard therapeutical modality. Hence this study suggests that this compounds could induce apoptosis may have good therapeutical value.

Chapter-7

Summary and Conclusion

Tumour metastasis is the major cause of treatment failure in cancer patients and is a principal contributing factor to cancer morbidity and mortality. The formation of metastatic foci is a continuous process that can begin early in the growth of the primary tumour and increases in frequently with tumour duration and tumour burden. Thus the design of more effective therapies to treat metastatic cancer requires better understanding of the molecular events and cellular processes that are involved in the 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 current available antimetastatic therapy have low efficiency, low response rate and produce a number of side effects such as nephrotoxicity, acute cochlear ototoxicity and peripheral neuropathy and immunosuppression (Bruce, 1993). Thus it has become necessary to develop a second generation of drug which can interfere with process of metastasis and these drugs should be equally effective and less toxic.

In the present study we have evaluated the effect of some naturally occurring polyphenolic compounds such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, 13cis-Retinoic acid and β-carotene on the experimental metastasis. A preliminary study about the antimetastatic activity of Curcumin and Catechin was done in our laboratory previously (Menon, Kuttan and Kuttan, 1999). In this study we investigated the antimetastatic mechanism of Curcumin and Catechin. Compounds such as Tetrahydrocurcumin, Piperine, 13cis-Retinoic

acid and β- carotene were subjected to a detailed analysis in *in vitro* and *in vivo* metastatic models.

Administration of compounds such as Tetrahydrocurcumin, Piperine, 13cis-Retinoic acid and β-Carotene inhibited the metastatic tumour colony formation in the lungs after 21 days of tumour induction. There was also a corresponding increase in the life span of metastatic tumour bearing animals, treated with these compounds. Administration of Tetrahydrocurcumin, Piperine, 13cis-Retinoic acid and β carotene could normalize the hydroxyproline content of the lungs indicating the inhibition of lung fibrosis. There is an increased expression of surface sialic acid on circulating tumour cells, which facilitates their invasive behaviour. In melanoma, both secretion of glyco proteins and shedding of gangliosides have been demonstrated (Hersey, 1985) and the metastatic property of tumour 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 Piperine, 13cis-Retinoic acid and β carotene. The elevated levels of lung uronic acid and hexosamine content also significantly 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 tumour bearing animals. Increase of GGT has been reported during the growth of tumour cells (Hanigan,1994). But treatments with compounds such as Tetrahydrocurcumin, β carotene and 13cis-Retinoic acid by simultaneous modality could reduce the serum gamma glutamyl transpeptidase (GGT) level.

Adhesion and Invasion are two major steps in the process of metastatic dissemination. Metastatic tumour cells disseminating through the blood must penetrate the capillary basement membrane, which is a mechanical barrier for invasion. Tumour 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 tumour cells (Burrow,1991).

β-Carotene and 13cis-Retinoic acid 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 compounds such as Piperine and Tetrahydrocurcumine did not inhibit the adhesion of B16F-10 melanoma cells.

Metalloproteinases have been implicated in the denaturation of the basement membrane during the metastatic invasion of tumour cells. Several studies of the tumour 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, Graber and Muschel, 1994). Treatment of Curcumin, Tetrahydrocurcumin, Piperine, and 13cis-Retinoic acid were inhibited the production of type IV collagenase as seen from zymographic analysis but β-Carotene could not inhibit the production of type IV collagenase. Effects of Curcumin and Catechin on the inhibition of invasion of B16F-10 cells were reported previously from our lab (Menon, Kuttan

and Kuttan, 1999). Piperine, Tetrahydrocurcumin, 13cis- Retinoic acid inhibited the invasion of B16F-10 melanoma cells in a dose dependent manner.

Various proinflammatory cytokines such as IL-1 β, IL-6, GM-CSF, TNF-α and IL-12p40 are pleiotropic cytokines and act as autocrine growth factors for tumour cells (Dendorfer, Oettgen and Liberman,1995; Neurath,Becker and Barbulescu,1998; Yoneda, Kuniyasu and Crispens, 1998; Hensel et al,1987). These 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 (Hensel et al,1987; 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 (Oroz et al,1993). It has been reported that IL-6 level is high in cancer, which promote the production of other cytokines (Dendorfer, Oettgen and Liberman, 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 are homologous to extracellular domains of IL-6Rα and GM-CSF receptors. IL-12p40 can acts as a proinflammatory cytokine, which can activate the tyrosine kinase-2 and STAT proteins (Trinchieri,1995; Oppmann, Lesley and Blou,2002). Treatment of B16F-10 cells with Curcumin, Tetrahydrocurcumin, Catechin, Piperine, 13cis-Retinoic acid inhibited the expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, GM-CSF

and IL-12p40. Treatment with these compounds such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine and 13cis-Retinoic acid significantly inhibited the production of proinflammatory cytokines such as IL-1 β, IL-6, GM-CSF and TNF-α. These compounds were found to inhibit nitric oxide (NO) production by activated peritoneal macrophages *in vitro* as well as *in vivo* models. The proinflammatory action of NO seems to play an important role in signal transduction that occurs in many diseases including cancer.

We have checked the inhibitory potential of these compounds on the activation and nuclear translocation of transcription factors. 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,2000; Karin, Liu and Zandi,1997). We could find that Curcumin, Tetrahydrocurcumin, Piperine, Catechin and 13cis-Retinoic acid could inhibit the nuclear translocation of sub units 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, TNF-α and IL-12p40 as well as the expression of degradative enzymes such as matrix metalloproteinases (MMPs). Thus the inhibition of activation or nuclear translocation of NF-κB, AP-1 and CREB could inhibit the expression of proinflammatory cytokines and collagenases essential for tumour progression and metastasis.

Apoptosis and programmed cell death has become interest as an intervening target in cancer chemoprevention. 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; Umansky, Korol and Neliporich, 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, Black and Thomson, 1994). Compounds such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, and 13cis-Retinoic acid were found to inhibit the expression of antiapoptotic bcl-2 gene in Dalton's lymphoma ascites 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 Dalton's lymphoma ascites cells were subjected to treat with these compounds.

This study is focused on the effect of some natural products, such as Curcumin, Tetrahydrocurcumin, Catechin, Piperine, β-Carotene and 13cis-Retinoic acid which are found in the indigenous system of medicine and the diet. The result not only indicates the effectiveness of these natural products in the inhibition of metastasis and the promotion of apoptosis; it also reveals 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, and some of them are rich in fruits and vegetables. This may indicate that a diet with adequate spices or fruits and vegetables not simply the consumption of a specific phytochemical, may effectively reduce the risk of several serious diseases including cancer. Thus these compounds either alone or in combination with any chemotherapeutical agent may help to prevent metastasis or tumour progression in clinical patients.

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Abbreviations

AP-1	-	Activated protein
ATF-2	-	Activator of transcription factor
BAD	-	Bcl-2 associated death promoter
CARD	-	Caspase recruit domain
CRE	-	cyclicAMP response element
CRB	-	CRE binding protein
ECM	-	Extracellular matrix
EGF	-	Epidermal growth factor receptor
EGTA	-	Ethylene glycol-bis(2aminoethylether)tetra acetic acid
ELAM	-	Endothelial leukocyte adhesion molecule
ERBB	-	Epidermal growth factor receptor
ERK	-	Extracellular regulatory kinase
FKHR	-	Member of the fork head family of transcription factor
GM-CSF	-	Granulocyte monocyte colony stimulating factor
HSP	-	Heat shock proteins
ICE	-	Interleukin-1β converting enzyme
IFN	-	Interferon
IRF	-	Interferon regulatory factor
IL	-	Interleukin
ILP	-	IAP like protein
IKK	-	Inhibitory kapp- B kinase
JNK	-	Jun N terminal kinase
LMP-1	-	Latent membrane protein-1
MAPK	-	Mitogen activated protein kinase
MEK	-	MAPK/ERK kinase

M-CSF	**-**	**Macrophage-Colony stimulating factor**
MMP	**-**	**Matrix metalloproteinases**
MIHA	**-**	**Mammalian IAP homologue A**
MTT	**-**	**3-(4,5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium bromide**
NF-κB	**-**	**Nuclear factor-kappaB**
NIAP	**-**	**Neuronal apoptosis inhibitory factor and surviving**
PARP	**-**	**PolyADP Ribosyl phosphorylase**
PI3K	**-**	**Phosphatidyl inositol 3 kinase**
PKC	**-**	**Proteinkinase-C**
13Cis-RA	**-**	**13cis-Retinoic acid**
STAT	**-**	**Signal transducers and activators of transcription**
TBP	**-**	**Tata binding protein**
THC	**-**	**Tetrahydrocurcumin**
TNF	**-**	**Tumour necrosis factor**
TRAIL	**-**	**Tumour necrosis factor mediated apoptosis inducing ligand**
VCAM	**-**	**Vascular cell adhesion molecule**
XIAP	**-**	**X-linked IAP**

List of publications

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