ROLE OF NATURAL PRODUCTS ON THE TUMOUR SPECIFIC ANGIOGENESIS BY ENDOTHELIAL CELLS

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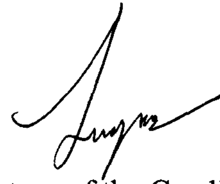
DECLARATION

I, Leyon Varghese 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.



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CERTIFICATE

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

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INTRODUCTION

Cells within a tissue display functional variation over time. These variations generally reflect change in the extrinsic characteristics. On occasion, however, functional variations occur because of intrinsic modification in the cells. The consequence of these modifications is the formation of a pathological out growth within the tissue known as a tumour (Coleman et al 1992). Even if cancer doesn't originate necessarily in normal stem cells, tumour growth apparently depends on a small population of stem like cells that may differ from the bulk of the tumour population in ways that make them even resistant to therapy. "Cancer is cells that grow too much, but only one in a million has the ability to sustain growth," says stem cell biologist John Dick of the university of Toronto. The neoplastic process appears to be a problem of growth and differentiation and its properties are stable and heritable. New treatment modalities involving immunotherapy and agents that promote normal cell maturation remains experimental and is under intense investigation. Research employing modern technology in molecular genetics and immunotherapy promises a new array of anticancer agents.

Tumourigenesis is a multi-step process in which multiple genetic alterations, usually over a span of years, have a cumulative effect on the control of differentiation, cell division and growth. Infact "cancer development is a long term process," says Sauvageau, "you can't have a cancer development in a cell/cell population that is short lived." (Jean Marx 2003). Observations of human cancers and animals models argue that tumour development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic

changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Foulds 1954, Nowell 1976).

However, in addition to the genetic and epigenetic changes that occur during transformation, another discrete step is required to allow tumour propagation and progression – the induction of tumour vasculature, termed the 'angiogenic switch' (Hanahan and Weiberg 2000, Folkman J 2000). Like normal tissues, tumours require an adequate supply of oxygen, metabolites and an effective way to remove waste products (Papetti M et al 2002). These requirements vary, however, among tumour types, and change over the course of tumour progression (Hlatky L et al 2002). The angiogenic switch can occur at different stages of the tumour progression pathway, depending on the tumour type and the environment. It has been shown that dormant lesions and in some instances pre-malignant lesions also initiate neovascularisation which allows them to progress further (Folkman J 2000, Hanahan D et al 1996). Gaining access to the host vascular system and the generation of a tumour blood supply are rate-limiting steps in tumour progression. The fact that tumours are dependent on blood supply has inspired many researchers to search for antiangiogenic molecules and to design antiangiogenic strategies for treatment.

Tumours may be either benign or malignant. Malignant tumours are more aggressive and have the characteristics – immoralization, transformation and metastasis (Hahn and Weinberg 2002). The principle cause of death from cancer however is the growth of metastases of malignancies. Metastatic development is determined by both host factors and intrinsic properties of the tumour cells (Fidler

and Bucana 1977). To establish metastases, tumour cells must invade the surrounding local tissue and eventually penetrate into blood vessels or lymphatics, be arrested in the capillary bed of distant organs, exrtavasate into organ parenchyma, vascularize and proliferate to form distinct foci of metastatic tumour growth (Fidler and Kripke 1977, Sugarbaker EV 1981, Weiss L 1985).

Several classes of proteins and proteases are involved in the tethering of cells to their surroundings in a tissue that are altered in possessing invasiveness or metastatic capabilities. The affected proteins include cell-cell adhesion molecules (CAMs) – notably members of immunoglobulin and calcium dependent cadherin families – and integrins, which link cells to extra cellular matrix substances. Enzymatic digestion of the basement membrane allows carcinoma cell access to connective tissue (Kohn and Liotta 1995). A positive association with tumour aggressiveness has been noted for a variety of class of enzymes including heparanases, serine, thiol and metal - dependent enzymes. Urokinase plasminogen activator, Cathepsin B etc are also been closely linked to the metastatic phenotype and elevated levels of these enzymes in the serum is indicative of the metastatic potential (Sinha et al 1993, 1995). It is interesting to note that majority of these proteins that are necessary for metastasis is also important in the process of angiogenesis. Indeed, a cascade including all these enzymes is probably involved in the invasive process, and more than one enzyme is necessary but may not be sufficient for the success of metastasis and also for angiogenesis (Liotta and Stevenson 1991)

The tumour mass whether primary or secondary, like any other tissue, is composed of parenchymal cells and stroma. The parenchymal cells may be poorly

differentiated, rapidly proliferating and have little or no organization and the host is induced supply stroma for it. Although parenchymal cells represent the proliferating 'cutting edge of neoplasm' the growth and evolution of neoplasm are critically dependent on their stroma. An adequate stromal blood supply is a requisite, and the stromal connective tissue provides the framework for the parenchyma. This host response is mediated by angiogenic factor (Folkman J 1985, 1993), which are synthesized by the parenchyma of the tumour and stimulate proliferation of all stromal cells including fibroblasts and vascular cells. Tumours, which can grow beyond a minimum size of 2mm, synthesize and secrete angiogenic factors. These factors stimulate endothelial cell proliferation in local capillary blood vessel and induce the out growth of nascent capillary loops which ramify into the tumour cell mass. Since angiogenesis is critical for growth and spread of tumours, much attention is focused on the use of angiogenesis inhibitors as adjuncts to other form of therapy (Ramzi et al 1999). Already available evidence indicates that different types of tumour cells use distinct molecular strategies to activate angiogenic switch. This raises the question of whether a single antiangiogenic therapeutics will be sufficient to treat all tumour types, or whether an ensemble of such therapeutics will be needed to develop, each responding to a distinct program of angiogenesis that has been developed by a specific class of human tumours. Success has been achieved in treating fairly large tumours in mice by administration of several natural as well as synthetic compounds. Results of ongoing research and clinical trials are eagerly awaited. The present study is aimed to evaluate certain plant products, naturally occurring polyphenolic compounds and their synthetic analogues regarding their antiangiogenic activity.

Chapter 1

Review of literature

A group of coordinated cellular processes, not just a single product is responsible for invasion and metastasis, the most life threatening aspect of cancer. However, the unrestrained growth and metastasis in particular require several genetic changes including the phenotypic change of the tumour to be angiogenic (inducing neoangiogenesis), in order to establish the tumour colony. Earlier belief was that angiogenesis is essential to the late stages of carcinogenesis. It has now widely accepted the idea, that the acquisition of angiogenic activity by a previously non-angiogenic cell population may be associated with an increased risk of neoplastic transformation and enable tumours to grow larger than 1-2 mm in diameter, invade and travel to distant site (metastasis) in the body. (Sharma RA et al 2001). Thus, before relevant growth factors and specific genetic abnormalities had been identified, it was recognized that the "switch" to an angiogenic phenotype involved over expression of one or more positive regulators of angiogenesis, mobilization of proteins from the ECM, recruitment of host cells such as macrophages or a combination of these processes (Folkman J 1995).

INTRODUCTION TO ANGIOGENESIS

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100-200μm of blood vessels- the diffusion limit of oxygen. For multicellular organism to grow beyond this size, they must recruit new blood vessels. A new vessel formation can occur by two means- vasculogenesis and angiogenesis. In vasculogenesis a primitive vascular network is established and it occurs during the embryonic development from endothelial cell precursors called angioblasts. Angiogenesis on the other hand is the formation of new blood vessel out of preexisting capillaries and is a key factor in many physiological

processes and in certain pathological conditions. The normal physiological processes such as embryonic development, wound healing and female reproductive cycle are those dependent on new vessel formation for the supply of oxygen and nutrients (Razmi SC et al 1999). The pathologic processes such as ischemic diseases, chronic inflammation and cancer also largely depend on neo-angiogenesis. These diseases may be benefited from the therapeutic inhibition of angiogenesis and the initial recognition of angiogenesis being a therapeutically interesting process began in the area of oncology in early 1970s.

Structure And Functions Of Normal Endothelial Cell

The endothelial cells form a monolayer in every single blood vessel in the circulatory system, called endothelium and are actively involved in several regulatory processes in the body. Endothelium is in direct contact with blood and subsequently located are pericytes, smooth muscle cells, fibroblasts, and extra cellular matrix. When the integrity is maintained endothelium exert anticoagulative properties via synthesis of thrombomodulin, tissue factor pathway inhibitor and tissue type plasminogen activator (t-PA). On activation or damage of endothelium, endothelial cells quickly release proteins like von Willebrand factor (vWF)- which promotes platelet adhesion and aggregation, and plasminogen activator inhibitor-1. In addition Tissue Factor expression by endothelium leads to extrinsic blood coagulation pathway (Verstrae M 1995). Another important feature of endothelial cell is their ability to direct cells of immune system to specific sites in the body. Constitutively expressed or cytokine inducible adhesion molecules (E-selectin, ICAM-1 etc) and chemo attractants act in concert to recruit the immune cells to lymphoid organs or inflammatory sites (Carlos and Harlan 1994). Lastly,

endothelial cells are actively involved in vascular remodeling during ovulation, wound healing, diabetic retinopathy, and neo- angiogenesis.

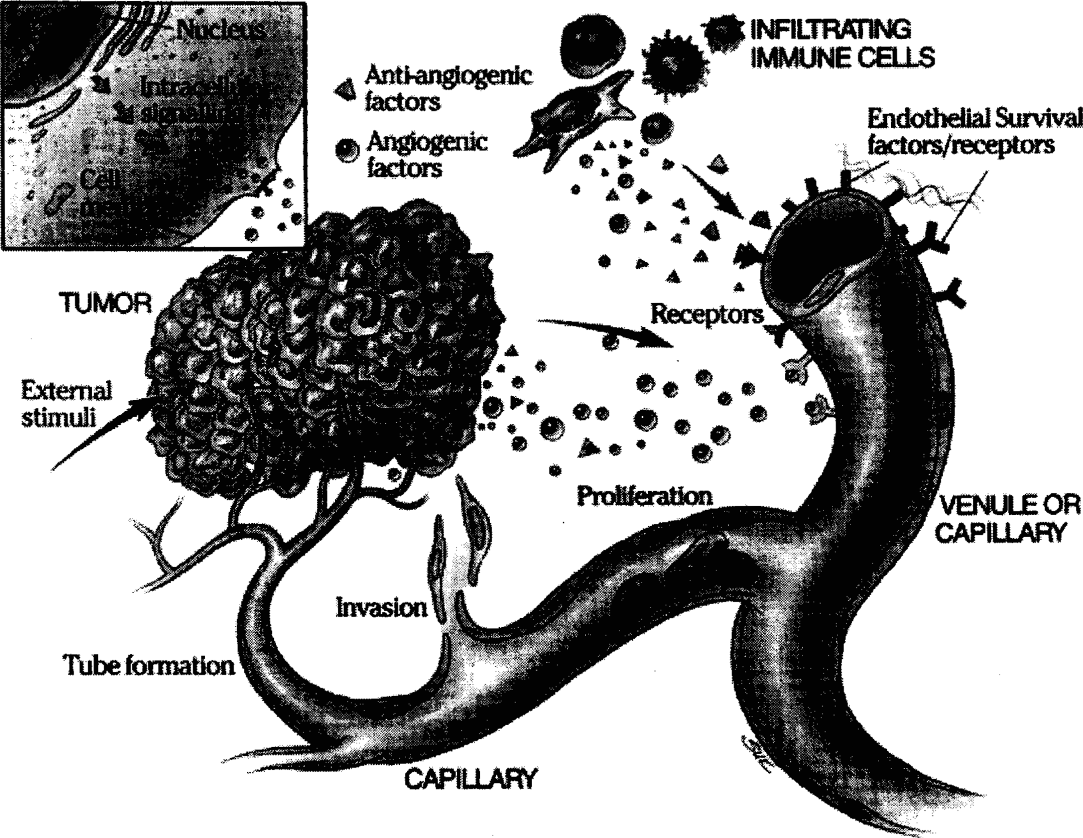
Induction Of Angiogenesis

The process of angiogenesis consists of several sequential and independent steps. It begins with local degradation of the basement membrane surrounding the capillaries, followed by invasion of stroma by the underlying endothelial cells in the direction of angiogenic stimulus. Endothelial cell migration is accompanied by its proliferation and their organization into three-dimensional structures that join with other similar structures to form a network of new blood vessels (Figure 1.1).

It is now widely accepted that angiogenesis is balanced between pro- and anti- angiogenic molecules present in the body. The 'angiogenic switch' is 'off' when the effect of proangiogenic molecules is balanced by that of antiangiogenic molecules and is 'on' when the net balance is tipped in favor of angiogenesis (Fukumura D et al 1998) Both pro- and anti- angiogenic molecules can emanate from endothelial cells, stromal cells, blood, extra cellular matrix and cancer cells. These molecules may mediate multiple steps in the overall process of angiogenesis and also can affect the functions of diverse cell types that are not only involved in angiogenesis. A more refined definition of an angiogenic factor is – a factor that selectively alters the characteristics of endothelial cells and associated perivascular structures (e.g. pericytes, vascular smooth muscle cells) but does not affect the functions of other cell types. Various signals that trigger this 'angiogenic switch' have been identified. These include metabolic stress (low pH, low O_2), immune/inflammatory responses (Nitric Oxide, Cytokines), and genetic mutations (Activation of Oncogene, deletion of oncosuppressor gene) (Kerbel RS 2000,

Figure 1.1

The process of tumour angiogenesis



Carmelite P 1999). How they can inter play between environment and genetic mechanisms, influence angiogenesis and tissue growth is very complex and largely unresolved matter.

Initiation Of Angiogenesis

Angiogenesis is readily initiated in response to ischemic or hypoxic conditions. In all types of angiogenesis, endothelial cell activation is the first process to take place. Vasodilation mediated by nitric oxide (NO) is a prerequisite for endothelial cells to enter angiogenic cascade. Recent findings have shown that hypoxic condition activates 'hypoxia inducible transcription factor' (HIF1-α), which functions as master switch to induce expression of several angiogenic factors. These factors include Vascular Endothelial Growth Factor (VEGF), Nitric Oxide Synthase (NOS), Platelet Derived Growth Factor (PDGF), Angiopoetin-2 (Ang-2) and others (Semenza GL 1998). It is suggested that VEGF is a major player in angiogenesis initiation based on its ability to induce vasodilation via endothelial nitric oxide production (Ziche M et al 1997a). VEGF also affects endothelial cell proliferation and this effect can be partly attributed to nitric oxide and cGMP (cyclic GMP) mediated activation of MAP kinase family (May also be due to altering the permeability of endothelial cells, Ziche M et al, 1997b). Until recently, VEGF was only growth factor proven to be specific and critical for blood vessel formation. It is a homodimeric heparin binding glycoprotein that exits in at least four isoforms, due to alternative splicing of primary mRNA transcript (Thomas KA 1996). The receptors for VEGF are expressed almost exclusively on endothelial cells. A recent exploration discovered some other vascular endothelium specific growth factors and these include –Placental Growth Factor (PlGF)

(included in VEGF family), four members of angiopoetin family and at least one member of the large ephrin family (Yancopoulos GD et al 2000). Numerous nonspecific angiogenic molecules that affect not only the growth of endothelial cells but also other cell types have been identified. These factors include Fibroblast Growth Factor (FGF), Transforming Growth Factor-β (TGF-β), Epidermal Growth Factor (EGF), Platelet-Derived Growth Factor (PDGF), Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF), angiogenin the CXC chemokine Interleukin-8 (IL-8), Macrophage Inflammatory Protein-1 (MIP) and Platelet Factor-4 (PF-4)(Moore BB et al 1998). A rule is that all these factors must be used in perfect harmony, in a complimentary and coordinated manner to form functional vessels.

IMPORTANT ANGIOGENIC FACTORS AND THEIR MOLECULAR MECHANISMS

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor-vascular permeability factor (VEGF/VPF) was initially detected as a factor secreted by tumor cells into tissue culture medium or ascites fluid *in vivo*. (Senger DR et al 1983) The factor was identified as a glycoprotein of molecular weight 34 to 42 kD and was termed VPF. It was later demonstrated that this permeability factor not only increased permeability but also stimulated division of endothelial cells (Connolly DT et al 1989) and the term VEGF became more pronounced. Later studies on the basis of amino acid sequence analysis, it became evident that VEGF is a homodimeric heparin-binding glycoprotein that exists in at least four isoforms due to alternative splicing of the primary messenger RNA (mRNA) transcript. The isoforms are

designated as VEGF (121), VEGF (165), VEGF (189), and VEGF (206), according to the number of amino acids that each protein contains (Thomas KA 1996). And the single gene for this VEGF is located on chromosome 6 (Mattei et al 1996). Recent findings indicate that mice lacking PIGF (Placental growth factor) exhibit deficiencies in certain models of adult vascular remodeling, raising the interesting possibility that the activity of PIGF may also be attributed to these settings (Carmelite P 2000). It is thus included as the fifth member in the VEGF family. It is of interest to note that receptors for VEGF (VEGFRs) are expressed almost exclusively on endothelial cells, limiting its effects at the endothelium only. Rarely, expression of the various VEGF receptors has been demonstrated on cells of neural origin, Kaposi's sarcoma cells, hematopoietic precursor cells, and other rare tumor cell types. (Ziegler BL et al 1999, Ferrer FA et al 1999). Recent studies have also shown that VEGF stimulates surfactant production by alveolar type II cells (Compernolle V et al 1002)

VEGF is a survival factor for endothelial cell both *in vitro* as well as *in vivo* (Gerber HP et al 1998b). VEGF induces a potent angiogenic response in a variety of *in vivo* models and (Leung DW 1989, Plouet J 1989) and promote endothelial cell growth *in vitro*. In *in vitro* systems VEGF prevents apoptosis induced by serum starvation and have shown that such activity is mediated by the phosphatidylinositol (Pl) –3-kinase-Akt pathway (Gerber HP et al 1998b, Benjamin 1999, Yuang R et al.1996). VEGF also induces expression of the anti-apoptotic proteins Bcl 2 and AP1 in endothelial cells (Gerber HP et al 1998a). VEGF induced permeability enhancing activity underlies significant of this molecule in inflammation and other pathological circumstances (Dvorak HF 1995). VEGF induces vasodilatation *in vitro* in a dose dependent fashion as a result of

endothelial cell derived nitric oxide (Ku OD et al 1993), and produces tachycardia, hypotension and a decrease in cardiac output when injected intra venous in conscious instrumented rats (Yuang R et al 1996).

Several major growth factors, including EGF, TGF α and β, KGF, IGF-1, FGF and PDGF up regulates VEGF mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment (Neufeld G et al 1999, Ferrara and Davis-Smyth 1997, Semenza G 1998). Inflammatory cytokines such as IL-1α and IL-6 also induces expression of VEGF in several cell types, including synovial fibroblast, suggesting that VEGF may be a mediator of angiogenesis and permeability in inflammatory disorders (Neufeld G et al 1999). Very recent studies have shown, however, that myeloid cell activation and infiltration, key aspects of acute inflammatory responses, require HIF1-α but are largely independent of VEGF (Cramer T 2003)

Various members of the VEGF family have overlapping abilities to interact with a set of cell-surface receptors that trigger responses to these factors. The main receptors that seem to be involved in initiating signal transduction cascades in response to the VEGFs comprise a family of closely related receptor tyrosine kinases consisting of three members (Eriksson and Alitalo 1999). The fms-like tyrosine kinase (Flt-1 i.e. VEGFR-1) and fetal liver kinase 1/kinase insert domain-containing receptor (Flk-1/KDR, i.e. VEGFR-2) are high-affinity VEGF/VPF receptors. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin-like domains in extra cellular domain, a single trans membrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (Shibuya M

et al.1990, Terman BI et al 1991). VEGFR-3 (fms-like-tyrosine kinase (Flt-4)-) is a member of the same family of RTK binding to VEGF-C and VEGF-D (Karkkainen MJ 2002), which is mostly associated with lymphangiogenesis. In addition to these, there are a number of accessory receptors such as the neuropilins which seem to be involved primarily in modulating the binding to the main receptors, although roles in signaling have not been ruled out (Soker S et al 1998).

Both Flt-1 and Flk-1/KDR (Flk-1 has 85% homology with the human homologue, KDR) have been shown to be important regulatory systems for vasculogenesis and physiologic angiogenesis. (Veikkola T et al 2000). However, the interaction of VEGF/VPF with Flk-1/KDR is believed to be the more important interaction for tumor angiogenesis, as it is essential for induction of the full spectrum of VEGF/VPF functions.(Ferrara N et al 1989). In fact intracellular signal transduction pathways in endothelial cell through VEGFR-1 dimerization lead to the permeability enhancement, cellular proliferation and migration. Whereas VEGFR-2 seems to mediate the major growth and permeability actions of VEGF, VEGFR-1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGFR-2. Thus, mice engineered to lack VEGFR-2 fail to develop a vasculature and have very few endothelial cells (Shalaby F et al 1995), whereas mice lacking VEGFR-1 seem to have excess formation of endothelial cells, which abnormally coalesce into disorganized tubules (Fong GH et al 1998). Mice engineered to express only a truncated form of VEGFR-1, lacking its kinase domain, appear rather normal, consistent with the notion that the primary role of VEGFR-1 may be that of a decoy receptor (Hiratsuka S et al 1998).

Under conditions of serum starvation, the sustained activation of c-Jun (directly activate DNA polymerase like c-fos) NH_2 terminal kinase (JNK) (or stress activated protein kinase (SAPK) could be counteracted by VEGF-mediated activation of MAPK, leading to the prevention of apoptosis in micro vascular endothelial cells (Gupta K et al 1999). VEGF-mediated signaling was also able to confer a proliferation inhibitory signal in endothelium through regulating cell cycle progression by p38 MAPK activation (Yu and Sato 1999) to induce endothelial expression of ECM-degrading enzymes and to recruit pericytes (Puri MC et al 1995, Lamoreaux WJ et al 1998). VEGFR-2 and endothelial NO synthase co-localized with caveolin in plasma membrane caveolae, suggestive of VEGF signaling events within the caveolar compartment of endothelium (Feng Y et al 1999). JNK kinases participate in cellular processes via the modulation of transcriptional activation factors such as AP-1. Other transcriptional activation factors down-stream of the VEGF signal transduction pathways shown to be functional in endothelial cells are nuclear factor of activated T cells (NFAT) and nuclear transcription factor ETS (Chen Z et al 1997). Whereas AP-1 and NFAT switch on genes regulating tissue factor expression upon VEGF activation, ETS regulates the expression of u-PA, MMPs, and integrin 3 (Iwasaka C et al 1996, Oda N et al 1999).

Fibroblast Growth Factors. (FGF-2)

Different operations in the overall process of angiogenesis may be regulated by different angiogenic factors (Kumar R et al 1998). Although Fibroblast Growth Factor (FGFs) was originally named after its fibroblast mitogenicity (Gospodarowicz D et al 1987), some FGF do not induce fibroblast

growth at all. Members of FGF family generally share 30-50% homology in amino acid sequence and have two conserved cysteine residues, and bind with high affinity to heparin. The FGF family consists of 21 structurally related polypeptides, of which FGF-1 (acidic FGF) and FGF-2 (basic FGF) and FGF-7 (Keratinocyte growth factor, KGF) are the most extensively studied. These molecules regulate diverse cellular functions such as growth, survival, apoptosis, motility and differentiation (Kanda et al 1997). FGF-1 and FGF-2 are the prototypic FGF members named because of their different isoelectric points. They share 55% homology in amino acid sequence, similar in size and is formed differently depending on translation extensions and truncations (15-18kDa for FGF-1 and 16-24kDa FGF-2). Both neither FGF-1 nor FGF-2 genes include a secretary signal sequence and the principal mechanism of their release into extra cellular fluid has not yet been resolved. Export from cells without compromising cell integrity or requiring cell death possibly follows a nonclassical, synaptotagmin-1-dependent exocytotic pathway (LaVallee TM et al 1998).

These two, FGF-1 and FGF-2, are chemotactic and mitogenic for endothelial cell and induce the release of agents that break down basement membrane facilitating angiogenesis (Galzie Z et al 1997). FGF transduce signals intracellularly via high affinity interactions with a number of cell surface receptors. The cellular effects are mediated via specific binding to these high-affinity tyrosine kinase receptors (Klein S et al 1997). In addition, low-affinity FGF receptors exist, that consist of polysaccharide components of heparan sulfate proteoglycans (HSPGs) on cell surfaces and in ECM. Binding to the latter receptors (low affinity receptors) has been proposed as a mechanism to stabilize and protect FGF from inactivation. Heparan sulfate on cell surfaces, on the other hand, plays a more

active role in displacing ECM-bound FGF-2 and subsequent presentation to the high-affinity signal transducing receptors (Miao HQ et al 1996). Of note is that angiogenesis seems exquisitely sensitive to small changes in factors such as FGF-2 and VEGF that drive the cascade of angiogenesis.

FGF-2 is the angiogenic factor mostly associated with increasing activity of degradative enzymes. Recently it was shown that a secreted FGF-2 binding protein could bind FGF-2 that is normally inactive due to strong adherence due to HSPGs in the ECM. Plasmin is believed to be the most important protease for the mobilization of FGF-2 from ECM pool. The displaced FGF-2 molecules were thus released to mediate biological functions. FGF-2 consists of two modifications, an 18kDa, low molecular weight and a 22-24kDa high molecular weight form. During angiogenesis binding of 18kDa form of FGF-2 to endothelium induces FGF-Receptor down regulation, increased motility, proliferation and protease activity and modulates integrin level. It is now known that FGF-2 (and TNF-α) induced αvβ3 dependant angiogenesis *in vivo.* 22kDa form of FGF-2 may act on endothelial cell proliferation after nuclear translocation in the endothelial cell (Gleizes PE 1995).

Four distinct FGF-Receptors from four separate genes have been identified: FGFR-1 (*flg*), FGFR-2 (*bek*), FGFR-3 and FGFR-4 (Bernard and Mathew 1994). The prototype FGF receptor has an extra cellular region containing three immunoglobulin (Ig) like domains, a trans membrane region and a cytosolic tyrosine kinase domain activated by ligand binding. FGFR-1 to FGFR-3 (but not FGFR-4) undergoes further complexity by differential RNA splicing such that a total of 145 unique FGFR may be produced (Galzie Z et al 1997). This alternative

splicing may modify all ligand binding specificity, signal transduction, and membrane attachment. For example one variant of FGFR-2 shows high affinity binding of FGF-1 and KGF but not FGF-2, while another variant binds FGF-1 and FGF-2 but not KGF. Some isoforms with truncated or absent tyrosine kinase may act as competitive antagonists.

Signal transduction occurs by ligand induced receptor dimerisation followed by tyrosine kinase cross-phosphorylation (transphosphorylation) at particular sites of FGFR cytoplasmic domains. After FGFR dimers are internalized by receptor-mediated endocytosis, three phospho-tyrosine residues then serve as highly selective binding site for specific cytoplasmic SH2 (src homology region 2) molecules, continuing the signal transduction pathway (Fantl N et al 1993).

In addition to initiating receptor signaling, FGF can be endocytosed and transported to the cell nucleus. This transport affects the cell cycle in the late G_1, stage, promoting transition to the S stage (Imamura T et al 1994). Entrance of FGF-2 into the nucleus correlated with phosphorylation of nucleolin and subsequent increases of rDNA transcription, likely to be mediated by the protein kinase CKII (Bouche G et al 1994). FGF-2 uptake by endothelial cell was furthermore shown to be a route of growth factor degradation and can therefore act to regulate FGF-2 activity (Gleizes PE et al 1995).

Receptor dimerization by FGF may also is facilitated by heparin. It results in protein kinase activity and receptor autophosphorylation. As with VEGFR signaling, this autophosphorylation enables adaptor proteins such as Grb2, Shc, and Nck to bind and subsequently activate the Ras/Raf- MAPK (Mitogen Activated Protein Kinase) pathway of endothelial cell proliferation activation

(Klein S et al 1997). p42 MAPK activation was also implicated in endothelial cell motility regulatory responses to FGF. p42 MAPK driven phosphorylation of cytoplasmic phospholipase-A_2 enabled arachidonic acid release upon FGF-2 activation of bovine aortic cells (Sa G et al 1995). Besides activating MAPK mediated cell proliferation, FGF-2 induced murine brain endothelial cell proliferation via a serine/threonine kinase that phosphorylates ribosomal protein S6 ($p70^{S6k}$). This proliferation activation route was restricted to endothelial cells cultured on fibronectin. When allowed to differentiate to form tube-like structures in collagen gels, $p70^{S6K}$ was not activated (Kanda S et al 1997).

Angiopoeitins

Angiopoietins and receptor tyrosine kinase Tie1 and Tie2 play critical roles in the later stages of angiogenesis. They are required for communication of endothelial cells with the surrounding mesenchyme to establish stable cellular and biochemical interactions (Maisonpierre PC et al 1997). There are four definitive members of the angiopoietins family have been identified. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are Tie2-specific ligands that activate or antagonize Tie2 signaling in endothelium, respectively. Using homology based cloning; two new members of the angiopoietins, Ang-3 (mouse-specific) and Ang-4 (human-specific) were identified. They are distributed differently in the respective species, where Ang-3 acts as an antagonist and Ang-4 as an agonist of receptor tyrosine kinase signaling. Their respective roles in vascular maintenance have not been established yet (Valenzuela DM et al 1999).

All of the known Angiopoietins bind primarily to Tie2 and it is unclear whether these Angiopoietins can in some way or other also engage Tie1 also. Tie1

function is believed to be related to endothelial cell differentiation and the establishment of blood vessel integrity. Tie2, on the other hand, is particularly important for vascular network formation (Dumont DJ et al 1994, Puri MC et al 1995, Sato TN et al 1995). Tie2 expression is restricted to the endothelial cells. Surprisingly, Tie2 was present on quiescent as well as angiogenic endothelium in the adult rat. Moreover, the receptor tyrosine kinase was constitutively phosphorylated in both types of vasculature. These data suggest that Tie2 has a dual function involving both angiogenesis and vascular maintenance (Wong AL et al 1997).

Angiopoietin-1 is an endogenously secreted glycoprotein of approximately 75 kDa. Its receptor, Tie-2, is generally restricted to the endothelium and of importance in angiogenesis during development, tumor growth, and wound healing (Sato TN et al 1995, Lin P et al 1997, Wong AL et al 1997, Stratmann A et al 1998). *In vitro*, Ang-1 stimulated tyrosine phosphorylation of Tie-2 in endothelial cells, inhibited serum starvation-induced endothelial apoptosis induced sprouting angiogenesis, and stabilized HUVEC network organization (Koblizek TI et al 1998, Korpelainen and Alitalo 1998, Kwak HJ et al 1999). When combined with other angiogenic factors such as VEGF or growth factor supplements containing FGF-1, the survival of both endothelial cells and vascular networks increased even more (Kwak HJ et al 1999, Papapetropoulos A et al 1999). Although being chemo tactic for endothelial cells and Tie2-transfected fibroblasts, no mitogenic responses of endothelial cells to Ang-1 could be observed (Koblizek TI et al 1998, Witzenbichler B et al 1998). In postnatal neovascularization, Ang-1 is likely to promote vascular network maturation. In contrast, Ang-2 rendered endothelium sensitive to angiogenic factors via induction of smooth muscle cell/pericyte loss

and hence destabilized the neovasculature (Maisonpierre PC et al 1997, Asahara T et al 1998). The observation that Ang-2 was able to phosphorylate Tie2 when expressed by fibroblasts indicates that in endothelial cells other regulatory mechanism(s) prevail leading to antagonistic activity. Whereas Ang-1 is widely expressed, Ang-2 is only found at sites of vascular remodeling. Here it may block vessel stabilization, maturation, or survival signals from Tie2 (Maisonpierre PC et al 1997, Korpelainen and Alitalo 1998). In human glioblastomas, a cell-specific up-regulation of Tie2, Ang-1, and Ang-2 during tumor progression was demonstrated in a pattern compatible with a role in tumor-induced angiogenesis (Stratmann A et al 1998).

The Ephrin

The Eph receptor tyrosine kinases comprise the largest known family of growth factor receptors, and use the similarly numerous ephrins as their ligands (Gale and Yancopoupos 1999). The ephrins are unlike ligands for other receptor tyrosine kinases in that they must be tethered to the membrane to activate their Eph receptors (Davis S et al 1994). Although initially characterized in the nervous system (Flanagan and Vanderheaghen 1998), recent knockout studies have suggested key roles for ephrin-B2 and its EphB4 receptor during vascular development (Wang HU et al 1998). Mouse embryos lacking ephrin-B2 and EphB4 suffer fatal defects in early angiogenic remodeling that are somewhat reminiscent of those seen in mice lacking Angl or Tie2 (Adams RH et al 1999, Gerety SS et al 1999). Moreover, ephrin-B2 and EphB4 display remarkably reciprocal distribution patterns during vascular development, with ephrin-B2 marking the endothelium of primordial arterial vessels while EphB4 marks the

endothelium of primordial venous vessels. These distributions suggested that ephrin-B2 and EphB4 are involved in establishing arterial versus venous identity, perhaps in fusing arterial and venous vessels at their junctions, and that defects in these processes might account for the early lethality observed in mouse embryos lacking these proteins (Adams RH et al 1999, Gerety SS et al 1999). Ephrin-B2 continues to selectively mark arteries during later embryonic development as well as in the adult, although this expression extends progressively from the arterial endothelium to the surrounding arterial smooth muscle to pericytes. The finding that angiogenic sprouting in the adult and in the tumours involves re expression of the ephrin- B2 arterial marker suggests that it may be important in these angiogenic settings.

In conclusion VEGF maintains its position as the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting, during development as well as in the adult. FGFs, Ang 1 and ephrin-B2 are subsequently required for further remodeling and maturation of this initially immature vasculature, with ephrin-B2 being particularly important in distinguishing developing arterial and venous vessels. Following vessel maturation, Ang 1 seems to continue to be important in maintaining the quiescence and stability of the mature vasculature. Disruption of this stabilizing signal coincides with re-initiation of vascular remodeling in the adult — as occurs in the adult female reproductive system or in tumours. VEGFs, FGFs, angiopoietins and ephrin-B2 apparently recapitulate their developmental roles during vascular remodeling in the adult, and administration of individual factors to the adult allows them to reprise these roles but not to trigger the entire process. Thus VEGF administration can initiate vessel formation in adult animals, but by

itself promotes formation of only leaky, immature and unstable vessels. In contrast, Ang 1 administration seemingly further stabilizes and protects the adult vasculature, making it resistant to the damage and leak induced by VEGF or inflammatory challenges. Altogether, it is becoming clear that precise understanding of the normal developmental roles of the VEGFs, FGFs, the angiopoietins and the ephrins will greatly aid in understanding how to manipulate these growth factor systems for therapeutic benefit.

ROLE OF EXTRA CELLULAR MATRIX IN ANGIOGENESIS

After proper activation of endothelial cells, endothelial proliferation into new areas of the body is achieved by degradation of basement membrane (BM) by matrix metalloproteinases (MMPs). These ECM endopeptidases are secreted as zymogens that become activated in the ECM compartment and subsequently degrade components of ECM (Stetler Stevenson 1999). By degrading the ECM, would allow endothelial cells to invade the stroma. Indeed cleavage of collagen type-1 is required for endothelial cell invasion of the ECM and for vessel formation (Seandel M et al 2001).

Proteases And Their Inhibitors

There are eight distinct classes of MMPs, five are secreted and three are membrane type (Table 1). The zymogene form of MMPs are kept inactive by an interaction between a cysteine-sulphydril group in the propeptide domain and Zinc ion bound to the catalytic domain: activation requiring proteolytic removal of the propeptide prodomain. Most of the MMPs are activated outside the cell by other MMPs or serine proteases (Egeblad and Werb 2002). To date, it is believed that

Table 1.1. The matrix metalloproteinase family.

Subfamily	Name	MMPs	Main substrates
Interstitial Collagenases	Fibroblast Collagenase	MMP-1	Fibrillar collagen
	Neutrophil Collagenase	MMP-8	Fibrillar collagen
	Collagenase-3	MMP-13	Fibrillar collagen
	Collagenase-4	MMP-18	Fibrillar collagen
Gelatinases	Gelatinase A	MMP-2	Gelatin, type IV collagen, fibronectin, elastin, laminin
	GelatinaseB	MMP-9	Gelatin, elastin, fibronectin, vitronectin
Stromelysins	Stromelysin-1	MMP-3	Gelatin, fibronectin, casein, laminin, elastin, MMP-2/TIMP-2
	Stromelysin-2	MMP-10	Same as above
	Stromelysin-3	MMP-11	Fibronectin, laminin, gelatin, aggrecan
	Matrilysin	MMP-7	Fibronectin, vitronectin, laminin, gelatin, aggrecan
Elastases	Metalloelastase	MMP-12	Elastin, gelatin, collagen IV, fibronectin, laminin, vitronectin, proteoglycan
Membrane-Type MMPs	MT1-MMP	MMP-14	proMMP-2, procollagenase 3
	MT2-MMP	MMP-15	pro-MMP-2
	MT3-MMP	MMP-16	proMMP-2
	MT4-MMP	MMP-17	Unknown
Other MMPs		MMP-19	
	Enamelysin	MMP-20	Amelogenin

MMP-2, MMP-9 and MMP-14 directly regulate angiogenesis, and MMP-19 might also be important, as it is expressed in blood vessels. However, MMP-2 and MMP-9 are more important in tumour angiogenesis (Kolb C et al 1997). Cleavage of type-IV collagen by MMP-2 exposes a cryptic αvβ3 integrin binding site within the collagen, which is necessary for the migration of endothelial cells and therefore angiogenesis (Xu J et al 2001). MMP-9 acts by increasing the bioavailability of proangiogenic factor VEGF, although it is not known exactly how (Bergers G et al (2000).

Plasmin, a serine protease, has broad trypsin like specificity and degrades fibronectin, laminin, the protein core of proteoglycan etc. found in ECM. It is generated from Plasminogen by the action of Plasminogen activators u-PA and t-PA. It also activates certain MMPs. It is believed to be the most important protease for the mobilization of FGF-2 – a potent proangiogenic factor-from the ECM pool (Griffioen and Molema 2000). The low molecular weight FGF-2, upon binding to its receptor on endothelium, induces FGF-receptor down regulation, increases motility, proliferation, proteanase activity and modulate integrin levels. High molecular weight FGF-2 may act on endothelial cell proliferation after nuclear translocation in the cells (Gleizes PE et al 1995, Klein S et al 1997).

Some of the cleavage products of these proteases are good inhibitors of angiogenesis. Cleavage of plasminogen to generate plasmin also produces a peptide fragment called angiostatin (Dong D et al 1997, Cornelius LA et al 1998, Gomin-Rivas MJ et al 2000). A C-terminal fragment of the BM collagen typeXVIII is produced by the activity of MMPs called endostatin (Ferreras M et al 2000). Both of these fragments reduces endothelial cell proliferation and there by

inhibit angiogenesis. MMP activity and thereby angiogenesis is again inhibited by one another family of molecules called Tissue Inhibitor of Metalloproteinases (TIMPs). Even though the main inhibitor of MMPs in tissue fluids is $\alpha 2$ macroglobulin, TIMPs will also bind to MMPs inhibiting its proteolytic activity (Egeblad and Werb 2002).

Integrins

Integrins are transmembrane proteins composed of α and β subunits. Combinations of different integrins on cell surface allow cells to recognize and respond to a variety of ECM proteins (Varner JA 1997). The two important integrins associated with angiogenesis are $\alpha v\beta 3$ and $\alpha v\beta 5$. The integrin $\alpha v\beta 3$ is minimally expressed on resting endothelium but significantly up regulated on activated endothelium and is believed to play a curtail role in angiogenesis. The peptide and antibody inhibitors of $\alpha v\beta 3$ induced endothelial cell apoptosis suggesting a role for this integrin in endothelial cell survival during angiogenesis (Brooks PC et al 1994a,b). When FGF-2 or TNF-α induced, $\alpha v\beta 3$ dependant angiogenesis, VEGF or TGF-β initiated an angiogenesis dependant on $\alpha v\beta 5$ (Friedlander M et al 1995). Integrin mediated cell adhesion impacts two key aspects of growth regulation. 1) it can influence the activity of the basal cell cycle machinery consisting of cyclin-dependant kinase complexes and 2) they play a pivotal role in anchorage dependant cell death or 'anoikis' (Frisch and Ruoslahti 1997, Howe A et al 1998)

VASCULAR MATURATION

Endothelial cell interaction with ECM and mesenchymal cells is a prerequisite to form a stable vasculature. Therefore after endothelial cell proliferation, maturation and formation of tube structures, surrounding vessel layer composed of mural cells (pericytes and smooth muscle cells) need to be recruited. Endothelial cell may accomplish this via the synthesis and secretion of PDGF, a mitogen and chemo attractant for a variety of mesenchymal cells. Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be a cell-cell contact dependant process. On endothelial cell-mural cell contact a latent form of TGF-β, produced by both endothelium and mural cells, is activated in a plasmin-mediated process. Activated TGF-β can induce changes in myofibroblast and pericytes, which may contribute to the formation of a quiescent vessel, ECM production and maintenance of growth control (Griffioen and Molema 2000). The coinciding investment of growing capillaries by pericytes with the deposition of BM and cessation of vessel growth during wound healing also indicates vessel growth regulation by pericytes (Hirschi and D'Amore 1997). FGF-1 is also implicated in endothelial cell differentiation leading to vascular formation. Besides inducing plasminogen activator and endothelial cell proliferation and migration, FGF-1 receptor signaling resulted in endothelial tube formation in collagen (Kanda S et al 1996).

ROLE OF IMMUNE SYSTEM IN ANGIOGENESIS

Although their relative role is not yet fully elucidated, it is well appreciated that the immune system plays an important role in the regulation of angiogenesis.

Multiple studies indicate that leukocyte can induce vascular proliferation and specific leukocyte derived cytokines have been identified to induce angiogenesis Normal endothelial cells contribute to the recruitment of immune cells to the site of inflammation by the expression of adhesion molecules (Carlos and Harlan 1994) and an intense inflammatory reaction is often associated with an increased risk of metastasis, suggesting that angiogenesis induced by inflammation may contribute to the tumour progression and metastasis. Macrophages have been recognized for a number of years as important angiogenesis effecter cells (Sunderkotter C et al 1994). They may influence new capillary growth by producing factors that act directly to influence angiogenesis linked endothelial functions. *In vitro* studies have shown that macrophages (Mϕs) produce more than twenty molecules that is reported to influence endothelial cell proliferation, migration and differentiation (Polverini and Leibovich 1984). A second mechanism by which Mϕs modulate angiogenesis is by modulating the extra cellular matrix (ECM), either through the production of ECM components or through the production of proteases that alter the composition of ECM (Sunderkotter C et al 1994). Mϕs has also been shown to express angiogenesis inhibitors like thrombospondin-1 (TSP-1), when treated with chemopreventive agent retinoic acid (Polverini PJ 1996). Different families of adhesion molecules play a role in the process of endothelial cell-leukocyte interaction. These include 1) immunoglobulin super family members such as ICAM 1, VCAM 1 and PECAM 1 (CD31); 2) selectins; 3) a group consisting of CD34 and L selectin binding glycoprotein (Kuzu et al 1992); and 4) CD44, the lymphocyte homing receptor which is expressed only on activated endothelial cells (Griffion AW et al 1997). Expression of endothelial cell adhesion molecules is controlled by cytokines such as TNF, IL-1, and IFN. A link between leukocyte-

endothelial cell adhesion molecules required facilitating leukocyte recruitment, and expression of endothelial cell adhesion molecules required to facilitate leukocyte recruitment is differentially regulated in angiogenesis. Although inflammatory signals increase expression, angiogenic stimuli often down regulate adhesion molecules involved in leukocyte-endothelial cell interaction. The net adhesion molecule receptor make-up on endothelium is hence determined by both signals (Kitayama J et al 1994).

Inflammation And Angiogenesis

The relation between angiogenesis and leukocyte interaction in chronic inflammation is well studied. In the first acute phase of inflammation, functional changes in the vasculature such as dilation, increased permeability and endothelial cell activation occurs. In the second sub acute phase capillaries and venules remodel with extensive endothelial cell mitotic activity (Majno G 1998). Upon chronic stimulation, both increases in capillary density and vascular dilation can be observed, although these responses can differ significantly between species (Thurston G et al 1998). A local inflammatory reaction consisting of T-lymphocytes and macrophages often is associated with invasive cutaneous melanoma, and an intense inflammatory reaction often is associated with an increased risk of metastasis, suggesting that angiogenesis induced by inflammation may contribute to melanoma progression and metastasis (Gutman H et al 1994). In many chronic inflammatory diseases in human, neovascularization can be identified in inflamed lesions. The increased levels of VEGF that correlated with disease activity in patients with inflammatory bowl diseases indicate the role of this cytokine in promoting inflammation (Bousvaros A et al 1999). All these

observations indicate that in a variety of conditions, inflammation and angiogenesis exists at the same time, and several of the inhibitors of angiogenesis can act as antiinflammatory as well and vice versa.

TUMOUR ANGIOGENESIS

It could be noticed that the process of angiogenesis may be differentially regulated in various disease settings. Most of the experimental data on angiogenesis control published so far deal with angiogenesis on cancer. These suggest that the tumour vasculature and angiogenesis is not as well organized and coordinated as in physiological angiogenesis. The observation that angiogenesis occurs around the tumour was made nearly 100 years ago (Goldman E 1907, Ide AG et al 1939, Algire and Chalkiey 1945). The hypothesis that tumour produces diffusible 'angiogenic' substance was put forward in 1968 (Greenbtatt and Shubik 1968, Ehrmann and Knoth 1968). It was in 1971, Folkman proposed that tumour growth and metastasis is angiogenesis dependant and hence blocking angiogenesis could be a strategy to arrest tumour growth. Eventually it became evident that a tumour mass greater than 0.5mm in diameter fails to enlarge further with out vascularisation because hypoxia induces apoptosis via activation of p53 gene (Razmi SC et al 1999). Hence any further increase in size requires the proliferation and morphogenesis of vascular endothelial cells and tumour cells have mechanism to induce the host endothelial cell to enter the angiogenic cascade.

Tumour vessels are structurally and functionally abnormal. They lack several characteristics that normal endothelial cell acquire during growth. For example they may lack functional perivascular cells, which are needed for protection against any mechanical damage or hormonal imbalance (Benjamin LE

et al 1999). A homogenous layer of endothelial cells does not always form the vessel wall, instead may heterogeneously lined with cancer cells also (Jain RK 1988, Chang YS et al 2000). The presence of cancer cells in tumour vessel has significant implication for metastatic spreading of tumour. In terms of ultra structure also, tumour vessels are abnormal. Cytokines and angiogenic molecules secreted by cancer and immune cells can modulate the expression of cellular adhesion molecules and other surface markers on the tumour endothelium. For example VEGF and TNF-α up regulates whereas FGF and TGF-β, down regulate adhesion molecules on endothelial cells (Jain RK et al 1996).

Recent findings have postulated a possibility that, many tumours, and metastases in particular, do not initiate in an avascular manner. Rather, tumour cells can initially home in on and grow by co-opting existing host vessel (Holash J et al 1999), and form a perivascular cuff on it. In response to co-option, host vessel mounts a defense- they regress, choking off the tumour and resulting in a secondarily avascular and hypoxic tumour. However successful tumours seem to overcome host vessel regression by inducing robust new angiogenesis. Angiopoetin-2 and VEGF inductions correlates remarkably well with the above process (Zagzag D et al 1999). Soon after tumour co-option, host vessel start expressing high autocrine levels of Ang-2. Consistent with the possibility that autocrine Ang-2 can destabilize vessel, the co-opted vessel begin to die, the tumour becomes secondarily avascular and hypoxic, and resulting in marked induction of tumour derived VEGF. These high levels of VEGF correlates with cessation or regression of the destabilized co-opted vessel, and onset of sprouting angiogenesis form these vessels (Yancopoulos GD et al 2000). The possibility that tumour vessel Tie-2 receptors are blocked continuously by Ang-2 and thus have an

imbalance towards VEGF may well explain the earlier mentioned observations that tumour vessels fail to mature, exhibit poor association between endothelial cell and supporting cells and are characterized by their leaky and hemorrhagic state.

Sprouting Angiogenesis

During sprouting angiogenesis, vessels initially dilate and become leaky in response to VEGF, Ang-1 and the junctional molecules. VE-cadherin and Platelet endothelial Cell Adhesion Molecule (PECAM/CD31) tighten vessel and their action needs to be overcome during angiogenesis. Ang-2 and proteinases mediate dissolution of the existing basement membrane and interstitial stroma matrix. Numerous molecules affect endothelial cell proliferation, migration and assembly, including VEGF, Ang-1 and FGF-2. Cell-matrix receptors such as the $\alpha v\beta 3$ and $\alpha 5$ integrins mediate cell spreading and migration. Maturation of nascent vessel involves formation of a new vessel in the tubular form with pericytes and smooth muscle cells investment. PDGF-BB recruits smooth muscle cells where as signaling by TGF-β1 and Angl/Tie-2 stabilizes the interaction between endothelial cell and smooth muscle cells (Carmelite and Jain 2000). It is important to notice that, certain molecules that initially induce angiogenesis are subsequently (proteolytically) processed to angiogenesis inhibitors, there by preventing a negative feedback.

THERAPEUTIC INHIBITION OF ANGIOGENESIS TO CONTROL TUMOUR GROWTH

Experimental and clinical data indicate that early in their growth most of the tumours do not induce angiogenesis. Over a period of time, ranging from months to years, tumours become more aggressive and acquire greater

invasiveness and malignant potential and switch to an angiogenic phenotype (Razmi SC et al 1999b). Thus tumour angiogenesis, although influenced by micro environmental conditions (eg. hypoxia), can be viewed as being, to large extent genetically driven. As such, angiogenesis is a means by which genetic alterations in cancer cells transcend cellular boundaries and secure an external –host dependent- support mechanism for tumour cell survival. This is consistent with the observation that when neovasculature is compromised, tumour cells are prone to undergo apoptosis, mainly as a result of hypoxia and metabolic stress (Rak J et al 2002), and tumour regression results. Taken together, the anti angiogenic strategy to arrest tumour growth, can be designed as 1) a chemeprenventive agent (delivered prior to or at the time of tumour inoculation in experiments) 2) adjuvant therapy (delivered when tumour is at relatively small volume or along with other treatment modalities) or 3) a therapeutic modality (delivered to the host with established tumours). A more recent advancement is antiangiogenic gene therapy, which offers the potential for cancer patients to manufacture their own antiangiogenic proteins to arrest the tumour growth (Feldman and Libutti 2000).

Antiangiogenic agents currently used in clinics and laboratories can be categorized into several broad classes based on the biologic activity of the compound used. The mode of action of these compounds range from inhibition of MMPs to be cytostatic for endothelial cells. Folkman and Kerbal postulated that the angiogenic inhibitors could be grouped into direct and indirect inhibitors (Kerbel and Folkman 2002). Direct angiogenesis inhibitors such as angiostatin, vitaxin and others, prevent vascular endothelial cell from proliferating, migrating or avoiding cell death in response to a spectrum of pro angiogenic proteins including VEGF and bFGF. Direct angiogenesis inhibitors are the least likely to

induce acquired drug resistance because they target genetically stable endothelial cell rather than unstable mutating tumour cells (Kerbel RS 1991).

Indirect angiogenesis inhibitors generally prevent the expression of or block the activity of a tumour protein that activates angiogenesis, or block the expression of its receptor on endothelial cell. Many of these tumour cell proteins are the products of oncogene that drive the angiogenic switch (Rak J et al 2002). An example for this is EGF receptor inhibitors such as 201839(Iressa), which prevent expression of VEGF, bFGF, TGF-α and IL-8 by tumour cells as well as possibly other inhibitors of oncogene products.

Ways To Interfere With Angiogenesis

A broad spectrum of strategies for modulation of angiogenesis has been described. Over more than forty antiangiogenic compounds have entered in clinical trials over which some of them are well characterized but others are not characterized regarding their mode of action and possible side effects.

Drugs that inhibits endothelial cell proliferation directly: -

The most successful approach to modulate angiogenesis, to date, is the use of agents that specifically inhibits growth of endothelial cells. O-chloroacetylcarbamoyl fumagillol or AGM-1470/TNP470 an analogue of the fungus derived antibiotic fumagillin (Ingber D et al 1990) is one among the first members that entered in clinical trials. The mechanism of action of this compound was found to be prevention of endothelial cell to enter G1 phase of the cell cycle, resulting in a decrease in proliferation (Castronovo and Belotti 1996)

Drugs that blocks matrix break down: -

Inhibition of proteases will prevent the ECM protein break down and thus inhibit angiogenesis. MMP inhibitors have probably been the most extensively studied category of molecules with antiangiogenic activity (Brown and Giavassi 1995). Batimastat, Marimastat and certain break down products of proteases (PEX) are known inhibitors of angiogenesis that are in clinical trials (Brooks PC et al 1998).

Drugs that inhibit endothelial specific integrin/survival signaling: -

When it was found that activated endothelial cells up regulate receptors for ECM components (Griffion AW et al 1997), interaction of endothelial cells with matrix was chosen as a target for inhibition of angiogenesis. This proved to be a relevant approaches by the demonstration that $\alpha v\beta 3$ integrin molecule, the biological function of which is binding of vitronectin and other RGD-containing matrix components (RGD = Arg-Gly-Asp, trypeptide moiety: infact $\alpha v\beta 3$ integrin mediate cellular adhesion to vitronectin, fibrinogen, laminin, collagen, vWF or osteopontin through their exposed RGD moiety) are, over expressed in angiogenically-stimulated blood vessels. Ligation of these with an antibody (LM609 in mice and Vitaxin in humans) interferes with endothelial cell growth (Brooks PC et al 1994a & b).

Drugs with non-endothelial cell specific mechanism of action: -

Recently a non-endothelial cell specific inhibitor of angiogenesis was described. Carboxy-amidotriazole (CAI) is an inhibitor of tumour cell motility: the mechanism of action being the inhibition of trans membrane calcium (Ca^{++}) influx.

The inhibition of Ca^{++} influx prevents the activation of focal adhesion kinase and RhoA. pathways. By interference in the biochemical pathways involved in endothelial spreading on ECM, the integrity of the vascular tube as well as stabilization of newly formed vessels was affected. Local administration of CAI inhibited capillary expression in CAM assay. *In vivo* studies confirmed the antiangiogenic effect of CAI for ovarian cancer (Kohn and Liotta 1995).

Drugs that block activators of angiogenesis: -

A separate method for modulation of angiogenesis is the interference with angiogenic factors such as VEGF or FGF and their receptors. Angiogenesis and subsequent tumour growth can be inhibited by blocking these factors this can be performed by treatment with blocking antibodies to these factors, antibodies to their receptors, with soluble receptors functioning as antagonists, dominant negative growth factor variants or VEGF anti sense constructs. SU5416 is a specific inhibitor of VEGFR-2 phosphorylation and interfering the growth factor signaling. SU6668 is a broad-spectrum inhibitor of angiogenesis and tumour growth, acting by interference with VEGF, FGF and PDGF receptors (Griffion and Molema 2000).

Several of the conventional chemotherapeutic agents are good inhibitors of angiogenesis as well. Doxorubicin by covalently conjugating to a peptide containing an RGD motif, Arap and co-workers constructed drug targeting conjugates specific for integrin αvβ3 and αvβ5 on endothelial cell of tumour vasculature (Arap et al 1998). Treatment of this conjugate caused vascular damage in tumours and had strong antitumour effect. Thalidomide is a drug that has now been tried in humans for arthritis and gastrointestinal ulceration. Although at

present the mechanism through which thalidomide induce these remission remains to be elucidated, it is believed to act via a combination of direct inhibition of TNF production and angiogenesis. It may, however, impair angiogenesis, possibly by down regulating endothelial cell integrin ($\alpha v\beta 3$) expression (Marriot MC et al 1999, Sands and Podolsky 1999), thus contributing to the curative effect in these pathologies.

Anticancer Activity Of Plant Products And Phytochemicals

Scientific studies using different solvent extracts of medicinal plants have shown to posses several pharmacological properties including antitumour/anticancer activities. Iscador, an extract of the plant *Viscum album* was found to inhibit 20-methylcholanthrin induced carcinogenesis in mice (Kuttan G et al 1996) and also inhibited lung metastasis induced by B16F10 melanoma cells (Antony S et al 1997). The plants used in the present study, *Boerhaavia diffusa* and *Tinospora cordifolia* are also well known for their medicinal properties, especially on the literatures of Ayurveda (Nadkarni AK 1999). It is of interest to note that many of the clinically used antineoplastic drugs (eg. Camptothecin, Taxol, Vincristin, Vimblastin etc) are of plant driveled metabolites and most of these compounds possess antiangiogenic activity as well. Several clinical trials on the use of nutritional supplements and modified diets to prevent cancer are ongoing. Phytochemicals (nonnutritive components of plant origin) that possess substantial anticarcinogenic and antimutagenic properties are also under intense investigation to prevent or treat cancer. Curcumin from turmeric, capsaicin from chillipepers, 6-gingerol from ginger, epigallocatechin-3-gallate from green tea, genistein from soyabeen, lycopene from tomatoes, sulphoraphane from broccoli, diallyl sulphide

from garlic, resveratrol from grapes, caffeic acid phenyl ester from honey, indol-3-carbinol from cabbage etc are well studied chemopreventive agents that target different cellular signaling molecules – that regulate cell proliferation and differentiation – to provide an effective preventive measure against canrcinogenesis. Numerous intracellular signal transuduction pathways converge with the activation of transcription factors NFκB and AP-1, which are ubiquitous eukaryotic transcription factors. These transcription factors mediate pleiotropic effects of both external and internal stimuli in the cellular signaling cascade, and they are the prime targets of diverse classes of chemopreventive phytochemicals (Surh YJ 2003).

Compounds Included In This Study: -

1. *Tinospora cordifolia* Miers: -

T. Cordifolia (Family-Manispermaceae) is an important medicinal plant cultivated throughout Indian subcontinent. It is extensively used in various Ayurvedic preparations for the treatment of various ailments such as jaundice, skin diseases, diabetes, anemia, emaciation and infections (Kirtikar and Basu 1933). Aqueous extract of *T. cordifolia* has shown significant radioprotection against γ-radiation induced damages in experimental animals (Goel and Premumar 2002). It has been shown that the aqueous extract of this plant stimulated the phagocytic and bactericidal activity of the neutrophils and macrophages (Rege NN et al 1999). The aqueous extract contains polyclonal B-cell activator, which may attribute to an acidic arabinogalactan polysaccharide. Various other biologically active compounds which have been isolated and characterized from *T. cordifolia* include

phenylpropanoids, norditerpene furan glycosides, diterpene furan glycosides and phytoecdysones (Chintalwar G et al 1999).

2. *Boerhaavia diffusa* Linn: -

In the traditional system of medicine *B. diffusa* (Family Nyctaginaceae) roots have been widely used for the treatment of dyspepsia, jaundice, enlargement of spleen, abdominal pain, and as an anti-stress agent (Chopra RN et al 1999). Oral administration of the alkaloidal fraction significantly inhibited SRBC induced delayed type hypersensitivity reactions in mice. Significant increase in antibody titre was observed by the treatment of this alkaloidal fraction whereas it failed to show any blastogenic responsiveness of murine splenocytes to mitogens Concavalin A (Con A) and Lipopolysaccharide (LPS) (Mungantiwar AA et al 1999). Boerhaavia is antiproliferative/antimitotic in nature and inhibited the growth of several cell lines of mouse and human origin, such as mouse macrophage, human monocytic cells, mouse fibroblastic cells, human embryonic kidney cells, mouse liver cells, human T cells (Jurkat cells), human erythroleukemic cells (K562), and mouse lymphoma cells (EL-4) (Mehrota et al 2002). Phytochemical research has demonstrated the presence of alkaloids and amino acids in B. diffusa (Garg S, 1978,1980). Two known lignans lilriodendrin and syringaresinol mono-β-D-glucoside, have been isolated from a methanol extract of the roots of B. diffusa. Lilriodendrin possess considerable calcium channel antagonistic effect (Lami N et al 1991).

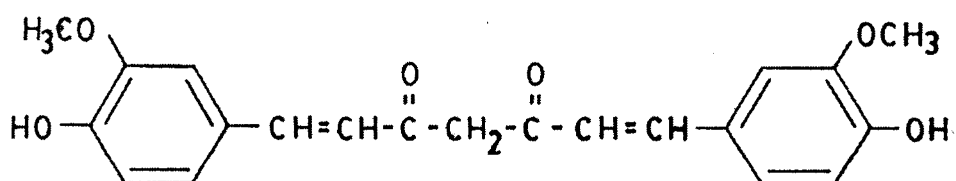
3. Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl) 1,6-heptadiene-3, 5-dione] and its natural derivatives (Figure 1.2, 1.3 & 1.4): -

Curcumin (diferuloylmethane/curcumin I) the phytochemical responsible for the colour of turmeric shows a wide range of pharmacological properties including antioxidant, antitumour, anticancer and antimetastatic activities. It is the most studied, active constituent of turmeric (*Curcuma longa*), which is now in phase II clinical trial for its anti-inflammatory property, and is planned for breast and colon cancer. Curcumin along with its two naturally occurring derivates, namely monodemethoxycurcumin (curcumin II) and bisdemethoxycurcumin (curcumin III) has been a subject of intense study to find out the structure – activity relationship. These studies have shown that curcumin I is considerably more active both as an antioxidant as well as an oxidative DNA cleaving agent (Ahsan et al 1999). In another study these curcumins showed good antioxidant, antiinflammatory and cytotoxicity to leukemia, colon, CNS, renal, melanoma and breast cancer cell lines. All three curcumins showed good inhibitory effect on inducible isoform of cyclooxygenase – COX II (Ramsewak RS et al 2000). Curcumin could inhibit IκBα phosphorylation in B16F10 melanoma cells through suppression of IKK activity that contribute to its antiproliferative, proapoptotic and antimetastatic activities (Philip S et al 2003)

4. Synthetic derivatives of curcumin: -

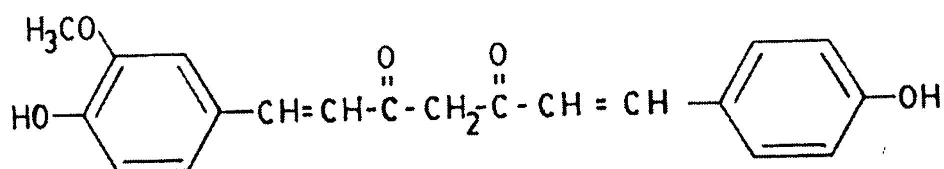
Considering the diverse pharmacological activity of curcumin, several synthetic derivatives of curcumin have been prepared and evaluated for their biological activity. Several of these synthetic curcumins have shown to possess antioxidant and free radical scavenging activity (Sreejayan and Rao 1997). The synthetic analogues such as Salicyl curcumin (Figure 5), Ansyl curcumin (Figure 6), Veretryl curcumin (Figure 8) and Dimethylamino curcumin (Figure 7) are some

Figure 1.2. Diferuloylmethane



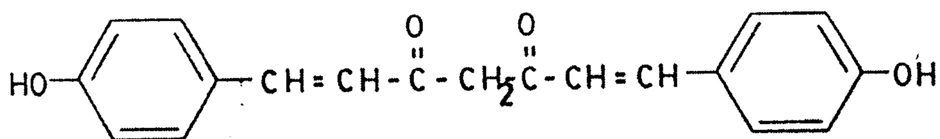
Curcumin-I

Figure 1.3. Monomethoxycurcumin



Curcumin-II

Figure 1.4. Bismethoxycurcumin



Curcumin-III

Figure 1.5. Salicyl curcumin

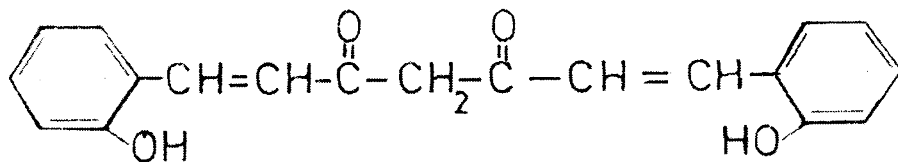


Figure 1.6. P-Anisyl curcumin

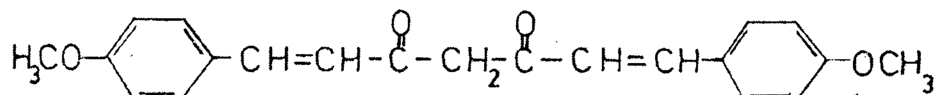


Figure 1.7. Dimethylamino curcumin

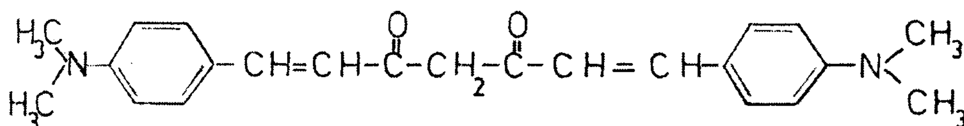
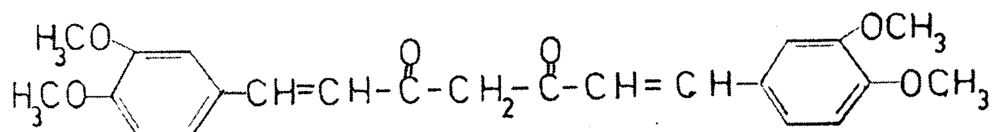


Figure 1.8. Veratryl curcumin



of them and studies have shown that they also possess some pharmacological properties like antiinflammatory (Anto RJ et al 1998) and antioxidant (Anto RJ et al 1996) activities. All these synthetic analogues of curcumin were also found to inhibit casein induced paw oedema (Anto RJ et al 1998) and possess antimutagenic properties (Anto RJ et al 1996a).

In the present study, we tried to evaluate the antiagiogenic activity of these pharmacologically well-known compounds. In oncology the primary goal of angio-suppression is not the cure, but the delay of tumour growth. The stop of tumour vessel reduces the tumour burden of the body, which might increase the efficacy of another, simultaneously applied adjuvant therapy. Chemotherapy, hyperthermia, radiation and immuno therapy are known to be much more effective, if the number of tumour cells is low. A combination of angio-suppression with such therapies might therefore increase the chance of cure. Angiogenesis inhibitors may be of endogenous or exogenous in origin. Proteolytic fragments such as angiostatin and endostatin, thrombospondin –1, platelet factor IV etc are examples for endogenous angiostatic molecules. The exogenous angiostatic molecules include, compounds derived from plants, microorganism or synthetic chemical molecules or derivatives of natural chemical molecules.

The heterogeneity in angiogenic stages in human tumour vasculature makes the antiangiogenic therapy alone is believed to be never sufficiently effective on its own. Combining antiangiogenic therapy with conventional tumour cell directed therapy: blockade of the formation of new blood vessels will hamper the availability of the chemotherapeutics in the tumour tissue. Whereas antiangiogenic therapies act on vessel in hypoxic tumour sites, chemotherapeutics would become

available in sites where the tumour vasculature is at rest. The question remains, whether the tumour cells in such a site are non-dividing cells, requiring few nutrients and being unresponsive to chemotherapeutics acting on cell regulatory process. On the other hand by lowering the tumour mass with antiangiogenic drugs, intra tumoural pressure may concurrently drop. As a result, the availability of chemotherapeutics can increase. In short, lack of proper knowledge on the mechanistic backgrounds of this potentiating effect prohibits any prediction on usefulness in cancer patients currently.

Chapter 2

Materials and Methods

A. MATERIALS

1. Test Compounds

A. Plant materials.

Authenticated plants such as *Boerhaavia diffusa* and *Tinospora cordifolia* were obtained from Amala Ayurveda Pharmacy, Thrissur, India. A partial purification of the polysaccharide fraction from the stem of *T. cordifolia* was prepared according to the protocol by Chintalwar et al (1999)

B. Natural curcumins.

Crude Curcumin, obtained by the alcoholic extraction of *Curcuma longa* was procured from Kancore (P) Ltd. Angamali, India. This was subjected to preparative TLC after dissolving in alcohol. Chloroform : Methanol (25 : 1) was the solvent system used for elution (Roughley 1973). Curcumin I (Mol. Wt.-368), Curcumin II (Mol. Wt.-338) and Curcumin III (Mol. Wt.-308) was thus separated and eluted in alcohol, the solvent was evaporated to yield the pure compounds (Anto RJ et al 1995).

C. Synthetic curcuminoids.

Synthetic Curcuminoids such as Salicyl curcuminoid (Mol. Wt.-308), Anisyl curcuminoid (Mol. Wt.-336), Veratryl curcuminoid (Mol. Wt.-396) and Dimethylamino curcuminoid (Mol. Wt.-320) was a kind gift from Dr. Subbharaju, Laila Impex, Banglore, India. The synthesis of these curcuminoids has already been accomplished (Dinesh Babu 1994).

2. Cells Used

L929 mouse lung fibroblast, B16F10 a highly metastatic mouse melanoma were obtained from National Centre for Cell Sciences, Pune, India. Human Umbilical Vein Endothelial Cells were isolated from the umbilical cord of neonatal according to the protocol of Jaffe et al (1973).

3. Animals

Sprague Dawley rat (180-200g), BALB/c mice and C57BL/6 mice (4-6 weeks old) were purchased from National Institute of Nutrition, Hyderabad, India.

4. Chemicals

Dulbecco's Modified Eagle Medium (DMEM)	:	Hi Media Laboratories, Mumbai, India.
Rosewell Park Memorial Institute Medium (RPMI 1640)	:	-do-
Medium 199	:	-do-
Trypsin	:	-do-
Casein	:	-do-
TBA (Thiobarbituric acid)	:	-do-
Fluid thioglycollate medium	:	-do-
L-Glutamine	:	-do-
ECM-Gel (From Engelbreth Holm-Swarm mouse sarcoma)	:	Sigma fine chemicals, St. Louis, USA.
Collagenase Type VIII (From Clostridium histoliticum)	:	-do-

Collagen solution Type I (From calf skin)	:	-do-
Gelatin-Type A (From porcine skin)	:	-do-
γ-GT substrate (L-Glutamic acid γ-*p*-nitroanilide / L-γ-Glutamyl-*p*-nitroanilide)	:	-do-
Glycyl-glycine	:	-do-
MTT (Methyl thiazol tetrazolium bromide)	:	-do-
Acrylamide	:	Sisco Research Laboratories, Mumbai, India.
N,N-methylene Bisacrylamide	:	-do-
Papain (Extracted from Papaya latex)	:	-do-
1, 4-Bis (5-Phenyloxazol-zyl) Benzene (POPOP)	:	-do-
2,5-Diphenyloxazole (PPO)	:	-do-
Hydroxyproline	:	-do-
N-acetylneuraminic acid	:	-do-
Glucosamine hydrochloride	:	-do-
Glucorono 4,6, lactone	:	-do-
Acetyl acetone	:	-do-
HEPES buffer	:	-do-
Folins reagent	:	-do-
Human recombinant FGF-basic	:	Pepro Tech, USA
Human recombinantVEGF	:	-do-
Fetal Calf Serum (FCS)	:	Biological Industries, Israel

^{3}H-Thymidine	: BRIT, Mumbai, India.
Crystal violet	: Romali, Mumbai, India.
Trypan blue	: -do-

5. Reagent Kits

Diffquick stain set	: Dade behring, USA
Gel code blue stain reagent	: Pierce, USA
Mouse ELISA kits for IL-1β, IL-6, TNF-α, GM-CSF and IL-2	: Pierce Endogen, USA
Mouse ELISA kits for VEGF and TIMP-1	: R&D Systems, USA
Mouse quantikine mRNA kit for VEGF	: -do-
Multiplex PCR kit for mouse inflammatory cytokines	: Biosource, USA
Cells-to-cDNA kit	: Ambion, USA

6. Tissue Culture Wares

Medium filtering assembly	: Millipore
Polycarbonate membrane filter (13mm, 8.0μM, PVP free)	: Whatman UK
Cellulose syringe filtering apparatus (0.2μM pore size)	: Sartorius, Germany
Tissue culture flask (25 cm^2)	: Tarson,Culcutta, India.
96-well flat bottom cluster plate	: -do-
24-well tissue culture plate	: Laxbro, Pune, India.

7. Instruments Used

Inverted microscope	: Leica, Germany.
Upright research microscope	: Meiji, Japan.

Horizontal Laminar flow hood	: Kemi,Ernakulam, India
Deep freezer, $^{-}70^{0}C$	: Remi, Chennai, India.
CO_2 incubator	: Napco, Canada.
Lyophilizer	: Labconco corp. USA
Spectrophotometer	: Elico, India
High speed cooling centrifuge	: Remi, Chennai, India.
Blind well chamber (Modified Boyden chamber)	: Nuclepore, Cambridge, USA.
ELISA-Reader	: Awareness Technology, Gujarat, India.
Disc electrophoresis tank	: Balaji Scientific Service, Chennai, India
Micro tome	: Lab Agencies, Ernakulam, India.
Liquid scintillation counter	: Rack Beta, Wallac, Finland.
Thermo-cycler	: MJ Research , USA

8. Reagents

A. Phosphate buffered saline (PBS)

NaCl	- 8.00g
KCl	- 0.20g
KH_2PO_4	- 0.20g
$Na_2HPO_4 . 2H_2O$	- 1.44g

Dissolved in distilled water, made up to 1000.0ml. pH was adjusted to 7.2 with 1N HCl. Sterilized by autoclaving at 15lbs for 15min.

B. Chord buffer

NaCl - 8.176g

KCl - 0.298g

Dextrose - 2.028g

Dissolved in one liter 0.001M phosphate buffer. PH 7.2. Sterilized by autoclaving at 15lbs for 15minutes.

C. Phosphate buffer (0.2M)

Solution-A. Na_2HPO_4 - 0.2M (27.80g of Na_2 HPO_4/litre)

Solution-B. NaH_2PO_4 . 0.2M (53.65g of NaH_2PO_4 $7H_2O$/litre)

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

D. PBS-EDTA

EDTA - 20.0mg

Dissolved in 100ml PBS and sterilized by autoclaving.

E. Trypsin solution

Trypsin - 200.0mg

Dextrose - 20.0mg

Dissolved in 100ml ice-cold PBS-EDTA and sterilized by filtration through 0.2μm membrane filter. Store at -20

F. Trypan blue

Trypan blue stain - 100.0mg

Normal saline (0.9%NaCl) - 100.0ml

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

G. Crystal violet

Crystal violet stain	- 50.0mg
20% Methanol	- 100.0ml

Crystal violet stain was dissolved in diluted methanol, filtered and used.

H. Scintillation fluid

PPO	- 2.50g
POPOP	- 0.25g
Naphthalene	- 100.0g

These reagents were dissolved in 1,4-Dioxan in a final volume of one litre. Kept in dark without exposing to light at any stage.

B. METHODS

1. Preparation Of The Plant Extracts

Air-dried plant materials were extracted with 70% methanol, by overnight maceration in 10volume of the solvent at RT. The solvent was then removed by vaccum evaporation and the residue was suspended in PBS containing 1% gum acacia. For *B. diffusa* whole plant was used but for *T. cordifolia* only stem was used for extracted in this manner.

2. Isolation Of The Polysaccharide From *T. Cordifolia.*

The polysaccharide (Pl) isolation was done according to the protocol of Chintalwar et al (1999). The air-dried powdered stem was extracted using methanol by stirring overnight at room temperature. This is to remove phenyl compounds ecdysoids etc. The residue was then extracted with boiling water for 1h and the extract was precipitated using acetone. This precipitate was dissolved in distilled water and the proteins were re-precipitated by adding equal volume of 15% TCA. The aqueous extract after removing the precipitate was dialyzed against distilled water and the dialysate was concentrated by lyophilization. The yield was about 0.01%. It was tested for the presence of polysaccharide by Molisch reagent and was confirmed using the test for uronic acid. (Chintalwar et al, 1999).

3.Sterilization Of Glass wares.

All glass wares and culture media filtration apparatus used for tissue culture purpose 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 oven dried at 80^0 C. These were then autoclaved at a pressure of 15 pounds/square inches for 15 minutes, dried and used for experiments.

4. Media Preparation And Sterility Checking.

10x medium of DMEM (9.98g/l) MEM (10.3g/l) and RPMI 1640 (10.3g/l) and 199 (10.9g/l) were prepared in autoclaved double distilled water. pH was adjusted to 7.2 using sodium bicarbonate. Additional supply of L-glutamine (2mM) was also provided. For culture of normal cells such as endothelial cells, an antioxidant – HEPES (10mM) was also supplemented in the medium. The medium was then

filter sterilized using a 0.2µm membrane filter. To check the sterility of the medium, 10ml sterile thioglycollate (29.8g/l) medium was inoculated with 1ml of the filtered medium and incubated at 37^0 for 6 days, and checked for visible contamination. Broad-spectrum antibiotics such as Penicillin (100U/ml) and streptomycin (100µg/ml) and Fetal Calf Serum (10%) were added to the medium prior to use.

5. Maintenance Of L929 Cell Lines In Tissue Culture.

To start with tissue culture techniques, mouse fibroblast 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 2ml of PBS-EDTA. 0.1 ml of trypsin solution containing 0.02% EDTA was added and incubated for 3-4 minutes at 37^0C and the bottles were tapped to dislodge the cells. MEM (5ml) containing 10% goat serum and antibiotics (complete medium) was added. Cells were dispersed to single cells by repeated pipetting and an aliquot of cell suspension was added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37^0C. The spent medium from every bottle was changed every 3rd day and cells were subcultured as detailed above every week.

6.Maintanance And Preparation Of Tumour Cell Line B16F10 For *In vitro* Studies.

To maintain the cells in culture, spent medium from confluent bottles was removed and the cells were washed three times with PBS. 0.1ml trypsin solution free of EDTA was added and incubated for 2-3 minutes at 37^0C. The bottles were then gently tapped and 10ml DMEM containing 10% FCS and antibiotics

(complete medium) was added and the cells were dispersed to make single cell suspension by repeated pipetting using a 10ml pipette. An aliquot of the cell suspension was added to fresh tissue culture bottles containing 10ml of complete medium and incubated at 37^{0}C. The spent medium was changed every 3rd day and was sub-cultured every week.

To perform an experiment, cells of sub-confluent cultures (70-80% confluence) were used. The spent complete-medium was replaced with serum free medium and kept overnight. The spent medium was again removed the monolayers were washed with PBS and the cells were harvested by mechanical dissociation using a cell scraper. The cell concentration was adjusted to required number and cell suspension with more than 90% viability were used for experiments.

7.Isolation And Maintenance Of Human Umbilical Vein Endothelial Cells (HUVECs)

Endothelial cells were collected from human umbilical cord vein, by the protocol proposed by Jaffe et al (1973). A sterile technique was followed in all manipulations of the cord. The cord was served from the placenta soon after birth, placed in a sterile container filled with cord buffer, and held at 4^{0}C until processing. Storage time averaged about 1h, and cords were discarded if held more than 3h. The cord was inspected and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt needle, and the needle was secured by clamping the cord over the needle with an umbilical cord tie. The vein was perfused with 100ml of cord buffer to wash out any blood and allowed to drain, and the other end was also tied. 1-2ml of 0.2% collagenase in cord buffer was then

infused in to the umbilical vein through the needle and the cord was placed in a water bath containing cord buffer and incubated at 37^0 Celsius for 15minutes. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30ml of cord buffer. The effluent was collected in a sterile centrifugal tube containing medium 199 with 20% FCS. The cells sedimented at 250g for 10minutes and the cell pellet were suspended by trituration in 5ml of fresh medium. The cell suspension was then added to gelatin pre-coated tissue culture flask. The flask were then incubated at 37^0C under 5% CO_2.

The cells were fed twice a week with a complete change of fresh culture medium. Endothelial cells were cultured in medium 199 containing FCS (20%), penicillin (100U/ml), streptomycin (100μg/ml), L-glutamine (2mM), HEPES (10mM) and VEGF (2ng/ml). On reaching confluence the cells were sub cultured by conventional trypsinisation.

8. Determination Of Cell Viability.

Cell viability was determined by trypan blue dye exclusion method. 0.9 ml of cell suspension was mixed with 0.1ml of 1% trypan blue, kept for 1-2 minutes and loaded on a haemocytometer. Viable cells exclude the dye, while non-viable cells take up the dye and appear blue coloured. The number of stained and unstained cells was counted separately.

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

9. Maintenance Of Experimental Animals And Tumour Line In Animals.

C57BL/6 mice and BALB/c mice were used for the experiments. They were originally procured from National Institute of Nutrition and were maintained in animal house, in well-ventilated cages. They were fed with pelleted mouse chow (Sai Durga feeds, Banglore, India) and water *ad libitum*.

B16F-10 melanoma cells were propagated in older C57BL/6 mice as transplantable solid tumours. 1×10^6 cells were injected subcutaneously to the hind limb of the mouse. After 10-15 days, the animal was sacrificed and tumour mass was dissected out and forced through a fine steel mesh using cold PBS, to get single cell suspension. 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 cell viability. 1×10^6 viable cells were injected in the same manner to another set of animals to maintain the tumour line.

10. Antiangiogenic Studies In Animals.

Studies on the antiangiogenic activities of the test materials were done in C57BL/6 mice. Angiogenesis was induced by injecting 1×10^6 B16F10 melanoma cells intradermally on the shaven ventral skin of the mice. After 9 days of tumour inoculation the animals were sacrificed, ventral skin was cut removed, washed in PBS and the number of tumour directed capillaries per cm^2 around the tumour was counted using a dissection microscope (Kishi et al 2000).

Blood of these angiogenesis induced animals were collected at two time points viz. day 1 and day 9, from the caudal vein in an aseptic manner. Serum was separated by centrifugation and used for cytokine profiling. Cytokines such as IL-

1β, IL-2, IL-6, TNF-α, GM-CSF, VEGF and an endogenous inhibitor of MMPs-TIMP-1 were assayed using respective ELISA kits.

11. Antiangiogenic Studies *In vitro.*

Rat aortic ring assay was used as the *in vitro* angiogenesis study model (Pan 2002). Dorsal aorta from a freshly sacrificed Sprague Dawley rat was cut removed in a sterile manner, rinsed in ice cold PBS to remove blood and any membranous tissue. It was then cut in to ~1mm pieces (transverse sections) using surgical blades. Each segment was placed in a collagen pre coated 24-well tissue culture plate. The rings were incubated for 24h at 37^0C in complete medium, afterwards exchanged for conditioned medium from B16F10 melanoma. The rings were further incubated for six days and then analyzed by phase contrast microscopy for any micro vessel out growth from the aorta. The test materials can be treated either to B16F10 melanoma from which the conditioned medium is taking to grow the aorta or directly to aorta along with conditioned medium.

12. Endothelial cell Tube formation assay (Gupta k et al 2002)

HUVECs were seeded (5×10^4) on the surface of ECM gel (25μl) coated well of a 96-well plate and incubated at 37^0C for 48h in CO_2 atmosphere in medium 199 suplemented with 20ng/ml VEGF and 20ng/ml bFGF. Various concentrations of extracts or curcuminoids was added along with this three wells were kept without any treatment and was the control. Supernatant was then removed cells were fixed and stained using Diff-Quik stain set. Tube formation was examined and the area of the capillary –like structure formed was photographed using an inverted microscope.

13. HUVEC motility assay (Guo HB et al 2002).

Endothelial cells (HUVEC) were grown to confluence in 96-well tissue culture plates coated with gelatin. A clear area was then scraped in the monolayer with a 200μl yellow tip by applying suction and fresh medium along with or without the test compounds were supplemented (Guo HB et al 2002). The culture were further incubated for 24h and then fixed using methanol and stained with crystal violet. Migration of cells into the wounded area was evaluated with an inverted microscope and photographed.

14. Antimetastatic Studies Using Animal Models.

In vivo antimetastaitc activity of the test materials was analyzed by injecting a highly metastatic tumour line B16F10 melanoma cells (10^6 in 50μl vol.) to C57BL/6 mice via lateral tail vein (Hill et al 1994). Animals were sacrificed on 21st day of tumour challenge, lungs and serum was collected. Antimetastatic activity was assed by analyzing various parameters such as a) counting the pulmonary metastatic colonies b) measuring biochemical parameters such as uronic acids (Bitter, Muir, 1962), hexosamines (Elson, Morgan 1933) and collagen hydroxyproline (Bergman, Loxley, 1970) in the lungs c) total sialic acids (Skoza and Mohos 1976) and γ-glutamyl transpeptidase activity in the serum (Szasz 1976) d) histopathological analysis of lungs, and e) determining the rate of survival of tumour bearing control and treated animals.

A. Estimation of protein (Lowry et al 1951).

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

Reagents

Solution A

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

Solution B

Folin's phenolreagent (1N)	- diluted 1:1 with distilled water

Procedure

20μl sample or different concentrations of BSA (150μg, 100μg, 50μg and 25μg, 0.0μg – as standard) were made up to 1.2ml with distilled water. To this, 6.0ml of solution A was added and then incubated at RT for 10minutes. The reaction mixture was vortex mixed and 300μl solution B was added, and incubated further for 30minutes at RT. Optical density of the blue colour developed was read at 660nm.

B. Estimation of hydroxyproline (Bergman and Loxley 1970).

Acid hydrolysis of protein yields amino acids, and chloramineT will oxidize hydroxyproline present in it. The coloured product is more stable in the presence of high concentrations of isopropanol.

Reagents

1.Oxidant solution

Sodium acetate ($3H_2O$) - 5.7g

Trisodium citrate ($2H_2O$)	- 3.75g
Citric acid	- 0.55g
Isopropanol	- 38.5ml
Distilled water	- 61.5ml

2. Ehrlisch's reagent A

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

3. Chloramine T - 1.75g/25ml oxidant solution (Prepared freshly)

Procedure

Homogenize 1g lung + 4ml isotonic saline. Seal the tubes with 6 N HCl and incubate at 110^0C for24h for hydrolyzing the sample.

20µl of the hydrolyzed sample after neutralization by evaporation was diluted to 3ml using isopropanol and to this 1ml oxidant solution was added. After 4minutes 2ml Ehrlisch's reagent A was added to the reaction mixture was heated for 21 minutes at 60^0C in water bath. Then the tubes were kept at room temperature for 1h and the absorbance was taken at 562nm.

C. Estimation of hexosamines (Elson and Morgan 1933).

The amino sugars (hexosamines) occurs in α or β pyranose form in many structural polysaccharides. Hexosamines have reducing properties with phenylhydrazine but not osazones, because the C-OH has been replaced by NH_2.

Reagents

Acetyl acetone (in 0.5M $Na_2 CO_3$)	- 2%
Ethanol	- 95%

p-dimethylaminobenzaldehyde - 1.33% (in 1:1 ethanol : Con. HCl mixture)

Procedure

Aliquots containing 10-50μg hexosamines were treated with 1ml of freshly prepared 2% acetyl acetone in glass stoppered tubes and kept in boiling water bath for 15minutes. After cooling in running tap water, 5ml of 95% ethanol and 1ml of Ehrlisch's reagent were added and mixed thoroughly. The purple red colour developed was read after 30minutes at 530nm. Water blank and standard glucosamine solutions of various concentrations were also treated similarly to get a standard curve. Hexosamine content of tissue sample was calculated by knowing the values of standard and expressed as μg/100mg dry tissue.

D. Estimation of uronic acids (Bitter and Muir 1962).

Acidic modifications of monosaccharides produced by the CH_2OH group to COOH group. C_6 uronic acids/hexuronic acids and their sulphated forms (eg. Iduronic acid 2-6-sulphate) occurs in α or β pyranose forms in many structural polysaccharides.

Reagents

Sulphuric acid reagent	- 0.952g sodium tetraborate in 100ml of Con.H_2SO4 (0.025M)
Carbazole reagent	- 0.125g carbazole in 100g absolute alcohol.
Acetate Buffer (0.1M)	- Solution A. 0.2M solution of acetic acid + Solution B. 0.2M solution of sodium acetate.

Procedure

Digestion of the tissue was carried out with crude papain (10mg/g wet weight of tissue) in 5ml of 0.5M acetate buffer (pH 5.5) containing 0.005M cysteine and

0.005 M disodium salt of EDTA at 65^0C for 24h. An aliquot (5ml) of sulphuric acid reagent was taken in tubes and cooled at -4^0C overnight. 0.1ml of sample (or standard glucaronolactone solution) containing 5-20μg uronic acids was layered on the acid. Tubes were closed with ground glass stoppers and the rack was shaken first gently and then vigorously. Tubes were kept in a boiling water bath for 10minutes and cooled at room temperature. To this 0.2ml of carbazole reagent was added, shaken, heated in a boiling water bath for 15minutes and cooled. The colour thus developed was read at 530nm. Uronic acid content of the tissues was expressed as μg/100mg wet tissue.

E. Estimation of total serum sialic acids (Skoza and Mohos 1976).

Acid hydrolysis of serum liberates sialic acids, which forms a coloured compound with thiobarbituric acid.

Reagents

H_2SO_4 - 0.2N

Periodic acid - 25µM in 62.5 mM H_2SO_4

Sodium arsenite - 0.2% in 0.5M HCl

Thiobarbituric acid - 0.6% (pH 9.0)

Dimethyl sulphoxide (DMSO)

Procedure

200µl of sample was mixed with equal volume of 0.2N H_2SO_4 and hydrolyzed for 1h at 80^0C. To this 50µl periodic acid was added and incubated for 30minutes at 37^0C. To this reaction mixture 50µl of sodium arsenite solution was added, followed by 100µl of thiobarbituric acid and was heated in a boiling water bath for 7.5minutes. Afterwards, 400µl of DMSO was added to intensify the colour and optical density was read at 549nm.

F. Estimation of γ-glutamyl transpeptidase activity (Szasz 1976).

Gamma glutamyl transpeptidase catalyses the transfer of γ-glutamyl moiety of a γ-glutamyl donor to a variety of acceptors (eg, Glycil glycine)

γ-Glutamyl-p-nitroanilide +

Acceptor (Glycil glycine) ⇆ p-nitroanilide (Donor residue) +

γ-glutamyl glycil glycine (Transfer product)

Reagents

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

Procedure

The standard assay mixture contained (1ml) 0.05M Tris HCl, 75mM NaCl, 2.5mM-L- γ-glutamyl-p-nitroanilide and 20mM glycil glycine along with 25μl sample. The rate of release of p-nitroaniline was measured by taking the absorbance of reaction mixture at an interval of 60sec. for 3minutes at 410nm using a spectrophotometer. A is calculated as; initial reading - final reading/3. The value was calculated using the following formula and expressed as U/L

$U/L = A \times 1114$

G. Histopathological analysis.

The tissue as soon as they are removed was fixed in 10% neutral buffered formalin for at least 24h. The tissues were dehydrated in alcohol series, cleaned in xylene and embedded in paraffin. About 5-6μm thick sections were taken using a microtome and the wax ribbon affix on a clean glass slide by gentle heating. The slides were then deparaffinated and stained with hematoxylin and eosin and mounted with a cover slip using DPX. These were then observed under the microscope for any histological changes (Culling CFA, 1976).

H. Determination of the rate of survival.

Animals were injected intravenously with B16F-10 melanoma cells ($1x10^6$). 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 treated animals survived and 'C' is the number of days untreated control animals survived after tumour inoculation.

15. Antimetastatic Studies *In vitro*.

A. Long term *in vitro* cytotoxicity studies using tissue culture techniques.

B16F-10 melanoma cells growing in log phase was used for this study. Cells were collected by trypsinization and 5×10^3 cells/well were seeded in to each well of a 96well flat bottomed tissue culture plate in 0.1ml complete medium and incubated at 37^0C in 5% CO_2. After 24hours of incubation, various concentrations of the test compounds were added in triplicate keeping 3 wells without any treatment as the control. The final volume of all the wells was made up to 200µl with complete medium and incubation was continued for further 48hours. 4 hour before termination of assay 20µl of MTT solution (5mg/ml) was added to each well. After completing the incubation the plates were centrifuged at 1000rpm for 10minutes the supernatant was removed and 100µl DMSO was added to each well. The plates were then incubated at room temperature for 15 minutes and the optical

density was measured at 545nm with reference of 630nm. The percentage dead cells was determined using the formula,

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

B. Cell proliferation studies using ^{3}H-thymidine uptake assay.

Cultures were set in flat bottom, 96-well plate as for the cytotoxicity studies, except that 10^4 cells were added to each well. 16h before termination of assay, 1.0μCi of ^{3}H-thynidine was added to each well and completed the incubation. The plates were then centrifuged at 1000rpm for 10minutes, the supernatant was removed and washed three times using PBS to remove unincorporated radioactive material. The cells were then dissolved in 0.2ml of 5N NaOH by incubating at 37^0C for 2h. The lysate were then transferred to scintillation vials containing 3ml scintillation fluid and kept overnight in dark (RT). The CPM was then taken using a liquid scintillation β-counter.

E. Collagen matrix invasion assay (Bootle-Wilbraham et al 2000).

Invasion of collagen matrix by tumour cells was carried out using modified, Boyden chambers (Blind well chambers). The lower compartment of the chamber was filled with diluted conditioned medium and polycarbonate filters were placed above this. The filters were coated with 25μg Type I collagen. Cells (1×10^4) were added to the upper chamber in 0.15ml of DMEM, and incubated for 16h at 37^0C in 5% CO_2 atmosphere. After incubation, the medium from upper chamber was removed and the cells on the upper side of the filter were removed by a cotton swab. The filter was then taken from the chamber placed on a clean glass slide in an upside down fashion. It was then fixed in methanol for 1-3 minutes and stained

for 3minutes with crystal violet. Cells that penetrated the polycarbonate filters were counted in 10 fields under a microscope and photographed.

Results were calculated as % inhibition of invasion using the formula,

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

F. Gelatin zymography (Kuo WH et al 2003).

Proteases liberated from the cells were initially resolved on SDS- poly acryl amide gels, which were incorporated with gelatin. Following incubation of the gel in the activation buffer, proteases separated on the gel will breakdown the gelatin and appears as transparent zones against a light back ground upon staining.

Reagents

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

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

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

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

3.Trypsin solution

Trypsin	- 75μg/ml (in reagent 2)

4. Separating Gel

29.2% acrylamide + 0.5% bisacrylamide	- 11ml
0.1M Tris-HCl, pH 8.8	- 1.2ml

20% SDS	- 0.15ml
10% Ammonium per sulphate	- 0.10ml
Gelatin (180mg/2ml distilled water, heated to dissolve)	- 2ml
Distilled water	- 6.505 ml
TEMED	- 0.045ml

Mix all the reagents and pour in to electrophoresis tubes at room temperature.

5. Stacking gel

29.2% acrylamide +0.5% bis acrylamide	- 1.67ml
0.1M Tris-HCl, pH 6.8	- 1.75ml
10%SDS	- 0.10ml
10% Ammonium per sulphate	- 0.10ml
Distilled water	- 6.36ml
TEMED	- 0.02ml

Mix and pour above the resolving gel at room temperature

6. Sample buffer (2x)

Glycerol	- 1.0ml
1M Tris-HCl, pH 6.8	- 0.25ml
20% SDS	- 1.0ml
Bromophenol blue (Tracking dye)	- 1.65mg

Made up to 5ml with distilled water

7. Running buffer

Tris base	- 3.0g
SDS	- 2.0g
Glycine	- 14.2g

Made up to 1000ml with distilled water

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

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

9. Triton X-100

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

10. EDTA solution

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

11.Destaining solution.

Methanol : Acetic acid : Water	- 50 : 10 : 40

12.Coomassie blue.

Coomassie blue	- 0.2g
Destaining solution	- 100ml

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

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

Procedure

The spent medium from sub-confluent (70-80%) cultures was removed, washed with serum free medium. The cells were further incubated for 24h in serum free medium (DMEM) at 37^0C. Zymography of this sample was performed using gelatin as the substrate in the gels. After the incubation, medium was collected,

centrifuged, and supernatant was used for zymographic analysis. After determining the protein concentration, supernatant (equivalent to 50μg protein) containing the proteases were activated with trypsin (5μl trypsin solution for 100μg protein) for 30 minutes at 37^0C. Trypsin treated and untreated samples (equivalent to 50μg protein) were mixed with an equal volume of sample buffer (2x), with out any educing agent or heat, loaded on 11% polyacrylamide gels containing 0.6% gelatin and electrophoresed at 4^0C with a constant current of 2mA per tube until the tracking dye (Bromophenol blue) reaches the periphery of the gels.

The gels were then taken from the tubes, washed with 2% Triton X-100 on a shaker at RT for three changes of 30 minutes each, to remove the SDS. This was followed by 2h washing with activation buffer and the gels were finally incubated in the same buffer at 37^0C for 18h, in the presence and absence of test compounds. Gels were then fixed and stained with Gel-cord Blue stain reagent and clear bands were visualized against a light background.

16.VEGF mRNA quantification assay.

B16F10 cells (1×10^6) were plated in 30mm tissue culture petri dishes and incubated at 37^0C overnight in 5% CO_2 atmosphere to get adhered. The cells were then treated with or without non- toxic concentration of extracts of Boerhaavia/Tinospora or curcuminoids for 4 hours. After incubation the medium was removed and the cells were lysed using the lysing solution provided in the quantikine mRNA kit. The lysate was then used for quantification of VEGF mRNA using the quantikine mRNA kit according to the manufacturer's procedure. Finally the colour developed was measured using an Elisa reader at 490 with a correction wavelength of 690 and the values for sample were determined from the

standard curve prepared. The values are expressed as attomoles of RNA /ml [1 attomol (10^{-18}mole)   600,000 molecules]

17. Statistical Data Analysis.

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

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

Where,

X - Mean of samples (x)

Y - Mean of samples (y)

nx - Sample size (x)

ny - Sample size (y)

'S' was found out from the equation

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

Where,

sx = Standard deviation of x

sy = Standard deviation of y

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

Chapter 3

In vivo angiogenesis study using extracts of B. diffusa and T. cordifolia in animal models.

Introduction

Natural products are rich source of pharmacologically active compounds in which plant materials deserves an important position. Medicinal plants serve as good source of pharmacological compounds and the plant based medicinal practices are effectively used in indigenous systems of medicine from time immemorial. Several scientific studies using different solvent extracts of many plants have been conducted and many of them are proven to be effective in curing diverse pathological conditions. The study has now been extended to the field of neoplasia, tumourigenesis, cancer chemoprevention etc. A recent finding in this area is the finding that tumours depend on the host vasculature for getting nourished and in fact they can induce the host to produce new vessel (angiogenesis) for the unrestrained growth of tumour. Numerous molecular players –that are secreted by the tumour cells itself or induce the host stromal cell to secrete–work in concert to form a functional vessel by the host endothelial cells. Several growth factors (VEGF, IL-2 etc) and cytokines (TNF-α, IL-1β etc) are important in this regard. Timely intervention of any agent that alters the profile of these angiogenic molecules can be antiangiogenic thereby inhibiting the vessel formation and hence restricting the tumour cells from further growth.

Materials and methods

Animals: - Four to six weeks old male C57BL/6 mice.

Cells: - B16F10, a highly metastatic mice melanoma.

Plant materials: - Authenticated *B. diffusa* L (whole plant) and *T. cordifolia* Miers (shoot only) were obtained from Amala Ayurveda Pharmacy, Thrissur, India. The

plants were dried at 45^0C and extracted using 70% methanol by overnight stirring at RT. The solvent was vaccum evaporated and the residue was suspended in 1% gum acacia.

TNP-470: - Takeda Neoplastic Product – 470 (TNP-470) was a kind gift from Dr. Ravi Varma, NCI, USA.

ELISA kits: - The quantitative ELISA kits used were mouse IL-1β, IL-2, IL-6, GM-CSF, TNF-α, VEGF and TIMP-1. The manufactures protocol was followed to estimate the level of the respective cytokine in the serum sample of angiogenesis induced animals.

Angiogenesis induction: - Detailed description is given in Chapter 2 Materials and Methods. Briefly, four groups of animals, comprising six animals in each group were shaven on the ventral side and 1×10^6 cells (B16F10 melanoma) were inoculated intradermally to the shaven area. This will induce the formation of new blood vessels towards the tumour site. Group I and II animals were treated simultaneously with five doses of Tinospora or Boerhaavia extract respectively. Group III animals received only 1% gum acacia and kept as vehicle control. The reference compound TNP-470 was administered to the group IV animals at a concentration of 30mg/kg subcutaneously to each animal for a total of five doses. After 9 days the animals were sacrificed, the ventral skin cut removed, washed in PBS and the number of tumour directed capillaries per cm^2 around the tumour was counted using a dissection microscope × 20(Kishi K et al 2000).

Results

<u>Effect of the Boerhaavia and Tinospora extracts on neo vessel formation *in vivo*: -</u>

All the B16F10 melanoma induced animals developed a palpable tumour and visible tumour directed blood vessels (Figure 3.1). The number of tumour directed capillaries were significantly reduced in the extracts treated animals compared to the control animals, which had an average of 30.8 ± 2.1 capillaries around $1cm^2$ area around the tumour (Table 3.1). Boerhaavia treatment reduced the number of capillaries to6.4 ± 1.3 and Tinospora treated animals had only 11.6 ± 1.4 capillaries. The reference compound, TNP-470 treated animals had only 4 ± 1.3 tumours directed capillaries.

<u>Effect of the plant extracts on the cytokine profile of angiogenesis induced animals: -</u>

After 24hr and 9 days of tumour cell inoculation, blood was collected from the caudal vein of each mouse. Serum was separated and used for the estimation of various cytokines using corresponding ELISA kits according to the manufacturers instructions.

<u>IL-1β</u>

After 24 hours of tumour challenge, the serum level of IL-1β was doubled in control (30 ±4.5pg/ml), *T.cordifolia* treated (30±4.2pg/ml) *B.diffusa* treated (30±3.7pg/ml) animals from the normal levels of 16 ± 4pg/ml (Table 3.2). But by 9th day the levels were normalised in the T.cordifolia (15±2.5 pg/ml) and *B.diffusa* treated animals (18±3.5 pg/ml) whereas in control animals the elevated level (30±3.7 pg/ml) sustained.

IL-6

The control (35±5.6 pg/ml), Boerhaavia (35±3.3 pg/ml) and Tinospora (36 ± 8.3pg/ml) treated animals had normal levels (35 ± 6.5 pg/ml) of serum IL-6 on 24th hour after tumour induction. By 9th day, the control animals had drastically elevated serum IL-6 levels (320 ± 9.5 pg/ml) whereas in both Boerhaavia (58 ± 8.8 pg/ml) and Tinospora (66 ± 6.8 pg/ml) treated animals the levels were significantly lower compared to the control (Table 3.3).

GM-CSF

In the control animals an immediate rise in the level of serum GM-CSF was found after 24 hours and the value reached 70 ± 5.6 pg/ml from the normal levels of 18 ± 3 pg/ml. By 9th day the values came down to 30 ± 3 pg/ml. All the treated animals showed an inhibitory effect in the elevation of the GM-CSF level after 24th hour, 28±2.5 pg/ml in *T.cordifolia* treated animals and 22±3.5 pg/ml in B.diffusa treated animals and the levels were normalized by 9th day - 18±2.6 pg/ml and 16±2.7 pg/ml in T.cordifolia and B.diffusa treated animals respectively. (Table.3.4).

TNF-α

There was a drastic increase in the serum levels of all the angiogenesis induced control animals (180 ± 6.3pg/ml) as compared to the normal ones (20 ± 3.2 pg/ml) after 24th hour of tumour induction. In the control animals the level was going on increasing to reach 630 ± 30 pg/ml, on the 9th day, where as in the Boerhaavia (180 ± 10.3 pg/ml) and Tinospora (150 ± 9.1 pg/ml) treated animals the levels were more or less similar as that on the 24th hour sample (Table.3.5).

IL-2

IL-2 levels showed a decrease in the control animals after 24th hour (16±2.1 pg/ml), which was normalized by 9th day (20±2.5 pg/ml). But all the treated animals could effectively overcome the initial dip in the in the serum levels of IL-2 induced by the tumour induction. Boerhaavia treatment elevated the levels to 78 ± 5 pg/ml by 9th day from 23±3.5 pg/ml at 24th hour and Tinospora treated animals had 25 ± 2 pg/ml serum of IL-2 on the same day. (Table 3.8)

VEGF

VEGF levels showed an elevation in both control (62 ± 9 pg/ml) as well as in B.diffusa (60 ± 11 pg/ml) and Tinospora (31 ± 6 pg/ml) treated animals after 24 hours compared to normal value of 16 ± 8 pg/ml (Table 3.6). After 9 days VEGF level was drastically elevated in control animals (150±12 pg/ml) whereas, Boerhaavia and Tinospora treatment could significantly inhibited the elevation to 22 ± 6 pg/ml and 59 ± 8 pg/ml respectively.

TIMP-1

The Tissue Inhibitor of Metalloprotease level in the serum of normal mice was 600 ± 36 pg/ml which was reduced by the induction of tumour cells to 350 ± 19 pg/ml in the untreated control animals. But Tinospora (750 ± 31 pg/ml) and Boerhaavia (1100 ± 39 pg/ml) treatment could elevate the TIMP levels significantly. Blood sample after 9 days of angiogenesis induction also showed a similar TIMP-1 profile in these animals. In control it was 360 ± 22pg/ml whereas Boerhaavia (1350 ± 34 pg/ml)

and Tinospora (825 ± 26 pg/ml) could maintain the initial elevation thereby negatively contributing to the formation of neovessels. (Table 3.7)

Discussion

Angiogenesis is a significant prognostic factor in melanoma. Several endogenous factors are there that control the process of neo vessel formation from the existing host vasculature (Rofstad and Halsor 2000). All the cytokines and growth factors estimated in this study are involved directly or indirectly in the process of neoangiogenesis. The necessity of IL-1β for *in vivo* angiogenesis driven by B16 melanoma has clearly been documented. IL-1β knock out mice showed lesser degree of micro vessel formation compared to its wild type when induced with B16 melanoma (Voronov E,et al 2003) indicating the importance of host derived IL-1β in angiogenesis. The antitumour mechanism of thalidomide is found to be related to reduced serum level of TNF-α in myeloma (Li J, et al 2002) and it is known to destabilize the TNF-α mRNA thereby preventing angiogenesis (Marriott JB et al 1999). IL-6 indirectly promotes tumour angiogenesis through the up regulation of VEGF-A load in the platelet aggregates on tumour endothelium (Salgado R, et al 2002, 2003). VEGF is the most potent angiogenic factor, induces its effect directly on endothelial function. For GM-CSF, even though it did not modulate endothelial functions related to inflammation (Bussolino F et al 1991), it induces endothelial cells to proliferate and migrate.

The levels of IL-2 (T-cell growth factor) and TIMP-1 were also estimated in this study. IL-2 was the first cytokine used clinically - that thought to be act solely through the immune system (Neville ME et al 2001)- for treating cancer, has also

reported to have antiangiogenic activity (Sakkoula E et al 1997). TIMPs that bound to MMP and preventing them from cleavage of extracellular matrix components will also inhibit the endothelial cell motility and invasion and thereby preventing angiogenesis.

In short, elevation of the cytokines such as IL-1β, IL-6, TNF-α, GM-CSF and VEGF are strictly proangiogenic and the treatment with the extracts of Boerhaavia and Tinospora could regulate the levels and inhibit angiogenesis Moreover the extracts were able to increase the production of antiangiogenic agents such as IL-2 and TIMP and could successfully shift the equilibrium towards an angiostatic condition.

Table 3.1

Effect of *T.cordifolia* and *B.diffusa* on tumour directed capillary formation.

Drug used	Dose	Mode (Simultaneous)	No of capillaries/cm^2 around tumour	% Inhibition compared to control
Control	-	-	30.2 ± 2.0	-
TNP 470	30mg/Kg	S.c.	4.0 ± 1.3*	85.8
T.cordifolia	200mg/Kg	Ip	11.6 ± 1.4*	46.2
B.diffusa	200mg/kg	Ip	6.4 ± 1.3*	80.0

Values are mean ± SD.

*P < 0.001 (Values are compared with control)

Animals were induced angiogenesis by injecting 10^6 B16F10 melanoma cells intradermally. All the animals were sacrificed 9 days after tumour challenge and number of tumour directed micro vessels per cm^2 around the tumour was counted using a dissection microscope.

Table 3.2

Effect of *T.cordifolia* and *B.diffusa* on the IL-1β profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of IL-1 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	16 ± 3.5	
Control	-	-	30 ± 4.5	30 ± 3.7
T.cordifolia	200mg/Kg	Ip	30 ± 4.2	15 ± 2.5
B.diffusa	200mg/kg	Ip	30 ± 3.7	18 ± 3.5

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-1β were estimated by ELISA method.

Table 3.3

Effect of *T.cordifolia* and *B.diffusa* on the IL-6 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of IL-6 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	35 ± 6.5	
Control	-	-	35 ± 5.6	320 ± 9.5
T.cordifolia	200mg/Kg	Ip	36 ± 8.3	66 ± 6.8
B.diffusa	200mg/kg	Ip	35 ± 3.3	58 ± 8.8

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-6 were estimated by ELISA method.

Table 3.4

Effect of *T.cordifolia* and *B.diffusa* on the GM-CSF profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of GM-CSF (pg/ml serum)	
			24th h	9th Day
Normal	-	-	18 ± 3.1	
Control	-	-	70 ± 5.6	30 ± 3.2
T.cordifolia	200mg/Kg	Ip	28 ± 2.5	18 ± 2.6
B.diffusa	200mg/kg	Ip	22 ± 3.5	16 ± 2.7

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of GM-CSF were estimated by ELISA method.

Table 3.5

Effect of *T.cordifolia* and *B.diffusa* on the TNF-α profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of TNF-α (pg/ml serum)	
			24th h	9th Day
Normal	-	-	20 ± 3.2	
Control	-	-	180 ± 6.3	630 ± 8.5
T.cordifolia	200mg/Kg	Ip	150 ± 8.3	150 ± 9.1
B.diffusa	200mg/kg	Ip	180 ±10.3	180 ± 9.5

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of TNF-α were estimated by ELISA method.

Table 3.6

Effect of *T.cordifolia* and *B.diffusa* on the VEGF profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of VEGF (pg/ml serum)	
			24th h	9th Day
Normal	-	-	16 ± 8	
Control	-	-	62 ± 9	150 ± 12
T.cordifolia	200mg/Kg	Ip	31 ± 6	59 ± 8
B.diffusa	200mg/kg	Ip	60 ± 11	22 ± 6

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of VEGF were estimated by ELISA method.

Table 3.7

Effect of *T.cordifolia* and *B.diffusa* on the TIMP-1 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of TIMP-1 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	600 ± 36	
Control	-	-	350 ± 19	360 ± 22
T.cordifolia	200mg/Kg	Ip	750 ± 31	825 ± 26
B.diffusa	200mg/kg	Ip	1100 ± 39	1350 ± 34

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of TIMP-1 were estimated by ELISA method.

Table 3.8

Effect of *T.cordifolia* and *B.diffusa* on the IL-2 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of IL-2 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	23 ± 3.2	
Control	-	-	16 ± 2.1	20 ± 2.5
T.cordifolia	200mg/Kg	Ip	25 ± 2.2	25 ± 2.2
B.diffusa	200mg/kg	Ip	23 ± 3.5	78 ± 5.2

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-2 were estimated by ELISA method.

Figure 3.1. Effect of *T.cordifolia* and *B.diffusa* on *in vivo* Angiogenesis. The animals were induced tumour directed neoangiogenesis by injecting B16F10 melanoma cells (10^6 cells/0.05ml vol) intradermally.

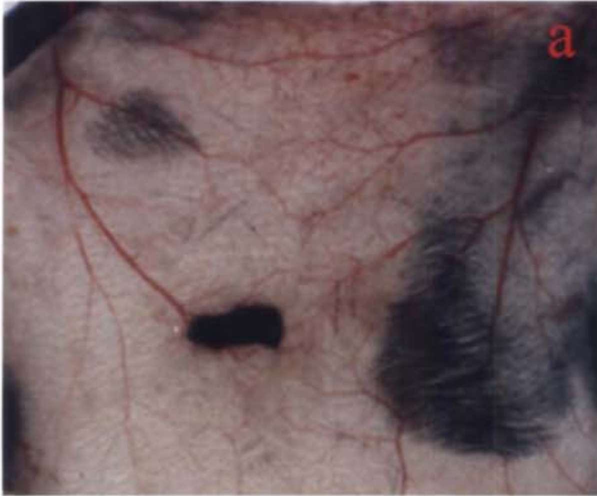
A) Vehicle treated control

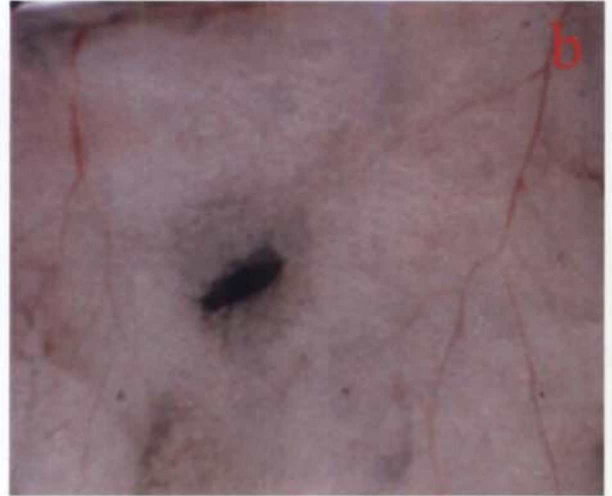
B) TNP-470 treated (30mg/Kg)

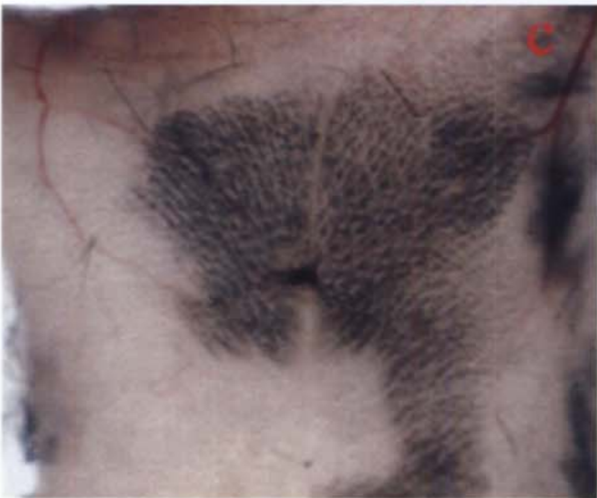
C) *T.cordifolia* treated at a concentration of 25mg/Kg

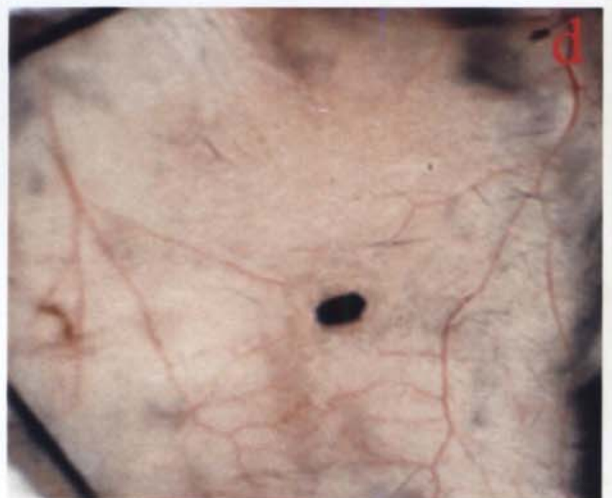
D) *B.diffusa* treated at a concentration of 25mg/Kg

Figure 3.1









Chapter 4

Antiangiogenic study of some curcuminoid derivatives in vivo

Introduction

Inflammation is considered as one of the major contributors of carcinogenesis. Substances produced by inflammatory cells - radicals and cytokines, not only cause direct damage to DNA but also exert tumour supportive effects such as deregulation of apoptosis, stimulation of angiogenesis etc. (Okada F 2002). One key new approach in pharmacological research appears to be, modulation of the inflammatory cascade, that links cancer initiation, promotion, progression, angiogenesis and metastasis. A 40-50% reduction in incidence of colorectal cancer is noticed in individuals taking non-steroidal anti-inflammatory drugs (Dempke W et al 2001).

Acute inflammatory response is a potent stimulus for PMN directed angiogenesis (McCourt M et al 1999). Chronic inflammation is also a contributor for neoangiogenesis (Kisley LR et al 2002). Altered levels of proinflammatory and proangiogenic factors are observed in various forms of cancer (Chen Z et al 1999) including melanoma (Lazar-Moinar E et al 2000). These cytokines include IL-1β, IL-6, GM-CSF, VEGF etc. Certain anti-inflammatory agents are proven to inhibit angiogenesis preferably by affecting COX mediated metabolism (Dicker AP et al 2001) and thereby altering the levels of proinflammatory cytokines. Antiangiogenic agents can also act as anti-inflammatory as well (Walsh and Pearson 2001).

Several natural products are being used as good anti-inflammatory agents without the risk of side effects from the time immemorial. Curcumin, diferulomethane, is the yellow pigment and active component of turmeric, *Curcuma longa* L. (Zingiberaceae). A small molecular weight compound,

curcumin constitutes 1% to 5% of the content of turmeric. It has a long history of safe use in Ayurvedic medicine, an indigenous system of medicine from India, particularly in the treatment of inflammatory disorders. In addition to its anti-inflammatory properties, curcumin is a potent antioxidant stronger than vitamin E preventing lipid peroxidation *in vitro* (Sharma OP 1976, Toda S et al 1985). Several of its derivatives, both natural and synthetic is also reported to have diverse biological functions. Considering its diverse pharmacological activity, in this study we tried to evaluate its effect in an *in vivo* angiogenesis model induced by a metastatic melanoma cell line.

Materials and mehods

Animals: - Four to six weeks old Male C57BL/6 mice.

Cells: - B16F10, a highly metastatic mice melanoma.

Study compounds: - Natural curcuminoids such as curcumin I (CI), II (CII) and III (CIII) were separated by preparative TLC from the crude curcumin preparation as detailed in chapter 2. The synthetic curcuminoids used were Salicyl curcumin (SC), Anisyl curcumin (AC), Veritryl curcumin (VC) and Dimethylamino curcumin (DC). The reference compound used for the *in vivo* capillary formation study was TNP-470.

ELISA kits: - The quantitative ELISA kits for mouse IL-1β, IL-2, IL-6, GM-CSF and TNF-α, VEGF and TIMP-1 were used to estimate the level of the respective cytokine in the serum sample of angiogenesis induced animals.

Angiogenesis induction: -

Angiogenesis was induced as described in Chapter 2. Briefly nine groups of animals, comprising six animals in each group were shaven on the ventral side and 1×10^6 cells (B16F10 melanoma) were inoculated intradermally to the shaven area. Group I was kept as control of the study and administered the 0.1% carboxymethyl cellulose (the vehicle for curcuminoids) and Group II as the standard reference (received TNP-470 as per the NCI recommended dosage). Group III to IX animals received the different curcuminoid derivatives at a concentration of 200µmoles/Kg per dose. A total of five doses were given to each animal at 24h interval. All the animals were sacrificed after 9 days, the ventral skin cut removed, washed in PBS and the number of tumour directed capillaries per cm^2 around the tumour was counted using a dissection microscope × 20(Kishi K et al 2000).

Determination of the effect of the curcuminoid derivatives on serum cytokine profile of angiogenesis induced animals: -

After 24hr and 9 days after tumour cell inoculation, blood was collected from the caudal vein of each mouse. The serum was separated and used for the estimation of various cytokines using corresponding ELISA kits according to the manufacturers instructions.

Results

Effect of the curcuminoid derivatives on tumour directed capillary formation *in vivo*: -

The number of tumour directed capillaries were significantly reduced in the treated animals compared to the control animals, which had an average of 30.8 ±

2.1 capillaries around 1cm^2 area around the tumour (Table 4.1). The reference compound treated animals showed at least 85% reduction in the neovessel formation and had an average number of 4 ± 1.3 capillaries. Treatment of natural curcuminoids such as Curcumin I (9.5 ± 1.5) Curcumin II (19.8 ± 2.1) and Curcumin III (14 ± 1.9) also had a reduced number of capillaries as compared to the synthetic curcuminoids such as AC (21 ± 2.6), VC (23 ± 3.1) and DC (25 ± 2.4). Among the synthetic curcuminoids studied SC (16 ± 2.5) showed the maximum inhibition in the formation of capillaries induced by the tumour (Figure 4.1 and 4.2).

Effect of the curcuminoid derivatives on the cytokine profile of angiogenesis induced animals: -

IL-1β

After 24h of tumour challenge, serum level of IL-1β was two fold higher in the control animals (30 ± 4.3pg/ml) than the normal levels (16 ± 3.5 pg/ml). Animal groups treated with Anicyl curcuminoid (31 ± 2.6 pg/ml) Veritryl curcuminoid (29 ± 2.5 pg/ml) and Dimethylamino curcuminoid (29 ± 3 pg/ml) were also found to have an elevated level of IL-1β and these animals maintained these levels on 9th day as well. But in the CI (20 ± 2.2 pg/ml) CII (25 ± 2.5 pg/ml) CIII (22 ± 3.1 pg/ml) and SC (24 ± 1.9 pg/ml) treated animals, the levels were much lower and by 9th day all these animals could normalize the serum level of IL-1β, except CII (28 ± 3.4 pg/ml) treated animals (Table 4.2).

IL-6

The initial level of serum IL-6 was more or less similar as normal levels (35 ± 6.5) for all the treated and control animals (35 ± 5.6 pg/ml). For CI (34 ± 3.6 pg/ml) CII (35 ± 4.3 pg/ml) CIII (38 ± 5.8 pg/ml) SC (38 ± 6.1 pg/ml) AC (40 ± 4.9 pg/ml) VC (41 ± 8.6 pg/ml) and DC (36 ± 5.6 pg/ml) treated animals the levels were not much deviated than the normal levels. But by 9th day, the levels were drastically elevated to reach 320 ± 9.5 in the control animals. Treatment of synthetic curcuminoids such as AC (188 ± 15.2 pg/ml) VC (202 ± 16.2 pg/ml) DC (196 ± 18 pg/ml) and CII (170 ± 12.6 pg/ml) were also not devoid of this elevation in IL-6 level on 9th day of tumour challenge. But the CI (70 ± 8.5 pg/ml) CIII (66 ± 10.5 pg/ml) and SC (78 ± 8.6 pg/ml) treated animals had much reduced levels of serum IL-6 (Table 4.3).

GM-CSF

Injection of B16F10 melanoma to the intradermal region of mice showed a tendency to immediately elevate the GM-CSF level in the serum as estimated from the 24th h serum sample. Control animals had a highly elevated level of GM-CSF (70 ± 5.6 pg/ml), which was significantly inhibited by the treatment with curcuminoids such as CI (20 ± 3.1 pg/ml) CII (29 ± 2.6 pg/ml) CIII (23 ± 1.9 pg/ml) and SC (30 ± 3.5 pg/ml) significantly inhibited this elevation. The other curcuminoids such as AC (42 ± 4.1 pg/ml) VC (53 ± 4.6 pg/ml) and DC (45 ±3.8 pg/ml) were also had a reduced level compared to the control. By 9th day this elevated level was not detected in any group of animals and all the animals had more or less same values as normal animals (Table 4.4).

TNF-α

As in the case of GM-CSF, an immediate elevation in the level of TNF-α was observed in all the angiogenesis induced animals (Table 4.5). The 24thh serum sample analysis showed that the maximum value for TNF-α was obtained again in the control animals (180 ± 6.3 pg/ml) followed by DC (170 ± 20.1 pg/ml) AC (155 ± 14.6 pg/ml) VC (150 ± 19.2 pg/ml) SC (115 ± 16.8 pg/ml) CIII (120 ± 15.8 pg/ml) and CII (105 ± 10.5 pg/ml) treated animals. TNF-α level was maximally inhibited in the CI (90 ± 6.8 pg/ml) treated animals. This pattern for TNF-α level was repeated in the serum sample after 9 days of tumour induction. The TNF-α level in the control animals after 9 days of angiogenesis induction was drastically elevated to 630 ± pg/ml. This was lowered in the animals treated with curcuminoids such as DC (550 ± 24.2 pg/ml) AC (450 ± 19.1 pg/ml) VC (375 ± 17.5 pg/ml) SC (165 ± 18.5 pg/ml) CIII (155 ± 18.4 pg/ml) and CII (173 ± 15.1 pg/ml). The maximum inhibition in the TNF-α level was obtained in CI treated animals (150 ± 13.2 pg/ml)

VEGF

Serum VEGF levels also showed an immediate elevation in control (62 ± 9 pg/ml) and all treated animals compared to the normal levels (16 ± 8 pg/ml), on the 24th h serum sample. For CI treated animals VEGF level was 30 ± 9 pg/ml and the treatment with DC (58 ± 6.4 pg/ml) AC (49 ± 6.1 pg/ml) VC (53 ± 7.5 pg/ml) SC (35 ± 3.9 pg/ml) CIII (30 ± 5.6 pg/ml) and CII (38 ± 8.0 pg/ml) also had higher levels of VEGF than the CI treatment but lower than the untreated control. Here again the pattern was similar in the 9th day serum sample analysis. For DC (118 ± 6.5 pg/ml) AC (98 ± 6.5 pg/ml) VC (120 ± 7.5 pg/ml) SC (58 ± 6.3 pg/ml) CIII (72

± 7.4 pg/ml) and CII (60 ± 5.6 pg/ml) treated animals the levels were much lower than the control of the same day whereas in the CI treated animals the levels reduced to 35 ± 4.3 pg/ml serum (Table 4.6).

IL-2

The IL-2 levels showed a decrease in the control animals on the 24th h (16 ± 2.1) and 9th day (20 ± 2.5 pg/ml) serum sample as compared to the normal levels (23 ± 3.2 pg/ml). But, interestingly all the curcuminoids such as DC (24 ± 3.3 pg/ml) AC (25 ± 2.6 pg/ml) VC (23 ± 2.2 pg/ml) SC (28 ± 3.0 pg/ml) CIII (24 ± 3.4 pg/ml) CII (21 ± 1.9 pg/ml) and CI (28 ± 2.0 pg/ml) treated animals could effectively inhibit this depression in IL-2 levels on 24th h serum sample. Similar results were also observed in the 9th day serum sample. In the control animals, serum IL-2 level was 20 ± 2.5 whereas the treatment with curcuminoids such as CI (35 ± 4.1 pg/ml), CII (25 ± 3.1 pg/ml), CIII (30 ± 2.9 pg/ml), SC (28 ± 1.8 pg/ml) AC (24 ± 2.3 pg/ml) VC (26 ± 3.1 pg/ml) and DC (25 ± 4.0 pg/ml) effectively inhibited the depression in the serum level of IL-2 (Table 4.8).

TIMP-1

Serum profile for TIMP –1of the angiogenesis induced animals showed a similar pattern as that of IL-2. The control animals had reduced levels of TIMP on the 24th h (350 ± 19 pg/ml) and 9th day (355 ± 23 pg/ml) serum samples compared to the normal (600 ± 36 pg/ml). Whereas all the curcuminoids such as DC (765 ± 32 pg/ml) AC (1050 ± 55 pg/ml) VC (1110 ± 42 pg/ml) SC (955 ± 50 pg/ml) CIII (850 ± 40 pg/ml) and CII (675 ± 35 pg/ml) treated animals had an elevated level of this inhibitor protein of MMPs on the 24th h serum sample. The serum sample

analyses of 9th day sample also showed a similar pattern and treatment with CI (1100 ± 55 pg/ml), CII (855 ± 35 pg/ml), CIII (975 ± 40 pg/ml), SC (1350 ± 58 pg/ml), AC (1150 ± 42 pg/ml), VC (1250 ± 51 pg/ml), DC (850 ± 31 pg/ml) elevated the TIMP-1 level compared to normal levels (Table 4.7).

Discussion

The concept that inflammation and carcinogenesis are causally linked has been proposed for more than a century, but it has recently taken on a new importance as anti-inflammatory agents that inhibit the formation of prostaglandins have been shown to be useful in chemoprevention. Non-steroidal anti-inflammatory drugs (NSAIDs), and especially the newer selective inhibitors of cyclooxygenase-2 (COX-2) such as celecoxib, inhibit colon carcinogenesis in experimental animals (Reddy BS et al 2000), and cause a significant reduction in the number of colorectal polyps in human subjects (Steinbach G et al 2000). Mechanistically, these studies are particularly interesting as the beneficial effects of COX2 inhibitors seem to be mediated by their effects on stromal cells of the intestine, especially their ability to suppress angiogenesis, which is part of the stromal reaction (Gupta and DuBois 2001).

COX 2 can be stimulated by inflammatory mediators, cytokines, growth factors, and tumour promoters and is inhibited by steroids and NSAIDs. Studies to date suggest a functional role for COX 2 and inflammatory eicosanoids in tumour-induced angiogenesis (Wallace JM 2002). Several mechanisms appear to contribute to the proangiogenic effect of COX 2. Increased production of eicosanoid byproducts (eg, PGE2, TXA2, PGI2) may potentially reduce endothelial cell apoptosis and directly stimulate endothelial cell migration (Gately

S 2000, Leahy KM et al 2000). Treatment with selective inhibitors of COX 2 effectively suppresses angiogenesis in *in vivo* models of many types of cancer (Sawaoka H et al 1999, Masferrar JL et al 2000, Jones MK, et al 1999, Yamada M et al 1999).

In addition to agents that act directly as inhibitors of COX2, the transcription factor Nuclear Factor κB (NF-κB), which regulates the activities of many genes that are involved in the inflammatory process, provides an excellent target for the development of new chemo preventive agents. For example, several natural products that have both anti-inflammatory and anti-carcinogenic activity — such as curcumin, resveratrol and caffeicacid phenethylester (CAPE) —block either the activation or transcriptional activity of NFκB (Bharti et al 2003). Pre treatment of human colonic epithelial with curcumin inhibited TNF-α induced COX-2 gene transcription and NFκB activation. In these studies curcumin inhibited IκB degradation by down regulation of NFκB inducing kinase (NIK) and IκB kinase (IKK) α/β (Plummer SM et al 1999). Curcumin has also proven to be antiangiogenic, and is preferably by affecting the MMP activity (Taloor D et al 1998). Studies using B16F10 cells have shown that it could inhibit the osteopontin induced pro-MMP-2 activation by modulating NFκB transcriptional pathway (Philip S et al 2001). The development of new inhibitors of NFκB is being widely pursued for treatment of diseases other than cancer (for eg. Inflammatory bowl disease or nuerodegradative disease) and illustrates the diverse applications of studies of regulation of gene transcription in modern pharmacology. However suppression of NFκB activity is not without risk as it can increase susceptibility to infections such as tuberculosis (Sporn and Suh 2002).

In the present study, administration of curcumin and some of its derivatives significantly inhibited neovessel formation induced by the induction of angiogenesis by B16F10 melanoma cells in the mice. The analysis of the serum cytokine profile shows a drastic decrease in the level of proinflammatory cytokine in the curcumin/curcuminoids treated animals compared to the control animals. Since the proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, are NFκB dependant (Schwarz and Murphy 2001), the inhibitory effect may be attributed to the inhibition of NFκB activation. As all these proinflammatory cytokines are relevant mediators of angiogenesis (Refer chapter 1), a reduction in the level of these will negatively contribute to the completion of neovessel formation. More over the inhibition of angiogenesis is further achieved by the increased production of antiangiogenic factors such as TIMP-1 and IL-2, both of which were showing an elevated level in the curcumin/curcuminoids treated animals compared to the untreated control.

Table 4.1

Effect of different curcuminoids on tumour directed capillary formation in mice.

Drug used	Dose	Mode (Simultaneous)	No of capillaries/cm^2 around tumour	% Inhibition compared to control
Control	-	-	30.2 ± 2.0	-
TNP 470	30mg/Kg	S.c.	4.0 ± 1.3*	85.8
C I	200µmoles/Kg	Ip	9.5 ± 1.5*	70.0
C II	200µmoles/Kg	Ip	19.8 ± 2.1	33.0
C III	200µmoles/Kg	Ip	14.0 ± 1.9#	53.0
Salicyl	200µmoles/Kg	Ip	16.0 ± 2.5#	44.0
Anisyl	200µmoles/Kg	Ip	21 ± 2.6	30.0
Veretryl	200µmoles/Kg	Ip	23 ± 3.1	23.3
Dimetylamino	200µmoles/Kg	Ip	25 ± 2.4	16.6

Values are mean ± SD.

*$p < 0.001$ (Values are compared with control)

#$p < 0.05$ (Values are compared with control)

Animals were induced angiogenesis by injecting 10^6 B16F10 melanoma cells intradermally. All the animals were sacrificed 9 days after tumour challenge and number of tumour directed micro vessels per cm^2 around the tumour was counted using a dissection microscope.

Table 4.2

Effect of different curcuminoid derivatives on the IL-1β profile of angiogenesis induced animals

Drug used	Dose	Mode (Simultaneous)	Amount of IL-1β (pg/ml serum)	
			24th h	9th Day
Normal	-	-	16 ± 3.5	
Control	-	-	30 ± 4.5	30 ± 3.7
C I	200µmoles/Kg	Ip	20 ± 2.2	18 ± 1.5
C II	200µmoles/Kg	Ip	25 ± 2.5	28 ± 3.4
C III	200µmoles/Kg	Ip	22 ± 3.1	18 ± 2.9
Salicyl	200µmoles/Kg	Ip	24 ± 1.9	20 ± 2.8
Anisyl	200µmoles/Kg	Ip	31 ± 2.6	30 ± 3.6
Veretryl	200µmoles/Kg	Ip	29 ± 2.5	31 ± 3.2
Dimetylamino	200µmoles/Kg	Ip	29 ± 3.0	30 ± 1.9

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-1β were estimated by ELISA method.

Table 4.3

Effect of *T.cordifolia* and *B.diffusa* on the IL-6 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of IL-6 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	35 ± 6.5	
Control	-	-	35 ± 5.6	320 ± 9.5
C I	200μmoles/Kg	Ip	34 ± 3.6	70 ± 8.5
C II	200μmoles/Kg	Ip	35 ± 4.3	170 ± 12.6
C III	200μmoles/Kg	Ip	38 ± 5.6	66 ± 10.5
Salicyl	200μmoles/Kg	Ip	38 ± 6.1	78 ± 8.6
Anisyl	200μmoles/Kg	Ip	40 ± 4.9	188 ± 15.2
Veretryl	200μmoles/Kg	Ip	41 ± 8.6	202 ± 16.2
Dimetylamino	200μmoles/Kg	Ip	36 ± 5.6	196 ± 18.0

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-6 were estimated by ELISA method.

Table 4.4

Effect of different curcuminoid derivatives on the GM-CSF profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of GM-CSF (pg/ml serum)	
			24th h	9th Day
Normal	-	-	18 ± 3.1	
Control	-	-	70 ± 5.6	30 ± 3.2
C I	200µmoles/Kg	Ip	20 ± 3.1	18 ±2.4
C II	200µmoles/Kg	Ip	29 ± 2.6	20 ± 2.6
C III	200µmoles/Kg	Ip	23 ± 1.9	18 ± 2.3
Salicyl	200µmoles/Kg	Ip	30 ± 3.5	22 ± 3.2
Anisyl	200µmoles/Kg	Ip	42 ± 4.1	20 ± 2.4
Veretryl	200µmoles/Kg	Ip	53 ± 4.6	21 ± 2.0
Dimetylamino	200µmoles/Kg	Ip	45 ± 3.8	19 ± 2.4

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of GM-CSF were estimated by ELISA method.

Table 4.5

Effect of *T.cordifolia* and *B.diffusa* on the TNF-α profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of TNF-α (pg/ml serum)	
			24th h	9th Day
Normal	-	-	20 ± 3.2	
Control	-	-	180 ± 6.3	630 ± 8.5
C I	200µmoles/Kg	Ip	90 ± 6.8	150 ± 13.2
C II	200µmoles/Kg	Ip	105 ± 10.5	173 ± 15.1
C III	200µmoles/Kg	Ip	120 ± 15.8	155 ± 18.4
Salicyl	200µmoles/Kg	Ip	115 ± 16.8	165 ± 18.5
Anisyl	200µmoles/Kg	Ip	155 ± 14.6	450 ± 19.1
Veretryl	200µmoles/Kg	Ip	150 ± 19.2	375 ± 17.5
Dimetylamino	200µmoles/Kg	Ip	170 ± 20.1	550 ± 24.2

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of TNF-α were estimated by ELISA method.

Table 4.6

Effect of different curcuminoids on the VEGF profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of VEGF (pg/ml serum)	
			24th h	9th Day
Normal	-	-	16 ± 8	
Control	-	-	62 ± 9	150 ± 12
C I	200μmoles/Kg	Ip	30 ± 9	35 ± 4.3
C II	200μmoles/Kg	Ip	38 ± 8	60 ± 5.6
C III	200μmoles/Kg	Ip	30 ± 5.6	72 ± 7.4
Salicyl	200μmoles/Kg	Ip	35 ± 3.9	58 ± 6.3
Anisyl	200μmoles/Kg	Ip	49 ± 6.1	98 ± 6.5
Veretryl	200μmoles/Kg	Ip	53 ± 7.5	120 ± 7.5
Dimetylamino	200μmoles/Kg	Ip	58 ± 6.4	118 ± 6.5

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of VEGF were estimated by ELISA method.

Table 4.7

Effect of different curcuminoids on the TIMP-1 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of TIMP-1 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	600 ± 36	
Control	-	-	350 ± 19	350 ± 19
C I	200µmoles/Kg	Ip	750 ± 42	1100 ± 55
C II	200µmoles/Kg	Ip	675 ± 35	855 ± 35
C III	200µmoles/Kg	Ip	850 ± 40	975 ± 40
Salicyl	200µmoles/Kg	Ip	955 ± 50	1350 ±58
Anisyl	200µmoles/Kg	Ip	1050 ± 55	1150 ± 42
Veretryl	200µmoles/Kg	Ip	1110 ± 42	1250 ± 51
Dimetylamino	200µmoles/Kg	Ip	765 ± 32	850 ± 31

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of TIMP-1 were estimated by ELISA method.

Table 4.8

Effect of different curcuminoids on the IL-2 profile of angiogenesis induced animals.

Drug used	Dose	Mode (Simultaneous)	Amount of IL-2 (pg/ml serum)	
			24th h	9th Day
Normal	-	-	23 ± 3.2	
Control	-	-	16 ± 2.1	20 ± 2.5
C I	200μmoles/Kg	Ip	28 ± 2.0	35 ± 4.1
C II	200μmoles/Kg	Ip	21 ± 1.9	25 ± 3.1
C III	200μmoles/Kg	Ip	24 ± 3.4	30 ± 2.9
Salicyl	200μmoles/Kg	Ip	28 ± 3.0	28 ±1.8
Anisyl	200μmoles/Kg	Ip	25 ± 2.6	24 ± 2.3
Veretryl	200μmoles/Kg	Ip	23 ± 2.2	26 ± 3.1
Dimetylamino	200μmoles/Kg	Ip	24 ± 3.3	25 ± 4.0

All the values are mean ± SD

Blood was collected from the angiogenesis induced animals (see Table 1) at the indicated time points after tumour challenge. Serum was separated by centrifugation, and levels of IL-2 were estimated by ELISA method.

Figure 4.1. Effect of natural curcumins on *in vivo* Angiogenesis. The animals were induced tumour directed neoangiogenesis by injecting B16F10 melanoma cells (10^6 cells/0.05ml vol) intradermally. All the curcumins were treated at a concentration of 200μmoles/Kg

A) Vehicle treated control

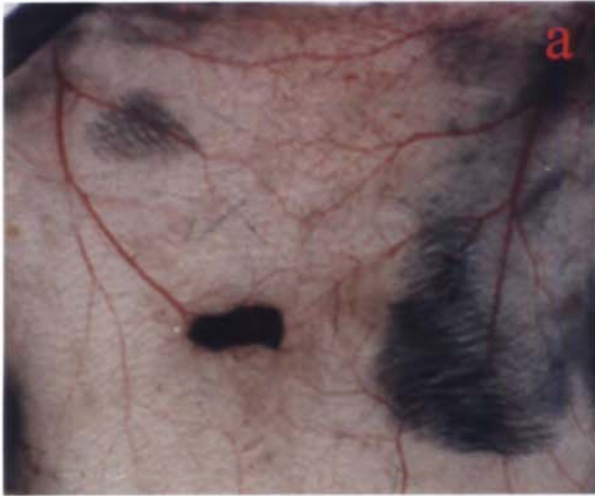
B) TNP-470 treated (30mg/Kg)

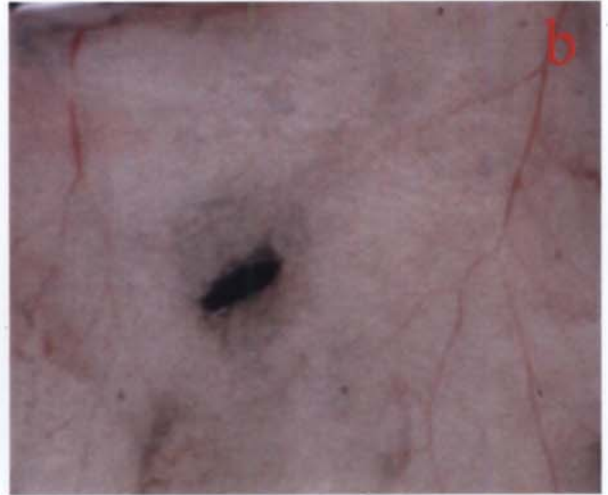
C) CI treated

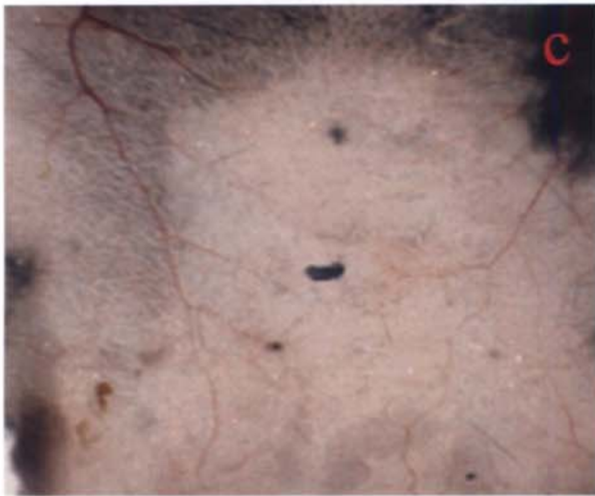
D) CII treated

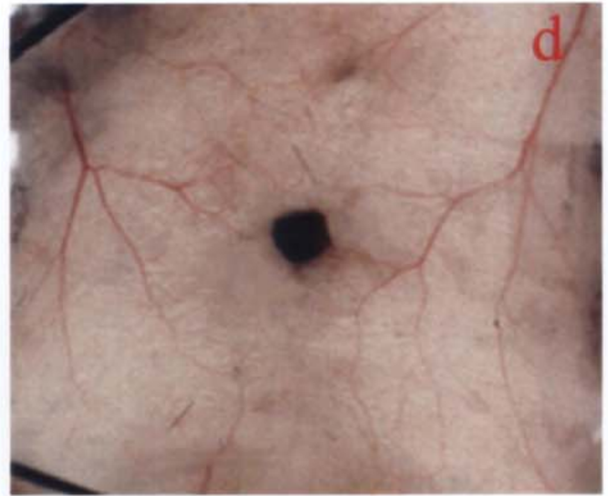
E) CIII treated

Figure 4.1









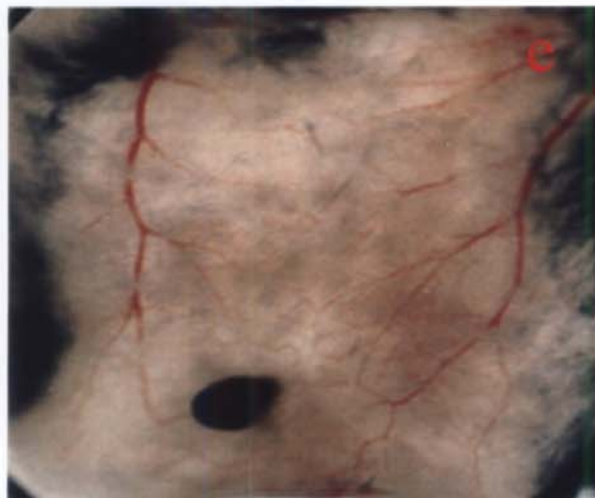


Figure 4.2. Effect of synthetic curcumin analogues on *in vivo* Angiogenesis. The animals were induced tumour directed neoangiogenesis by injecting B16F10 (10^6cells/0.05ml vol) melanoma cells intradermally. All the curcuminoids were treated at a concentration of 200µmoles/Kg

A) Vehicle treated control

B) TNP-470 treated (30mg/Kg)

C) SC treated

D) AC treated

E) VC treated

F) DC treated

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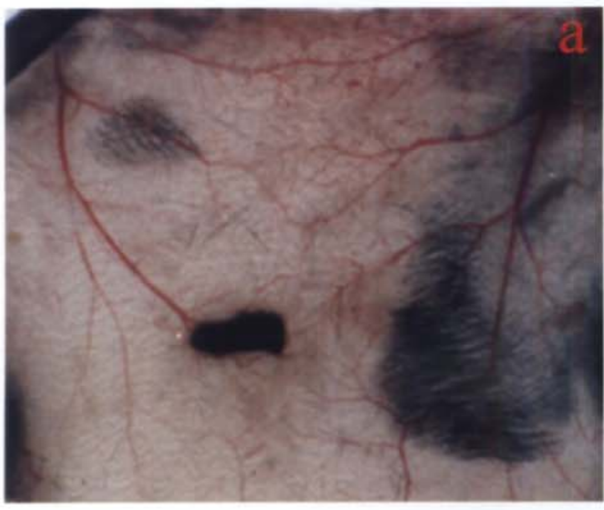
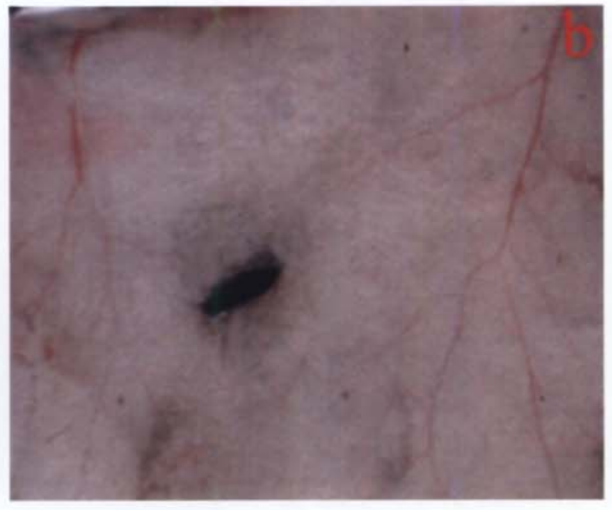
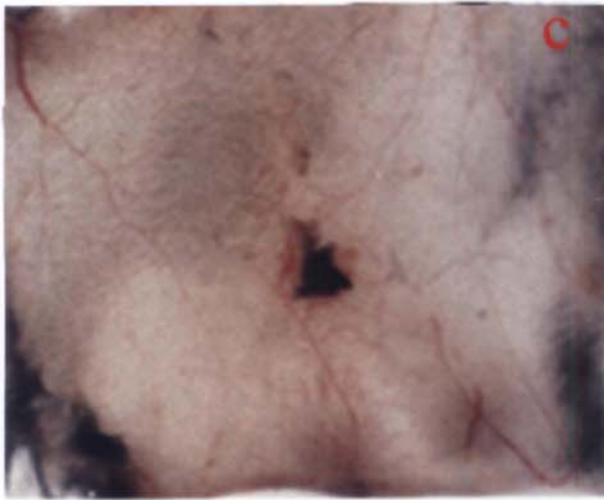
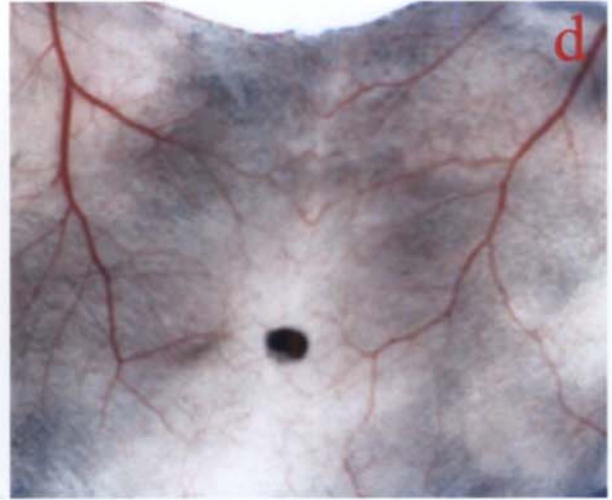
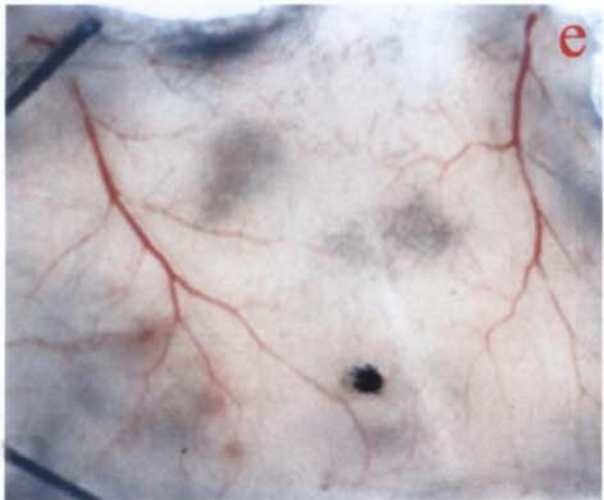
Figure 4.2

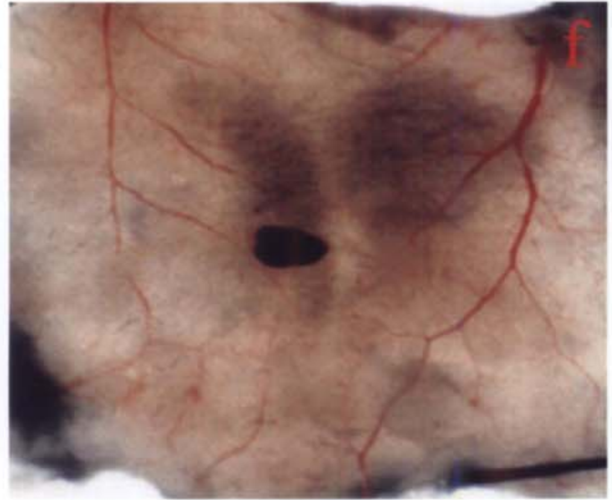












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Chapter 5

In vitro antiangiogenic activity studies using Boerhaavia, Tinospora and some Curcuminoid derivatives.

Introduction

Vascular remodeling in host tissues surrounding growing tumour is implicated in the successful development of tumour neovasculature (Zhang L et al 2003). The onset of angiogenesis appears to be the result of an imbalance between stimulatory and inhibitory factors that leads to the activation of previously quiescent endothelial cells (Langley R et al 2003). VEGF is regarded as one of the earliest and important signals to stimulate the multi-step cascade of tumour angiogenesis and is preferably by promoting endothelial cell proliferation and migration (Zhang L et al 2003). However, the activity of angiogenic factor is modulated by other factors that affect endothelial cell survival and attachment to surrounding structures (Stoeltzing O 2003). Activation of endothelial cell elicits a complex series of responses that include the elaboration of proteolytic enzymes, migration and proliferation. Endothelial cells begin to branch off from pre existing micro vessels and form capillary sprout structures that will ultimately coalesce and perfuse the tumour tissue (Langley R et al 2003). Given that endothelial cells play a central role in several rate-limiting steps of metastasis including angiogenesis, the endothelial cell behavior in the presence of different natural as well as synthetic compounds, has been the focus of intense investigation.

One of the major impediments to obtain a large number of endothelial cells from different tissues has been the inability to purify and propagate these cells in culture. For this Human Umbilical Vein Endothelial Cell (HUVEC) has been widely accepted as a model of study. In this study, we tried to evaluate the effect of *B. diffusa, T. cordifolia*, and some curcuminoid derivatives on the HUVEC proliferation, motility and invasion using nontoxic concentrations, in culture. We also tried to

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evaluate the micro vessel outgrowth pattern from a rat aortic ring when treated with these test materials.

Materials and Methods

Study materials: - Methanolic (70%) extracts of *B. diffusa* and *T. cordifolia*. Natural curcuminoids such as CI, CII, and CIII (Figure 1.2-4) synthetic curcuminoids such as Salicyl curcuminoid (SC), Anisyl curcuminoid (AC), Veratryl curcuminoid (VC) and Dimethylamino curcuminoid (DC) (Figure 1.5-8).

Cells and Tissues: - HUVEC (Isolated from umbilical chord as described in chapter 2) and B16F10 mice melanoma cells. Thoracic aortic ring sections of Sprague Dawley rat (Cultured as detailed in chapter 2).

Kit, Medium and Reagents: - Quantikine mouse mRNA estimation kit. DMEM with10%FCS and 199 with 20% FCS. HEPES, MTT, recombinant mouse VEGF, recombinant mouse bFGF, ^{3}H-thymidine, Matrigel (ECM), Collagen type-1 and Scintillation fluid (Preparation is described in chapter 2). Cells-to-cDNA kit, providing reverse transcription without RNA isolation and multiplex PCR kit of mouse inflammatory cytokine set.

Cell viability assay: -

HUVECs were plated into 96 well culture plates (5×10^3 cells/well) in a final volume of 200μl. The cells were treated with varying concentrations (0-10μg/ml) of curcuminoids or extracts of Boerhaavia or Tinospora for 24h. The chromogenic methyl thiazol tetrazolium bromide (MTT) dye, an indicator of metabolically active mass, was added to the cells and incubated for 4h at 37^{0}C. Cells were lysed and

intracellular formazan product was dissolved in DMSO. The absorbance was recorded at 570nm and percentage inhibition was plotted against untreated cells.

Cell Proliferation assay by ^{3}H-thymidine uptake: -

HUVECs (5×10^{4}) were plated and treated in triplicate with various concentrations of curcuminoids (0-1.0μg/ml) or extracts of Boerhaavia or Tinospora (0-10μg/ml) and 20ng/ml VEGF. The cells in each well were pulse-labeled with 1μCi ^{3}H-Thymidine/well (specific activity, 17000mCi/mmol) for 16h at 37^{0}C. The cells were harvested and the radioactivity associated with individual samples was measured in a liquid scintillation counter.

Endothelial cell motility assay: -

HUVECs (2×10^{5}) were seeded into tye-1 gelatin coated 96 well plate in 0.2ml medium. After reaching confluence in the well, a clear area was scraped in the monolayer with a 200μl yellow plastic tip by applying suction force. After being washed with serum free medium, the plates were further incubated for 24h in the presence and absence of various test compounds, and 20ng/ml VEGF, fixed in methanol thereafter and stained by crystal violet.

Invasion assay: -

Invasion of collagen matrix by endothelial cells was carried out using modified, Boyden chamber as described in chapter 2. In brief the lower compartment of the chamber was filled with diluted conditioned medium and type I collagen-coated polycarbonate filters were placed above this. Cells (1×10^{5}) were added to this along with 20ng/ml VEGF and incubated for 24h at 37^{0}C in 5% CO_2 atmosphere. Test

materials were added during this incubation period. After incubation, the medium from upper chamber was removed and the cells on the upper side of the filter were removed by a cotton swab. The filter was then taken from the chamber placed on a clean glass slide in an upside down fashion. It was then fixed in methanol for 1-3 minutes and stained for 3minutes with crystal violet. Cells that penetrated the polycarbonate filters were counted in 10 fields under a microscope and photographed.

VEGF mRNA expression studies: -

B16F10 cells (1×10^6) were plated in 30mm tissue culture petri dishes and incubated at 37^0C overnight in 5% CO_2 to get adhered. The cells were then treated with or without non-toxic concentration of extracts of Boerhaavia/Tinospora (10μg/ml) or curcuminoids (1.0μg/ml) for 4 hours. After incubation these cells were used for quantification of VEGF mRNA using the quantikine mRNA kit according to the manufacturer's procedure.

Pro-inflammatory cytokine gene expression studies using RT-PCR analysis

B16F10 melanoma cells (1.25×10^4/well) were seeded in a 96 well plate and incubated overnight. Then the cells were treated with/without a nontoxic concentration of the study compound for four hours in serum free medium (Boerhaavia/Tinospora -10μg/ml and curcuminoids -1.0μg/ml). After the treatment, the medium was removed and cells were washed with ice-cold PBS and used for mRNA isolation and cDNA synthesis. The cDNA was synthesized using a sophisticated 'Cell-to-cDNA' kit (Ambion International) by reverse transcription in a thermalcycler set at 42^0C for 60 minutes and then 92^0C for 5minutes to inactivate

reverse transcriptase enzyme. This cDNA samples can be then stored at $^-20^0C$ or can be used for further amplification.

From this cDNA synthesized from RNA using Oligo $(dt)_{18}$ primers, the genes of interest such as IL-1β (430bp), IL-6 (484bp), TNF-α (290bp), GM-CSF (375bp) and IL-12p40 (239) were amplified by PCR. The internal standard used for the comparison was mouse GAPDH (557bp). All these PCR primers and the master mix for thermal cycling were provided in a single multiplex PCR kit (Biosource). This kit has been designed to direct the simultaneous amplification of specific ORF (Open Reading Frame) region of the mouse GAPDH, IL-1β, IL-6, GM-CSF, TNF-α and IL-12p40 genes. For running the PCR the thermal cycler were set as following.

A) After initial denaturation step at 95^0C for 1min, run the following temperature profiles for 5 cycles: denaturing – 94^0C for 1min, annealing - 60^0C for 4min

B) Continue the cycle program as follows for 40cycles: denaturing – 94^0C for 1min, annealing - 68^0C for 2.5min

C) For the final step, incubate at 70^0C for 10min followed by soaking at 25^0C. This can be stored at $^-20$ for subsequent analysis.

The amplified products were then electrophorezed on 2.0% agarose incorporated with ethidium bromide (0.5µg/ml gel). Bands of the separated DNA were then visualized using a UV transilluminator and documented using a gel-documentation system.

Gelatin gel zymography analysis for MMP activity: -

HUVECs were cultured to sub confluence in 30mm petri dishes coated with gelatin. The cells were then treated overnight with nontoxic concentration of extracts of Boerhaavia, Tinospora (10µg/ml) or curcuminoid derivatives (1.0µg/ml). The medium was then replaced with 1.0ml serum free media containing 20ng/ml VEGF and 20ng/ml bFGF and further incubated for 24h. This conditioned media were collected, centrifuged and the supernatant was used for zymogram analysis as detailed in chapter 2.

Rat aortic ring assay: -

Aorta removed from a freshly sacrificed Sprague Dawley rat and rinsed in ice-cold PBS containing penicillin and streptomycin. Segmental ring ~1mm in width were cut from the aorta and embedded in a 50µg collagen coated 96well culture plate. Rings were incubated overnight at 37^0C in complete medium and then exchanged for conditioned media from treated or untreated B16F10 melanoma cells. Subsequently rings were incubated for 6days at 37^0C analyzed by phase contrast microscopy for micro vessel out growth.

Results

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on the viability of HUVECs: -

To establish the non-toxic doses of study materials to HUVEC, MTT assay was performed. Data (Figure 5.1, 5.2) shows that curcumin (CI) was least toxic and concentration up to 5µg/ml did not significantly affect the viability of the endothelial

cells. But a concentration of 10μg/ml had a toxicity of 8% compared to the untreated control. This result correlates with the earlier studies, where 24h incubation with 10μM curcumin showed a toxicity of about 15% (Sing AK et al 1999). Other curcumins such as CII (10%) CIII (35%) SC (30%) AC (22%) VC (19%) and DC (28%) at this concentration (10μg/ml) also showed toxic effects to HUVEC when treated for a long period of 48h. Extracts of Boerhaavia and Tinospora required 50μg/ml concentration to show any toxic effect.

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on the ^{3}H-thymidine uptake by HUVECs: -

Treatment with curcuminoids and Boerhaavia and Tinospora for 48h suppressed tritiated thymidine uptake by HUVEC in a dose dependent manner (Figure 5.3, 5.4). There was almost 80-85% inhibition of tritiated thymidine uptake within 16h by the treatment with curcumin (1μg/ml) where as more than 95% of the cells were viable at that time point as measured by MTT assay. Treatment with other curcuminoids such as CII (78%), CIII (85%), SC (80%), AC (67%), VC (56%), and DC (56%) at this concentration (1μg/ml) also inhibited the thymidine uptake significantly. HUVEC when treated with extracts of Boerhaavia and Tinospora (25μg/ml) inhibited the thymidine uptake by 64% and 60% respectively.

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on the HUVEC Motility/migration (Figure 5.5 a-t): -

HUVECs migrated into the denuded scar area when stimulated with VEGF and an inhibitory effect in this migration was observed when treated with curcumins such as CI, CIII, and SC and extract of Boerhaavia and Tinospora. The inhibitory

effect was not attributed to a cytostatic or cytotoxic effect because neither concentrations (0.5μ/ml and 1.0μg/ml for the curcumins and 10μg/ml and 25μg/ml for extracts) had any notable effect on HUVEC viability as estimated by MTT reduction method. The other curcuminoids (CII, AC, VC and DC) did not have any inhibitory effect in EC migration to the degelatinated area and looked like that of untreated control when visualized under microscope.

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on the HUVEC Invasion (Figure 5.6 a-j): -

HUVECs were seen to migrate across collagen-coated filters when induced with VEGF and bFGF in the invasion assay. Boerhaavia and Tinospora treatment significantly inhibited the cell invasion compared to the untreated control in a dose dependent manner. Curcumins such as CI, CIII, and SC also inhibited HUVEC invasion whereas other curcuminoids (CII, AC, VC and DC) did not had a profound inhibition in HUVEC invasion.

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on VEGF mRNA expression: -

Agents that inhibited the elevation in serum VEGF level of angiogenesis induced animals were used in this study. VEGF mRNA quantification studies showed that curcumin and some other compounds inhibited the RNA transcripts of mouse VEGF from B16F10 melanoma (Table 5.1). This indicates that these compounds possibly exert their action at a transcriptional level at least in tumour cells. Four hour treatment to the B16F10 monolayer with nontoxic concentration of extracts (Boerhaavia and Tinospora 10μg/ml) and curcuminoids (CI, CIII and SC 1.0μg/ml)

reduced the VEGF mRNA transcript level in the cells compared to the untreated control.

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on proinflammatory cytokine gene expression: -

Agents that showed an inhibition in VEGF mRNA transcription further evaluated for their effect on proinflammatory cytokine gene expression by means of RT-PCR analysis. Four hour treatment of the B16F10 monolayer with nontoxic concentration of extracts (Boerhaavia and Tinospora 10μg/ml) and curcuminoids (CI, CIII and SC 1.0μg/ml) reduced the levels of mRNA transcripts for IL-1β, IL-6, GM-CSF and TNF-α in the cells compared to the untreated control where as none of the treatment had a significant inhibition in IL-12p40 expression (Figure 5.11).

Effect of curcuminoids and extracts of Boerhaavia and Tinospora on the HUVEC proteases: -

In order to reveal the changes in the expression/activity of proteases, conditioned medium from treated or untreated HUVECs were analyzed by gelatin zymography (Figure 5.7, 5.8, 5.9). The major forms of proteases detected by gelatin zymography are type IV collagenases, MMP-2 and 9. Since these proteases secreted by the cells into the conditioned medium is mainly proenzymes (latent form requiring activation), and this assay is able to detect only active MMPs, an activation step (trypsin activation) is necessary to get the MMPs activated. Since the addition of EDTA to the substrate incubation buffer completely inhibits the proteolytic activity by chelating the Ca^{++}, it clearly demonstrates that it is a metalloproteinase, which is responsible for the gelatinilytic activity.

Conditioned medium from pretreated HUVEC (with Boerhaavia, Tinospora, CI, CIII and SC) showed no clear band in the zymograph indicating the inhibition of gelatinase production/activity by the treatment. When these agents were added to the substrate incubation buffer in which the gels after electrophoretic separation is incubating to get the enzymatic digestion, no clear bands were observed indicating the inhibitory effect of these compounds on gelatinase activity.

<u>Effect of curcuminoids and extracts of Boerhaavia and Tinospora on rat aorta angiogenesis, *in vitro*: -</u>

Treatment with curcuminoids and extracts of Boerhaavia and Tinospora at a concentration of 10μg/ml inhibited the micro vessel out growth from the rat aorta ring induced by the conditioned medium from the B16F10 melanoma cells (Figure5.10 a-t). It is interesting to note that aortic rings incubated with conditioned media from the pre-treated B16F10 cells also showed a marked reduction in vessel out growth when compared with rings incubated with conditioned medium from untreated B16F10 melanoma cells. This also correlates with the results of mRNA expression studies, where the treatment inhibited IL-1β, IL-6, GM-CSF, TNF-α and VEGF mRNA transcripts formation. These results indicate that the treatment effectively inhibited the proangiogenic factor from the melanoma cells.

Discussion

Angiogenesis plays a key role in tumour initiation and promotion leading to metastasis. The invasion, proliferation, migration and differentiation of endothelial cell leads to the formation of a new mature vessel. In order to study the mechanism of action of the study materials at a cellular level, in inhibiting the tumour specific

angiogenesis, we have used some *in vitro* systems. Using an *in vitro* rat aortic ring assay, it was observed that the *in vivo* neovessel formation inhibitors at nontoxic concentrations inhibited the production of proangiogenic factors from B16F10 melanoma cells because the conditioned medium from the treated melanoma cells induced little microvessel outgrowth compared to the untreated control. The direct treatment of these angiogenesis inhibitors also inhibited the micro vessel outgrowth from the aortic ring.

It is a common consensus that tumour cells are capable of producing VEGF that auto stimulates neo angiogenesis of neoplastic tissue and tumour growth. Recent studies have shown that tumour cell derived VEGF is important but not sufficient to auto-maintain the tumour growth. Cellular sources of VEGF other than tumour cells are multiple and VEGF secreted by tumour infiltrating macrophages, Fibroblasts, PMN and mast cells essentially support tumour angiogenesis (Barbera-Guillem et al 2002). The mRNA quantification and RT-PCR studies reveal that the treatment could also inhibit the production of tumour derived VEGF and proinflammatory cytokines indicating the action of these antiangiogenic agents at gene expression level. All proinflammatory cytokine level was reduced by the treatment whereas none of the treatment showed a significant inhibition in IL-12p40 expression. IL-12 is the major driving force to induce Th1 (T helper -T_H- subset) differentiation that is linked to several pathologies such as arthritis, tumour progression and functioning as an indirectly acting inflammatory cytokine.

The present study demonstrates that certain curcuminoids (CI, CIII and SC) and extracts of Tinospora and Boerhaavia inhibited the proliferation and migration of HUVECs induced by VEGF in a dose dependent manner without significantly

affecting the viability of the cell. Some authors have suggested a mechanism for the inhibition in proliferation by curcumin –is related to the inhibition of Thymidine Kinase (TK) activity and thereby arresting the cells in the early S-phase of cell cycle (Sing AK et al 1996).

Proteolytic degradation of the extra cellular matrix, extend of the tumour vasculature and metastasis correlate with the expression endopeptidases known as MMPs (Schnaper HW et al 1993). MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage. Therefore the regulation of MMPs occurs at three levels, gene expression, proenzyme processing and inhibition of enzymatic activity (Woo JH et al 2003). Inhibition of MMP activity will reduce the invasiveness of the cells and also the tube formation (Taloor D et al 1998). Our results also point that the curcuminoids and extracts that inhibited the MMP activity, as visualized from the zymogram analysis, had also inhibited the collagen matrix invasion by endothelial cell *in vitro*.

We do not know whether the increased/decreased level of MMP-2 and –9 resulted from an induction/inhibition in the expression of genes in the cells. Earlier studies using blotting techniques are suggesting that curcumin inhibited the RNA transcripts of 72kDa protease indicating that it inhibited MMP-2 accumulation induced by any stimuli at the transcriptional and post transcriptional level. Because VEGF, IL-6, IL-8 and MMPs are NFκB regulated genes (Pan et al 2002), we anticipate that the decreased secretion of these proteins may a direct consequence on curcumin's ability to inhibit NFκB activity. Multiple factors including hypoxia, NO, and inflammatory cytokines such as MCP-1, IL-1 and IL-6 are involved in controlling

VEGF expression in normal tissue repair and remodeling events (Barbera-Guillem et al 2002).

In short, the test material that showed an antiangiogenic activities in *in vivo* systems were also giving a correlating results in the *in vitro* experiments. They all were showing an inhibitory effect on endothelial cell proliferation, mobility and invasion induced either by VEGF or by bFGF. It is also interesting to note that these antiangiogenic agents can also exert their effect on the tumour cells as well. This was evidenced from the aortic ring assay where the treatments of tumour cells with these agents were inhibiting the proangiogenic factor production thereby negatively contributing for the success of angiogenesis. The mRNA quantitation studies and RT-PCR analysis further confirm these results.

Table 5.1

VEGF mRNA quantification studies.

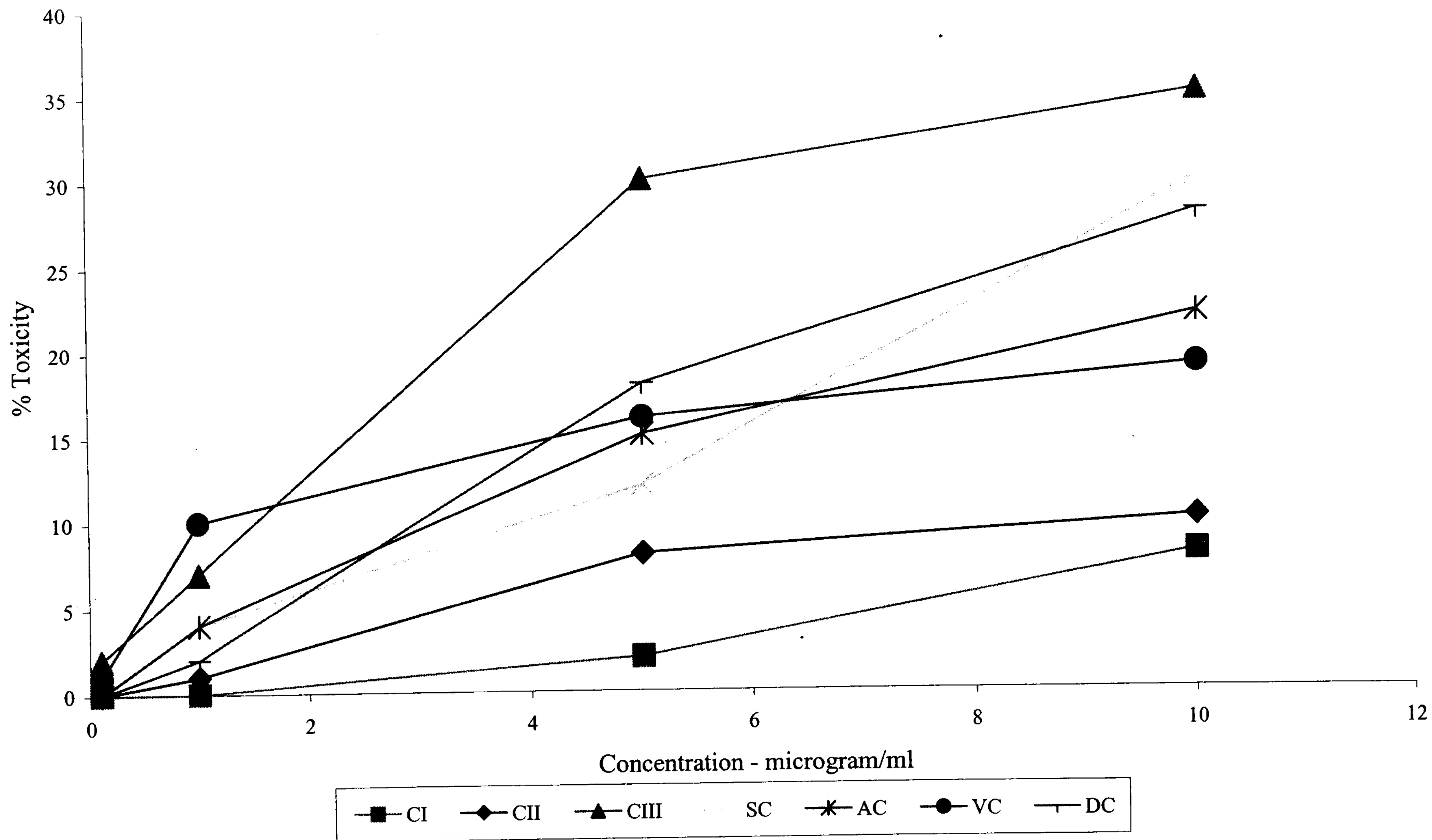
Treatment	Amount of mRNA (attomoles*/ml cell lysate)
Control cells (without any treatment)	28.9 ± 3.1
CI (1.0μg/ml)	10.3 ± 2.8#
CIII (1.0μg/ml)	11.2 ± 2.1#
Boerhaavia extract (10.0μg/ml)	9.5 ± 1.9#
Tinospora extract (10.0μg/ml)	10.2 ± 2.6#

*1 attomole (10^{-18}moles) = 600,000 molecules.

#$p > 0.001$

B16F10 cells (1×10^{6}) were treated with nontoxic concentration of the curcuminoid/extract for four hours. VEGF mRNA was isolated and estimated using the quantikine mouse VEGF mRNA kit according to the manufactures protocol.

Figure 5.1 Cytotoxic activities of curcuminoids to HUVEC



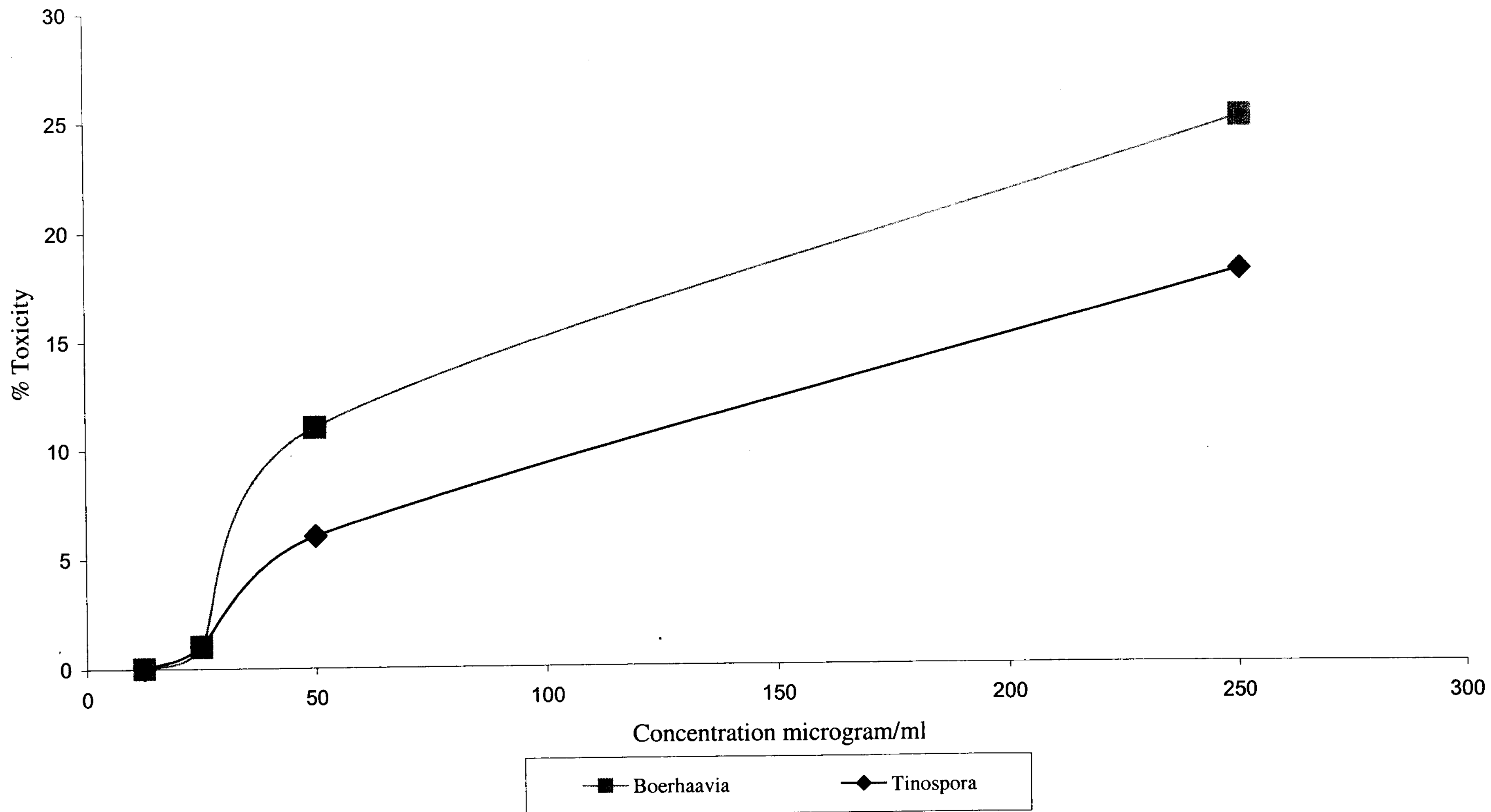
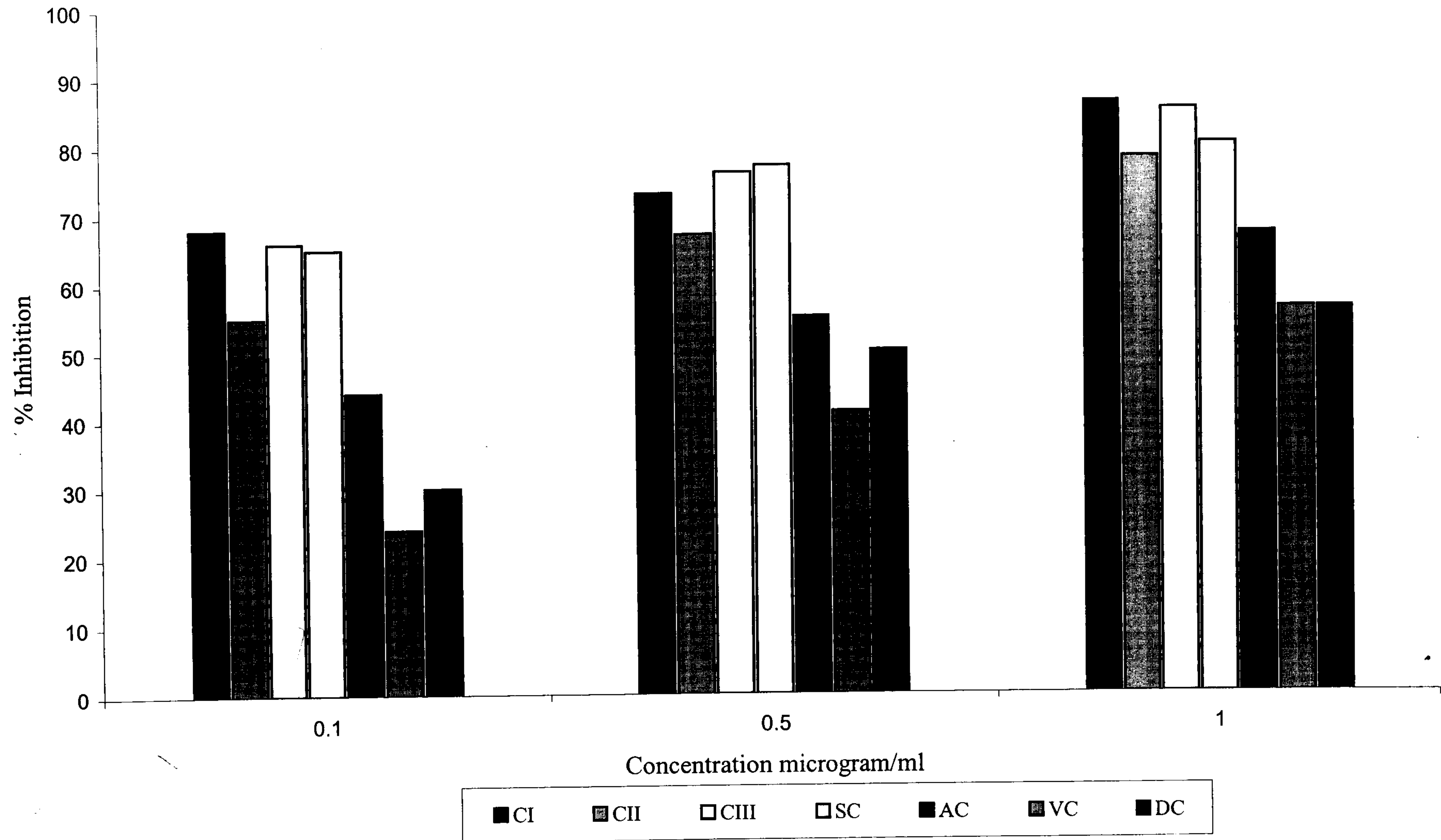


Figure 5.2 Cytotoxic activities of Boerhaavia and Tinospora to HUVEC
% Toxicity
30
25
20
15
10
5
0
0
50
100
150
200
250
300
Concentration microgram/ml
Boerhaavia
Tinospora

Figure 5.3 Inhibition in thymidine uptake by HUVEC



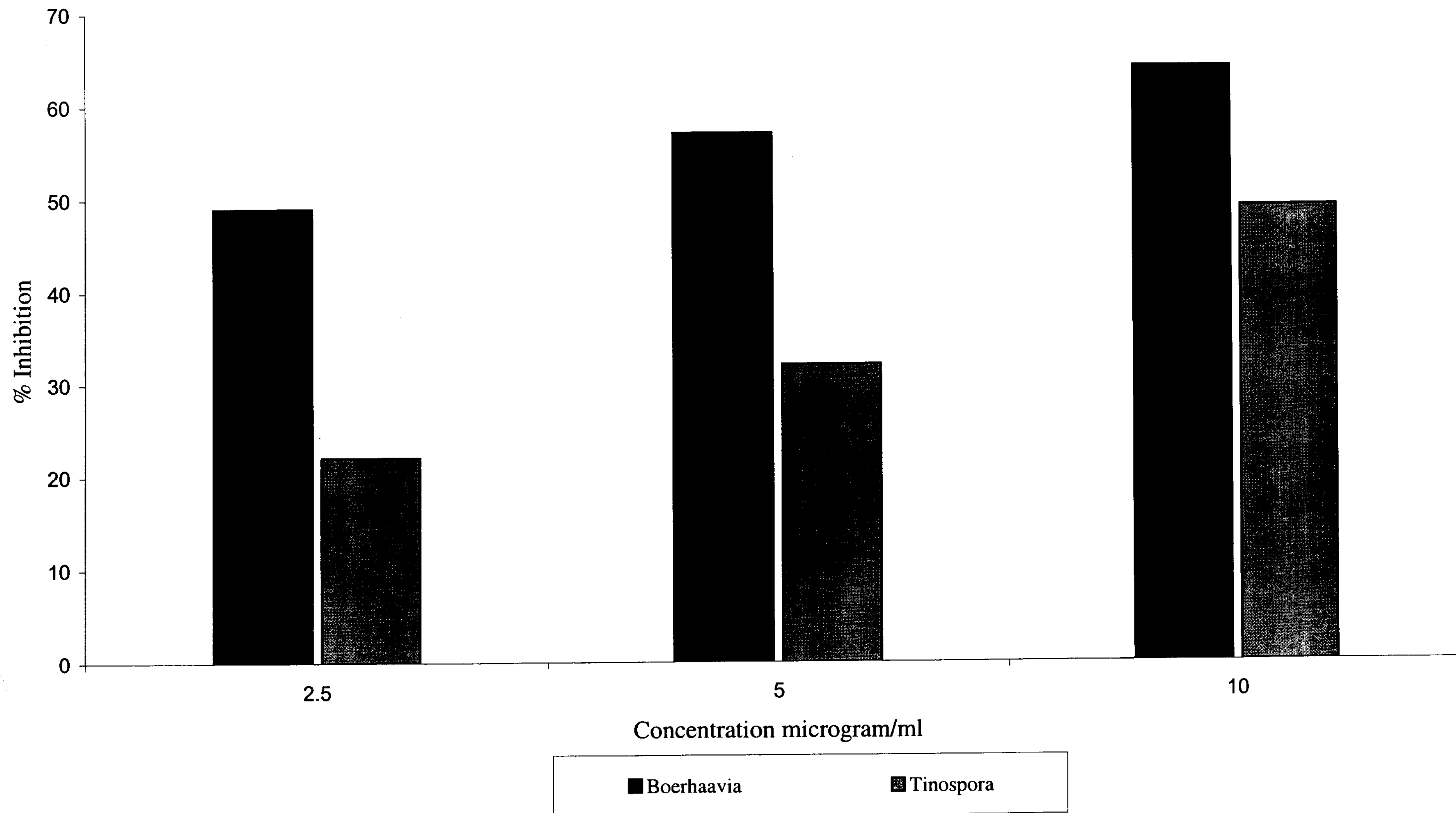


Figure 5.4 Inhibition in thymidine uptake by HUVEC

Figure 5.5 Inhibitory effects of Boerhaavia and Tinospora on HUVEC migration.

A) Control - '0' hour incubation

B) Control after 24h incubation in medium without any compounds

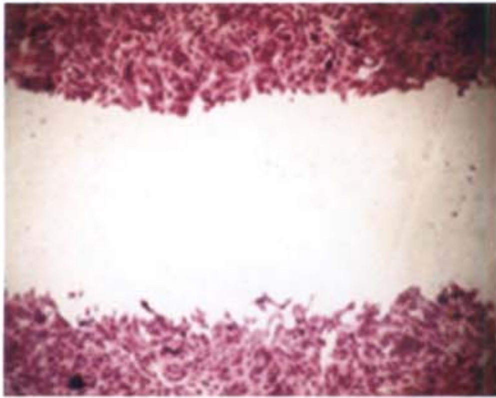
C) B.diffusa (5.0μg/ml)

D) B.diffusa (10.0μg/ml)

E) T.cordifolia (5.0μg/ml)

F) T.cordifolia (10.0μg/ml)

Figure 5.5



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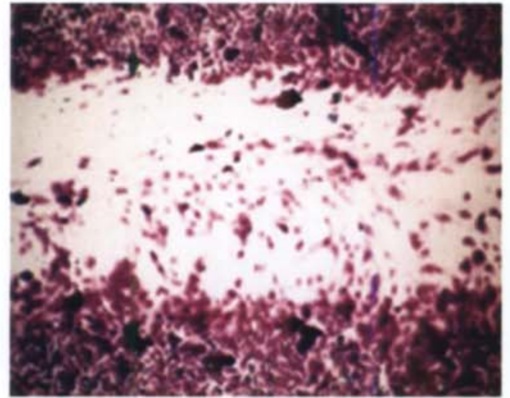

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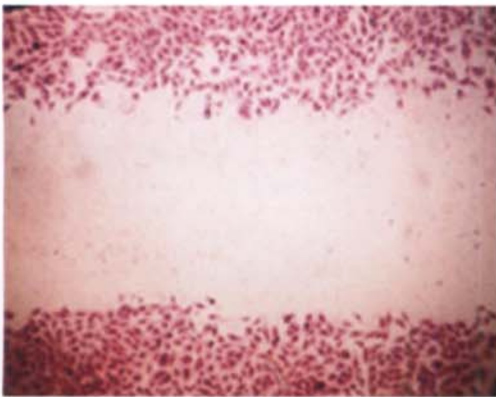

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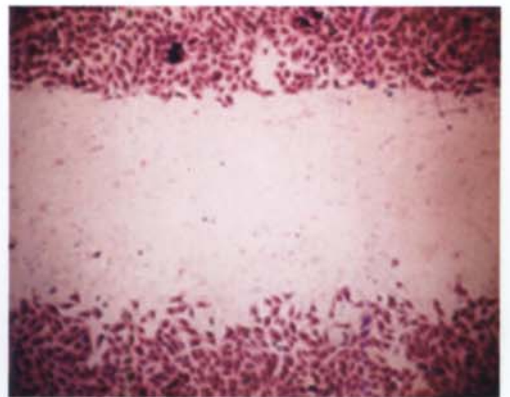

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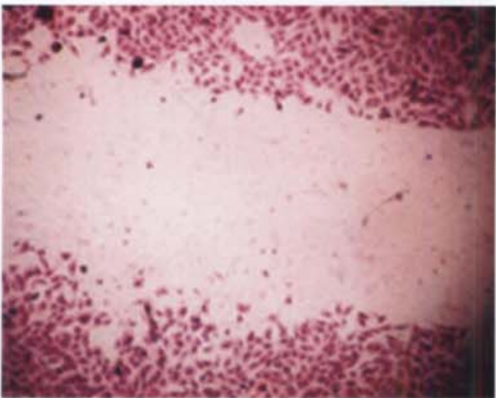

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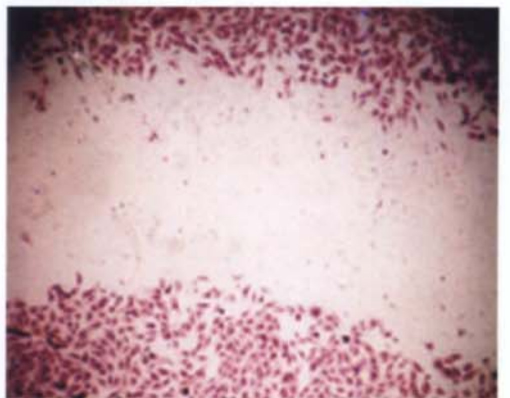


F

Figure 5.5 Inhibitory effects of natural curcumins on HUVEC migration.

A) Control - '0' hour incubation

B) Control after 24h incubation in medium without any compounds

G) CI treated (1.0μg/ml)

H) CI treated (0.5μg/ml)

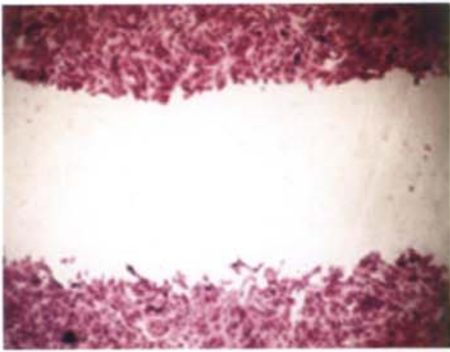
I) CII treated (1.0μg/ml)

J) CII treated (0.5μg/ml)

K) CIII treated (1.0μg/ml)

L) CIII treated (0.5μg/ml)

Figure 5.5



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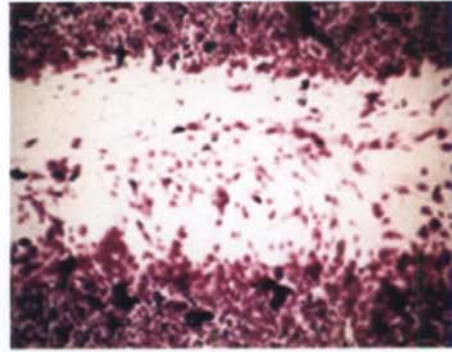

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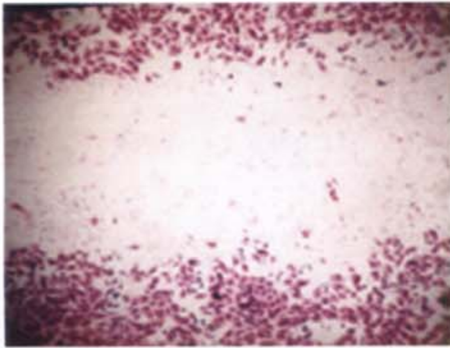

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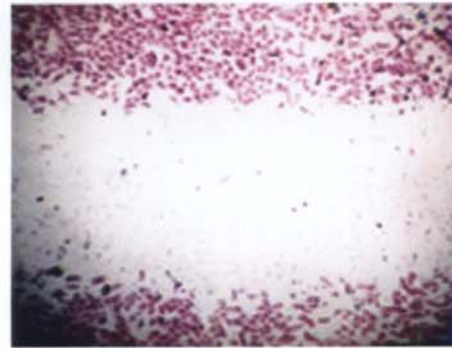

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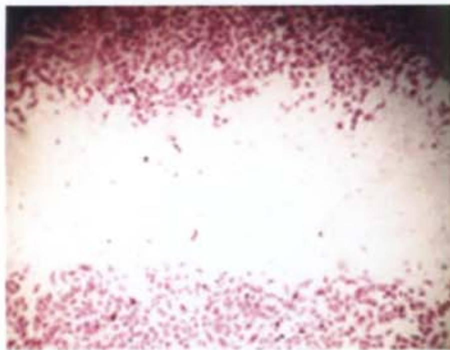

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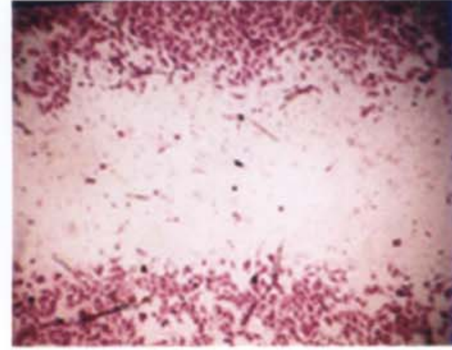

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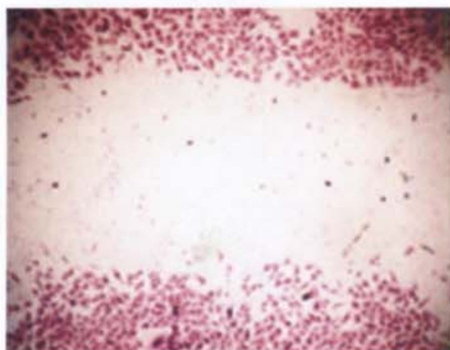

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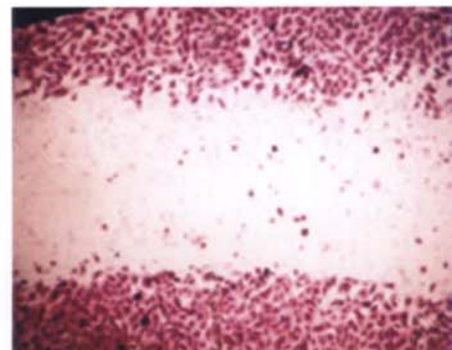


L

Figure 5.5 Inhibitory effects of synthetic curcuminoids on HUVEC migration.

A) Control - '0' hour incubation

B) Control after 24h incubation in medium without any compounds

M) SC treated (1.0μg/ml)

N) SC treated (0.5μg/ml)

O) AC treated (1.0μg/ml)

P) AC treated (0.5μg/ml)

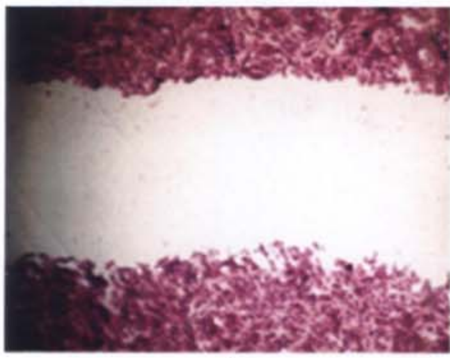
Q) VC treated (1.0μg/ml)

R) VC treated (0.5μg/ml)

S) DC treated (1.0μg/ml)

T) DC treated (0.5μg/ml)

Figure 5.5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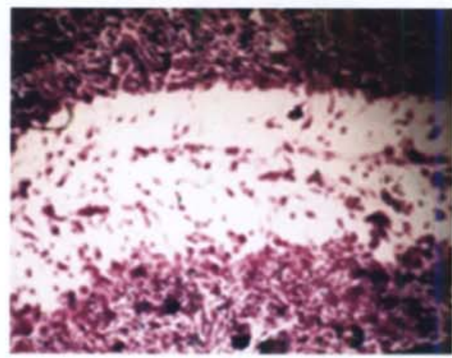
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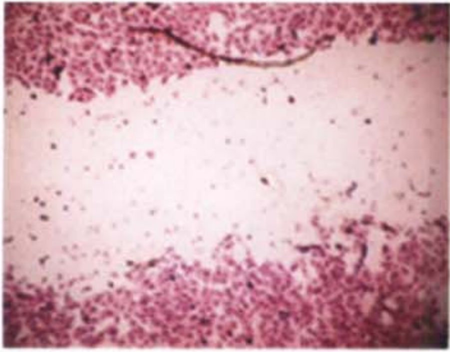
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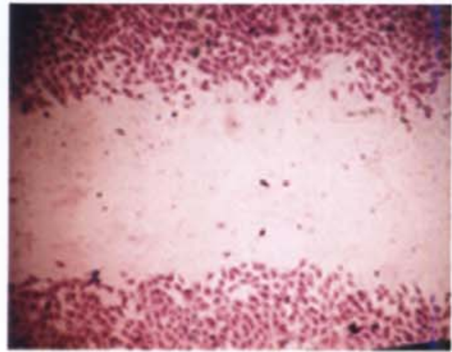
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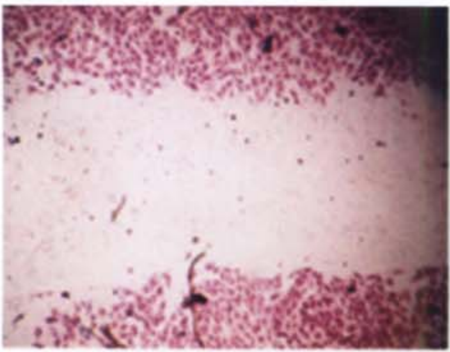
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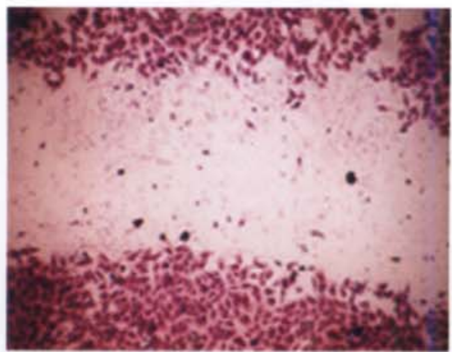
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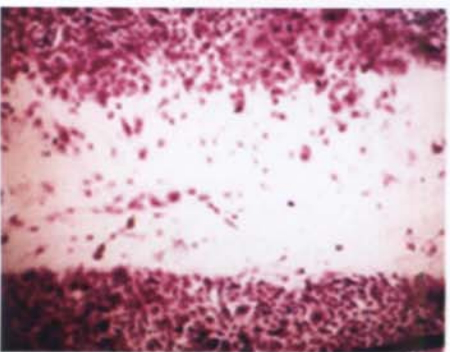
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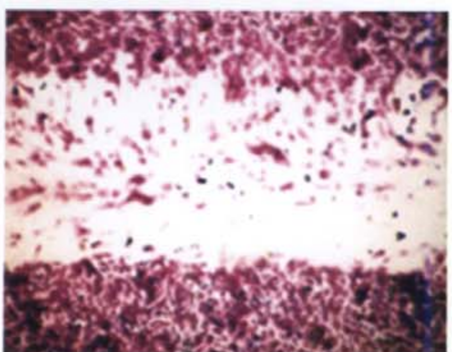
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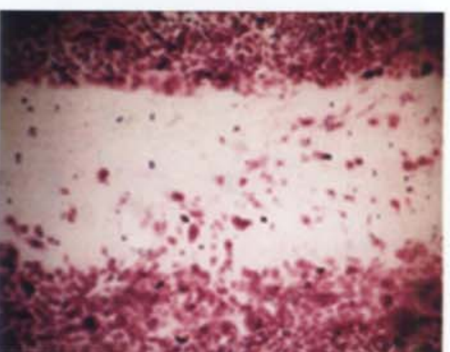
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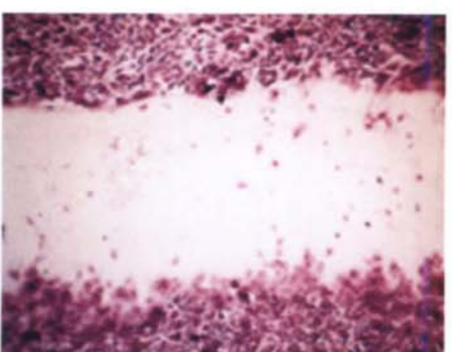

T

26

Figure 5.6 Inhibitory effects of Boerhaavia and Tinospora on HUVEC invasion through collagen matrix.

A) Control '0' hour incubation, without any treatment

B) Control after 24h incubation

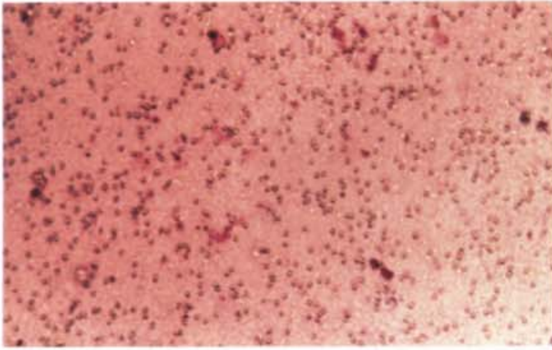
C) Boerhaavia treated (10μg/ml)

D) Boerhaavia treated (5μg/ml)

E) Tinospora treated (10μg/ml)

F) Tinospora treated (5μg/ml)

Figure 5.6



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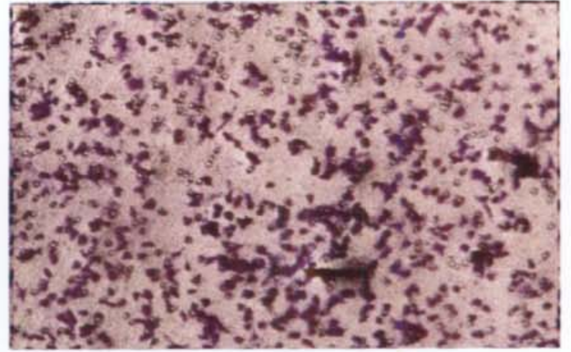

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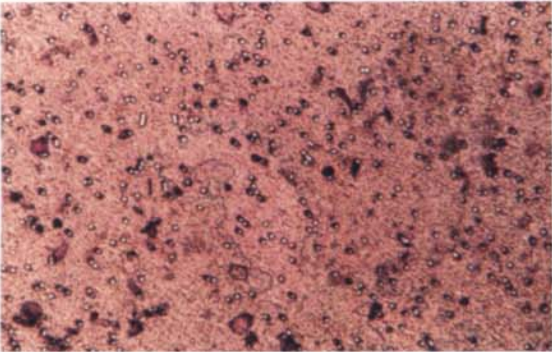

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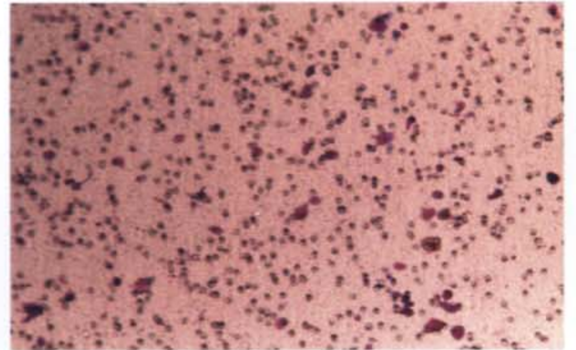

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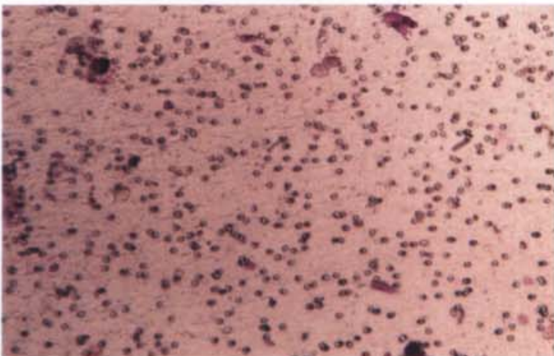

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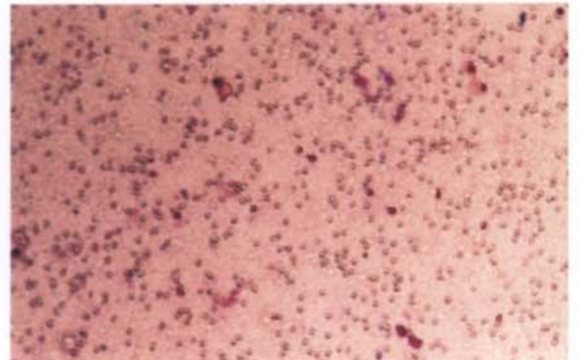


F

28

Figure 5.6 Inhibitory effects of natural curcuminoids on HUVEC invasion through collagen matrix.

G) CI treated (0.5μg/ml)

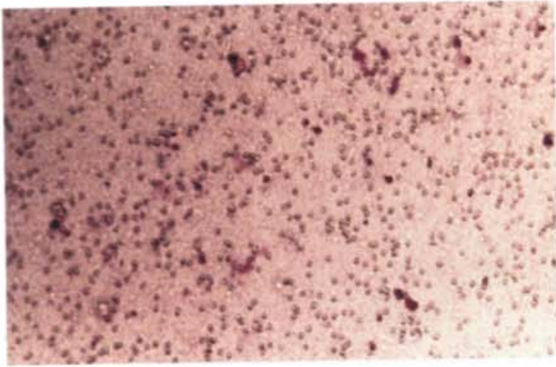
H) CI treated (1.0μg/ml)

I) CII treated (1.0μg/ml)

J) CIII treated (0.5μg/ml)

K) CIII treated (1.0μg/ml)

Figure 5.6



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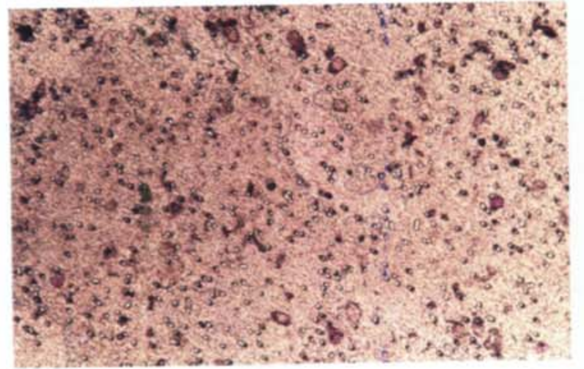

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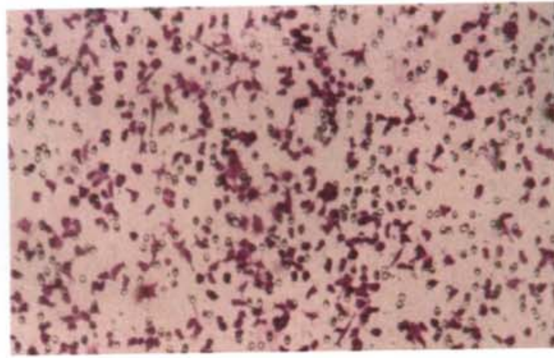

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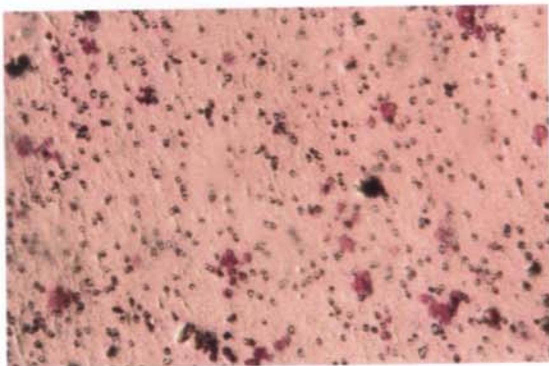

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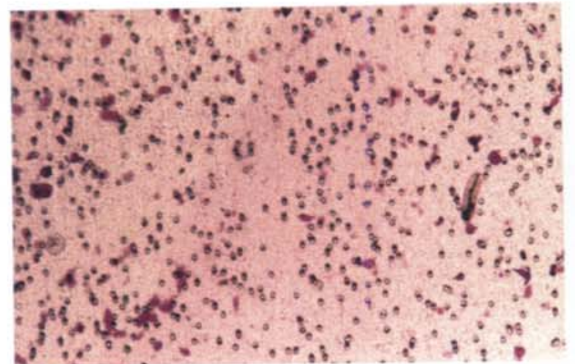


K

Figure 5.6 Inhibitory effects of synthetic curcuminoids on HUVEC invasion through collagen matrix.

L) SC treated (1.0μg/ml)

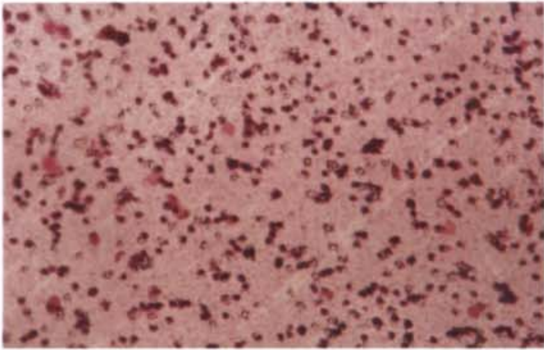
M) SC treated (0.5μg/ml)

N) AC treated (1.0μg/ml)

O) VC treated (1.0μg/ml)

P) DC treated (1.0μg/ml)

Figure 5.6



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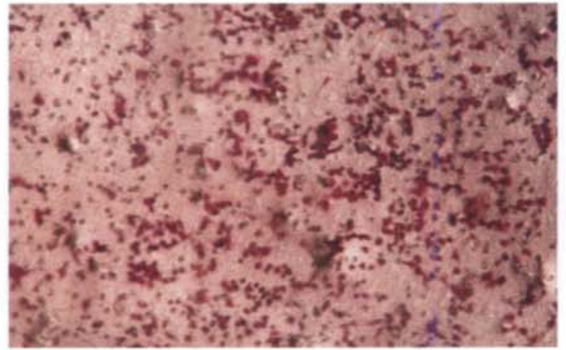

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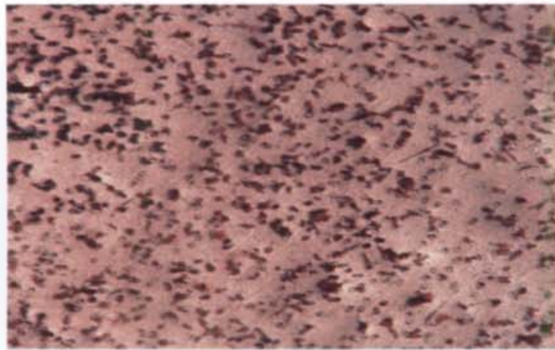

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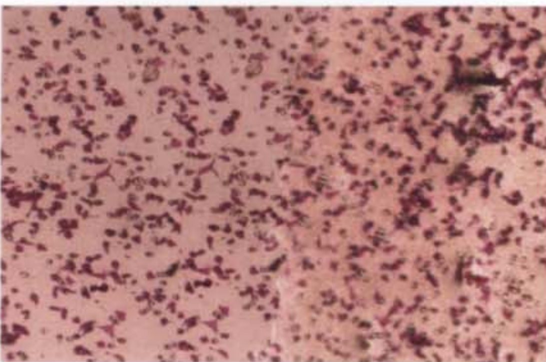

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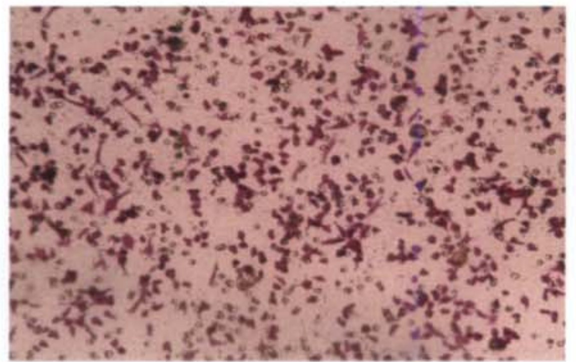


P

Figure. 5.7. Gelatin zymographic analysis

A. Without any trypsin activation prior to electrophoretic separation

B. Trypsin activation + EDTA in the substrate incubation buffer

C. Trypsin activated conditioned medium from untreated HUVEC

D. Trypsin activated conditioned medium from Boerhaavia extract treated (10μg/ml) HUVEC

E. Trypsin activation + Boerhaavia extract (50μg/ml) in the substrate incubation buffer

F. Trypsin activated conditioned medium from Tinospora extract treated (10μg/ml) HUVEC

G. Trypsin activation + Tinospora extract (50μg/ml) in the substrate incubation buffer

Figure. 5.8. Gelatin zymographic analysis

A,B and C –same as Figure 5.7

D. Trypsin activated conditioned medium from CI treated (1.0μg/ml) HUVEC

E. Trypsin activation + CI (10.0μg/ml) in the substrate incubation buffer

F. Trypsin activated conditioned medium from CII treated (1.0μg/ml) HUVEC

G. Trypsin activation + CII (10.0μg/ml) in the substrate incubation buffer

H. Trypsin activated conditioned medium from CI treated (1.0μg/ml) HUVEC

I. Trypsin activation + CIII (10.0μg/ml) in the substrate incubation buffer

Figure 5.7

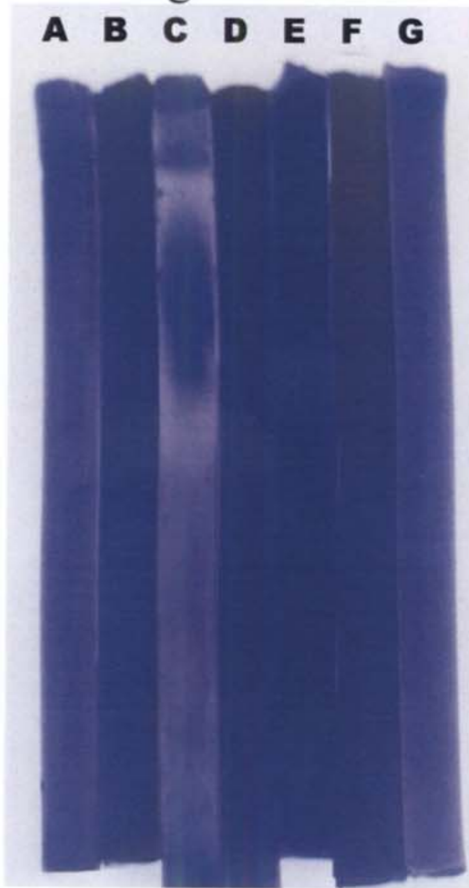


Figure 5.8

Figure. 5.9. Gelatin zymographic analysis

A,B and C –same as Figure 5.7

D. Trypsin activated conditioned medium from SC treated (1.0μg/ml) HUVEC

E. Trypsin activation + SC (10.0μg/ml) in the substrate incubation buffer

F. Trypsin activated conditioned medium from AC treated (1.0μg/ml) HUVEC

G. Trypsin activation + AC (10.0μg/ml) in the substrate incubation buffer

H. Trypsin activated conditioned medium from VC treated (1.0μg/ml) HUVEC

I. Trypsin activation + VC (10.0μg/ml) in the substrate incubation buffer

J. Trypsin activated conditioned medium from DC treated (1.0μg/ml) HUVEC

K. Trypsin activation + VC (10.0μg/ml) in the substrate incubation buffer

Figure 5.9

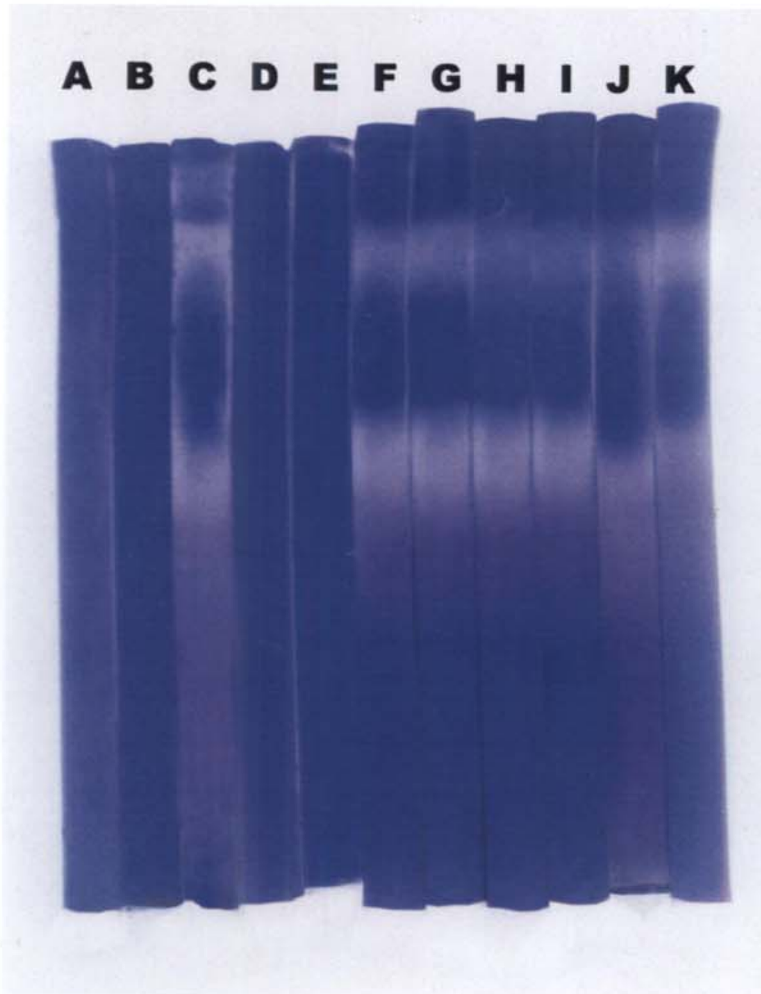


Figure 5.10. Effect of *B. diffusa* and *T.cordifolia* on *in vitro* Angiogenesis. Conditioned medium from normal semi confluent bottles of B16F10 cells act as the control. Treatment along with conditioned medium or the conditioned medium of pretreated B16F10 could reduce the micro vessel out growth from the aorta.

A) Without conditioned medium

B) Control – Conditioned medium alone

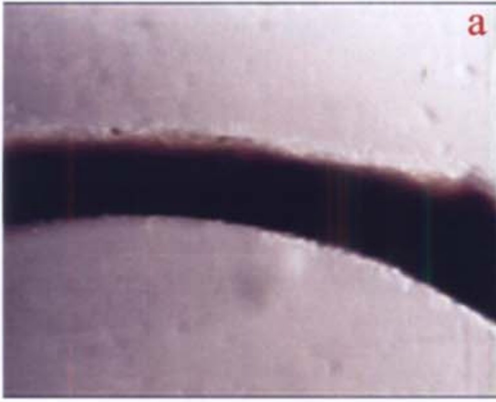
C) Conditioned medium from *B. diffusa* pre treated B16F10 (10µg/ml)

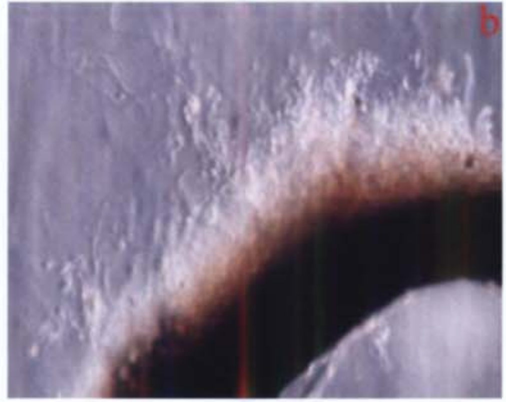
D) Conditioned medium plus *B. diffusa* extract-10µg/ml

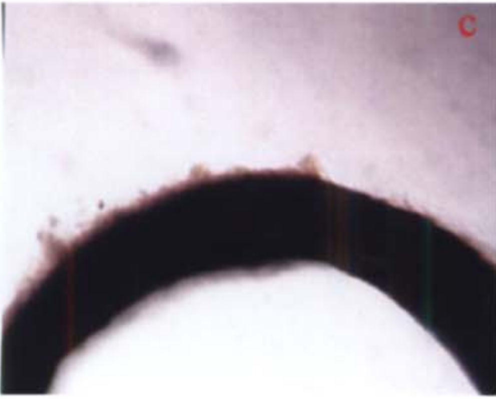
E) Conditioned medium from *T.cordifolia* pre treated B16F10 (10µg/ml)

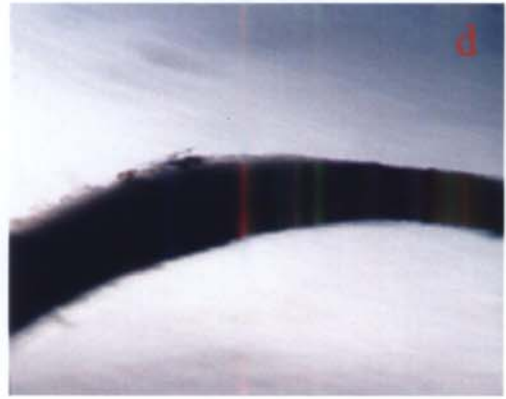
F) Conditioned medium plus *T.cordifolia* extract-10µg/ml

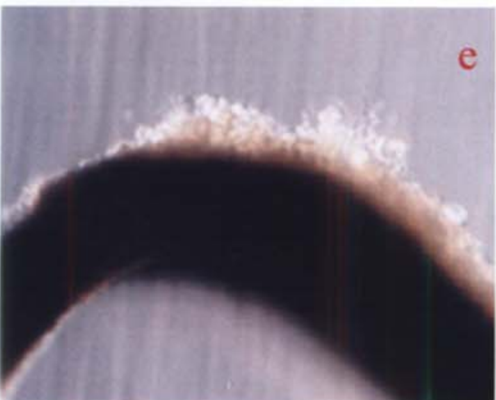
Figure 5.10











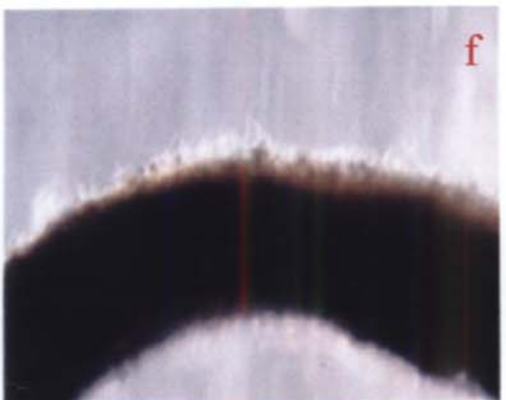


Figure 5.10. Effect of natural curcumins on *in vitro* Angiogenesis. Conditioned medium from normal semi confluent bottles of B16F10 cells act as the control. Treatment along with conditioned medium or the conditioned medium of pretreated B16F10 could reduce the micro vessel out growth from the aorta.

A) Without conditioned medium

B) Control – Conditioned medium alone

G) Conditioned medium from CI pre treated B16F10 (1.0μg/ml)

H) Conditioned medium plus CI-1.0μg/ml

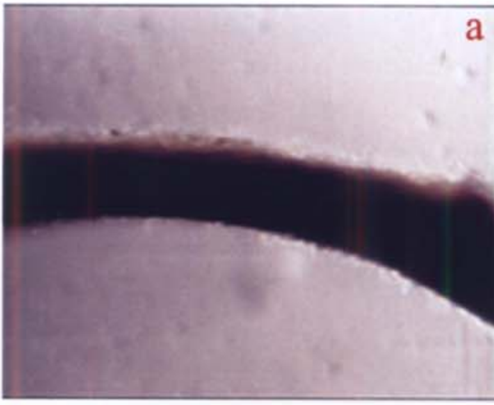
I) Conditioned medium from CII pre treated B16F10 (1.0μg/ml)

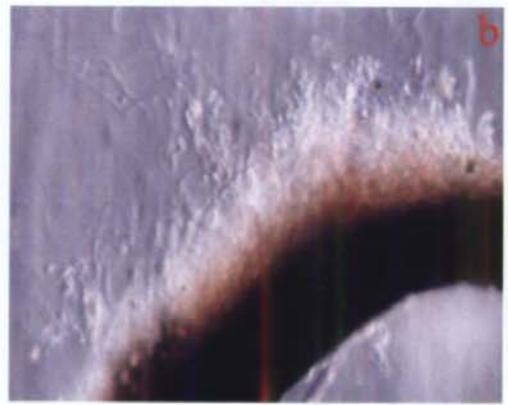
J) Conditioned medium plus CII –1.0μg/ml

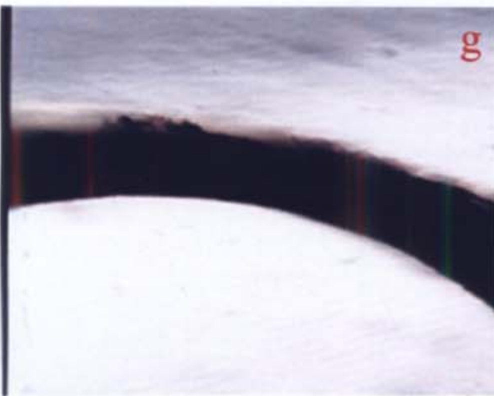
K) Conditioned medium from CIII pre treated B16F10 (1.0μg/ml)

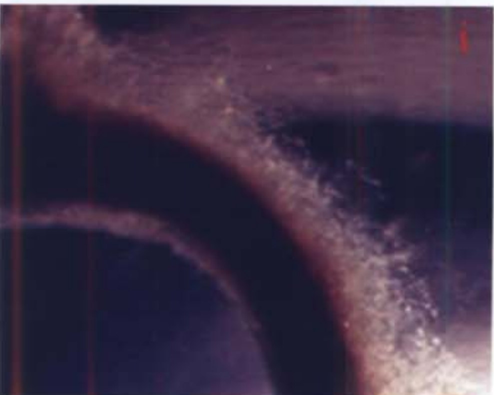
L) Conditioned medium plus CIII –1.0μg/ml

Figure 5.10









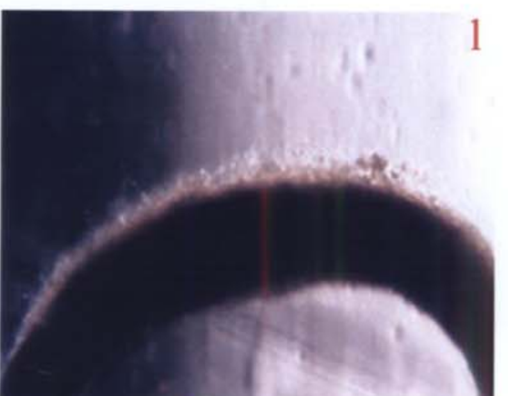


Figure 5.10. Effect of Synthetic curcumonoids on *in vitro* Angiogenesis. Conditioned medium from normal semi confluent bottles of B16F10 cells act as the control. Treatment along with conditioned medium or the conditioned medium of pretreated B16F10 could reduce the micro vessel out growth from the aorta.

A) Without conditioned medium

B) Control – Conditioned medium alone

M) Conditioned medium from SC pre treated B16F10 (1.0μg/ml)

N) Conditioned medium plus SC-1.0μg/ml

O) Conditioned medium from AC pre treated B16F10 (1.0μg/ml)

P) Conditioned medium plus AC –1.0μg/ml

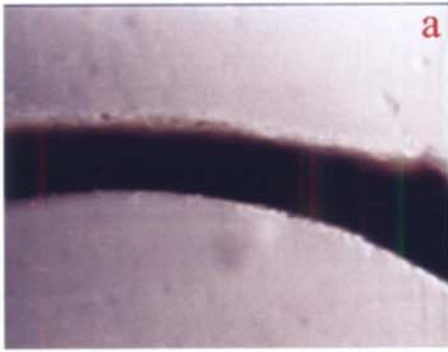
Q) Conditioned medium from VC pre treated B16F10 (1.0μg/ml)

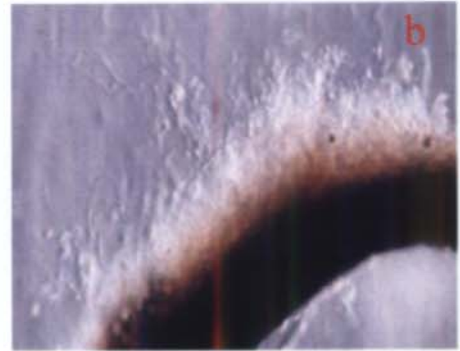
R) Conditioned medium plus VC –1.0μg/ml

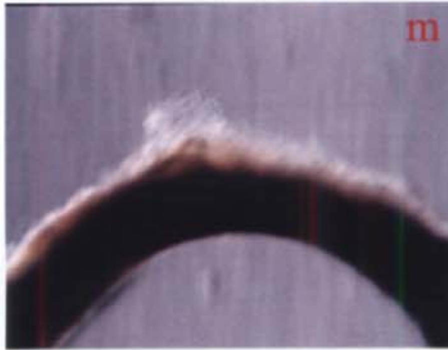
S) Conditioned medium from DC pre treated B16F10 (1.0μg/ml)

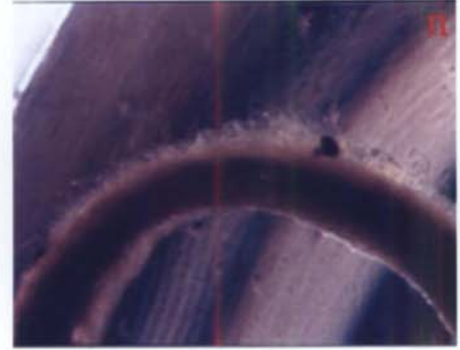
T) Conditioned medium plus DC –1.0μg/ml

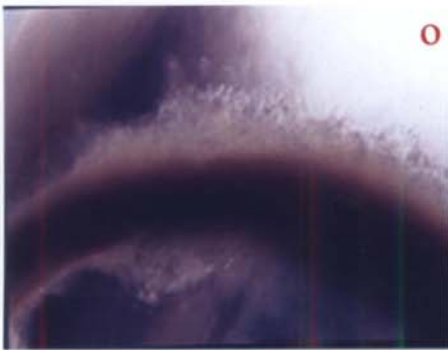
Figure 5.10

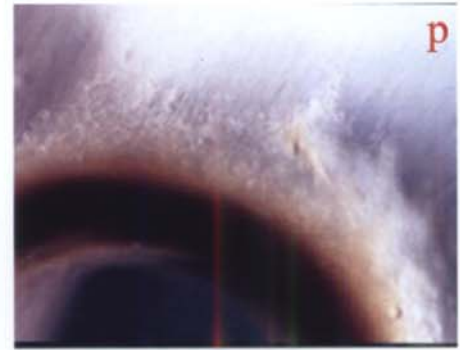


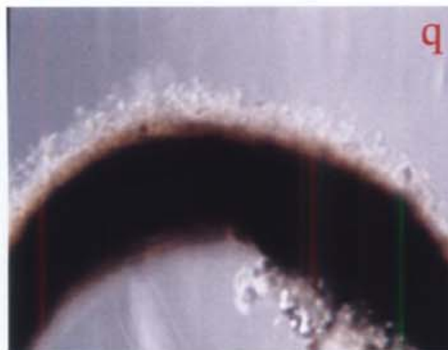


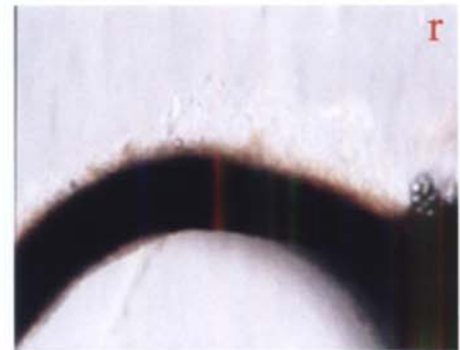


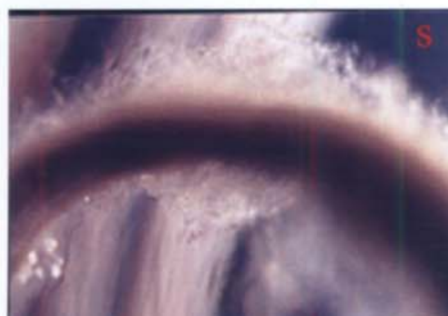












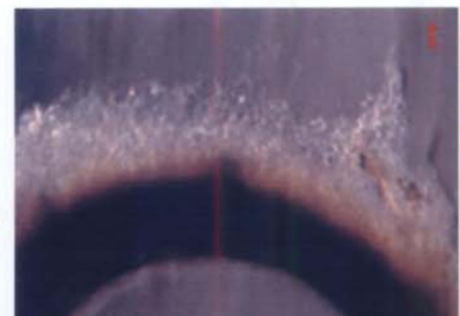
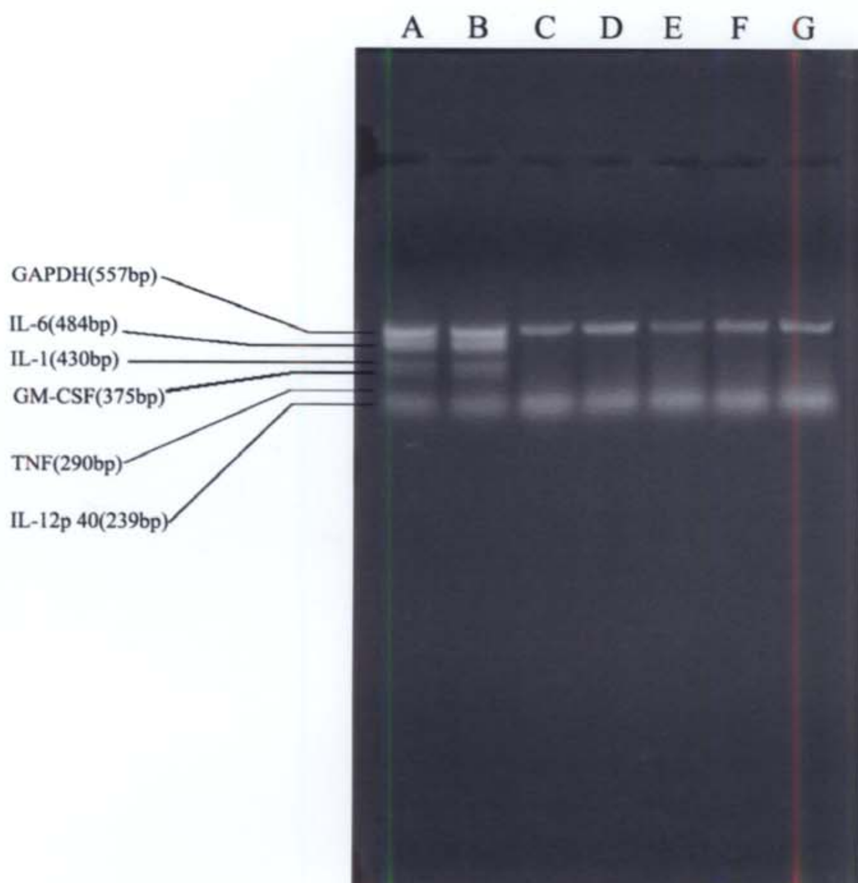


Figure 5.11. Gene expression studies using TR-PCR analysis

A) Positive control (provided in the Kit)
B) B16F10 melanoma cell sample without any treatment
C) B. diffusa treated (10.0μg/ml)
D) T. cordifolia treated (10.0μg/ml)
E) CI treated (1.0μg/ml)
F) CIII treated (1.0μg/ml)
G) SC treated (1.0μg/ml)

Figure 5.11



Chapter 6

Anti-metastasis studies using B. diffusa and T. cordifolia in animal models and in vitro.

Introduction

Metastasis has been defined earlier by Willis (1967) as the discontinuous growth of tumour cells. Indeed, metastasis is a multi step process (Mareel et al 1993) and can be abrogated, at least in theory, at any point in the cascade (Stracke and Liotta 1992). The interrelated events of the metastatic cascade are – disruption of the basement membrane, cell detachment, cell motility, invasion, penetration of the vascular system, circulating in the body fluids, arrest at distant site, extravasation and proliferation at the new site. To facilitate these diverse steps, tumour cells adopt a different pattern of gene expression as compared to its normal counterparts; some of them being up regulated (eg. Proteases like MMPs) and some one being down regulated (eg. Adhesion molecules like E-cadhering). Some authors found that disruption of some genes that regulate cell polarity and epithelial morphology, such as *dgl* and *cdc42* also promoted metastasis in conjunction with activating *Ras* mutations (Kristine Novak. 2003). Further screen will probably yield another genes that are important for induction of metastasis.

In this study the effect of aqueous methanol extract of *B. diffusa* and the polysaccharide fraction (Pl) from the dried stem of *T. cordifolia* is being evaluated in a metastasis model induced by B16F10 melanoma cells in C57BL/6 mice. In spite of the fact that metastasis is the most dangerous aspect of tumour progression, it has been difficult to study in mammalian models because of variations in tumour and host genotype (Kristine Novak. 2003). Using *in vitro* models, we cannot reproduce the exact *in vivo* situation that a metastatic cell comes across with during its course of spreading. But rather we can better understand, where and how do an external agent exert its action to prevent metastasis in the cascade of events. In this

chapter, we tried to elucidate at which step *B.diffusa* and *T. cordifolia* are exerting their effect in the cascade of metastasis. For this we studied the cellular functions such as proliferation, invasion and zymographic analysis using nontoxic concentrations of the compounds.

Materials and Methods

Chemicals: - Type 1 Collagen, Hydroxyproline, N–acetylneuraminicacid (NANA), Glucosamine hydrochloride, Glucuronicacid 4,6 lactone, Glycil glycine and γ-glutamyl-4-nitroanilide.

Animals: - Six to eight weeks old male C57BL/6 mice.

Cells and medium: - B16F-10, a highly metastatic melanoma cell line and the medium used for culture was DMEM with 10% FCS and antibiotics.

Tissue culture accessories: - 96-well flat bottom culture plate, Boyden chamber and polycarbonate filter.

Study materials: - Authenticated *T. cordifolia* and *B.diffusa* was used for this study. The polysaccharide fraction from *T. cordifolia* was isolated by the protocol of Chintalwar et al (1999) as detailed in chapter 2. In brief the powdered stem was extracted using methanol by stirring overnight at room temperature. The precipitate was then extracted with boiling water and the extract was precipitated using acetone. The proteins from this precipitate were removed by treatment with TCA. The sample was then dialyzed against distilled water and concentrated by lyophylization. The yield was about 0.01%. This polysaccharide preparation (PI) was given to the animals at a concentration of 0.5mg/dose/animal.

The air-dried powdered plant parts of *B.diffusa* were extracted using 70% methanol by stirring overnight at room temperature. The precipitate after removing the solvent by evaporation was re-suspended in PBS. The yield was about 10.2%. It was tested for the presence of alkaloids. In a previous study the extract did not showed any sign of toxicity up to an oral dose of 2g/Kg in mice (Chandan et al, 1991), and an oral administration of 250mg/Kg to pregnant Albino rats during the entire gestation period was found to be devoid of any teratogenic effect (Sing et al, 1991). Hence the concentration used in this study (25mg/Kg) might be devoid of any toxic side effects.

***In vivo* metastasis studies**

Determination of the effect of Pl and *B.diffusa* administration on the metastatic potential of B16F-10 cells: -

Highly metastatic B16F-10 melanoma cells (10^6 cells/0.05 ml PBS) was injected to each animal via lateral tail vein (day-0). The animals were divided into four groups, comprising fourteen animals in each group. Group I was receiving only the vehicle. Group II and III animals were treated Tinospora polysaccharide (Pl) and Boerhaavia extract respectively simultaneously with the induction of metastasis and continued for 10 days. In-group IV, Boerhaavia extract treatment started 10 days prior to the tumour induction.

Eight animals from each group were sacrificed after twenty-one days of tumour induction, the lungs were dissected out and blood was collected by heart puncture. The lungs were washed in PBS and used for morphological examination using a dissection microscope, and the blackish metastatic colonies were counted.

Then the tissue was subjected to the estimation of collagen hydroxyproline (Bergman and Loxley, 1940) hexosamines (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962).

The serum sialic acid levels of all the animals were determined according to the procedure of Skoza and Mohos (1976). The serum γ-GT (Szasz) levels were also assayed and expressed as units/litre.

Rest of six animals in all the groups were observed for their survival.

Histopathology of lungs: -

Lungs of the normal, treated and untreated control tumour bearing animals were fixed in 10% formalin for 24 hrs, washed and dehydrated using alcohol series and embedded in paraffin. 4μm sections were then stained with hematoxylin and eosin (H&E) mounted in DPX and examined under a microscope (×200).

***In vitro* metastasis studies**

Long-term cytotoxicity studies: -

B16F-10 melanoma cells (5×10^3 cells/well were0 seeded in to each well of a 96well plate in 0.1ml complete medium and incubated at 37^0C in 5% CO_2. After 24hours of incubation, various concentrations of the Boerhaavia extract or Pl were added in triplicate and incubation was continued for further 48hours. 4 hour before termination of assay 20μl of MTT solution (5mg/ml) was added to each well. After completing the incubation the supernatant was removed and 100μl DMSO was added to each well and the optical density was measured at 545nm with reference of 630nm.

Tumour cell proliferation assay: -

Tumour cell proliferation assay was carried out as described in Chapter 2. Briefly, 5×10^4 B16F-10 melanoma incubated with or without different concentrations of Boerhaavia extract (0-100μg/ml) and Tinospora polysaccharide (0-50μg/ml). 16h before the termination of the assay 1.0μCi of ^{3}H- thymidine was added to each well. After completing the incubation the cells were washed, and transferred to scintillation fluid and the amount of radioactivity incorporated was quantified.

Collagen matrix invasion assay: -

The invasion assay was carried out in Boyden chambers as described by Albini et al (1987). Briefly, the lower compartment of the chamber was filled with chemo-attractants diluted DMEM and a type-I collagen-coated polycarbonate filter membrane was placed on it. B16F-10 cells were then seeded on to the upper chamber. To test the effect of Boerhaavia and Tinospora polysaccharide on the invasion of B16F10 melanoma cells, they were added along with the cells to the upper chamber.

Gelatin Zymography: -

Gelatin Zymography was followed according to the procedure described in Chapter 2. After determining the protein concentration, supernatant containing the proteases of treated and untreated melanoma cells were subjected to zymographic analysis with or without trypsin activation. Gels were fixed, stained and clear bands were visualized against a dark background.

Results

In vivo metastasis studies

Effect of B. diffusa and T. cordifolia on the inhibition in lung colonisation of B16F-10 melanoma and increased survival by the treatment: -

Administration of the aqueous methanol extract and the isolated polysaccharide fraction could reduce pulmonary metastases formation of B16F-10 melanoma cells. Vehicle treated control animals had massive growth of tumour and was given an arbitrary-maximum countable number of 250 - as described by several other authors (Hill et al, 1994) (Table 6.1). It was reduced to 32 ± 2.8 (simultaneous) and 13 ± 2.8 (prophylactic) countable colonies by the Boerhaavia extract treatment. The survival rate of the animals also increased to 157% and 140% by the prophylactic and simultaneous treatment of the Boerhaavia extract respectively. In the polysaccharide treated animals it was reduced to 72 ± 10 and the survival time of this group was more than double that of control animals (Table 6.2).

Effect of *B. diffusa* and *T. cordifolia* on certain biochemical parameters of metastases bearing animals: -

Lung collagen hydroxyproline content

Effect of the Boerhaavia extract and polysaccharide treatment on the lung biochemical parameters is shown in Table-6.3. The lung collagen hydroxyproline content was drastically elevated (23.9 ± 1.6 μg/mg protein) in the control group compared to the normal level (0.9 ± 0.5 μg/mg protein) indicating the fibrosis of

lung tissue. This elevated level was reduced to 4.4 ± 0.6 and 3.8 ± 0.4 μg/mg proteins by the simultaneous and prophylactic treatment of the Boerhaavia extract respectively. This elevated level was reduced to 8.3 ± 2.0 μg/mg proteins by the simultaneous treatment of Pl.

Lung hexosamine content

The control animals had a high level of lung hexosamine content, (4.85 ± 0.20 mg/100 mg tissue, dry wt.) as compared to the normal animals (0.4±0.1 mg/100 mg tissue dry wt.) Simultaneous administration of the Pl could reduce this level to 0.94 ± 0.02-mg/100mg tissue dry wt. Both simultaneous and prophylactic administration of Boerhaavia extract could reduce this level to 0.46 ± 0.01 mg/100mg tissue dry wt. and 0.41 ± 0.03 mg/100mg tissue dry wt. respectively. (Table 6.4)

Uronic acid levels of the lung

As in the case of hydroxyproline and hexosamines, uronic acid levels in the lungs of control animals were (334.2 ± 22.7 μg/100 mg tissue wet wt.) also very high compared to the normal levels (32.2±2.0 μg/100 mg tissue, wet. Wt.). The Pl treatment (94.2 ± 17.4 μg/100 mg tissue) could reduce these elevated levels when administered simultaneously with the tumour cells (Table 6.5). Boerhaavia extract could reduce these elevated levels when administered simultaneously (83.2 ± 5.1 μg/100 mg tissue) with the tumour cells and also by prophylactic administration (82.6 ± 7.8μg/100 mg tissue)

Serum sialic acid levels

Effect of drug treatment on the serum biochemical parameters is shown in Table 6.6. The normal serum sialic acid level of C57BL/6 mice was 21.3 ± 1.5 μg/ml that was elevated to 102.2 ± 8.7 μg/ml serum in the metastases bearing animals. Simultaneous administration of Pl reduced the sialic acid levels to 40.7 ± 7.7 μg/ml serum. Simultaneous administration of Boerhaavia could reduce the sialic acid levels to 56.0 ± 2.2 μg/ml serums and prophylactic administration reduced the levels to 41.9 ± 1.9 μg/ml serum.

Serum γ-glutamyltranspeptidase activity

The γ-GT activity in the control animals was very high (35.3 ± 3.8 units/litre) as compared to the γ-GT level in the serum of the normal animals (8.2 ± 1.1 U/L). Simultaneous administration of Pl reduced the elevated levels to 12.5 ± 4.0 U/L. Simultaneous administration of the extract reduced the elevated levels to 10.6 ± 0.5 U/L and prophylactic administration reduced to 5.4 ± 0.5 U/L (Table 6.7)

Histopathological analysis of lung

The hematoxylin eosin (H & E) stained sections of lung tissues are shown in Figure 6.1 (×100). In the control animals massive tumour growth and fibrosis reduced the alveolar space thereby reducing the vital capacity of the lung. Same areas were characterized by necrosis around the alveolar passages and bronchioles. Administration of Boerhaavia extract and Tinospora Pl showed a reduction in

tumour mass around alveoli and pleura facilitating smooth alveolar function (Figure 6.1 A-E).

In vitro metastasis studies

Cytotoxicity studies

Boerhaavia extract showed cytotoxic activity towards melanoma cells even at concentration of 25μg/ml medium (12.4% cytotoxicity). Whereas polysaccharide fraction from Tinospora did not had a significant cytotoxic activity even at concentration of 100μg/ml (Table 6.8).

Thymidine uptake assay

The results of cell proliferation assay, using ^{3}H- thymidine assay indicate a dose dependent inhibition in the cell proliferation of the Boerhaavia extract treated cells whereas the treatment with polysaccharide did not have any inhibitory effect in the tumour cell proliferation. The concentration of the Boerhaavia extract needed for 50% inhibition in the thymidine incorporation was 25.0μg/ml (Figure 6.2)

Collagen Invasion assay

Boerhaavia treatment could effectively inhibit the tumour cell invasion (85% inhibition in cell invasion compared to control) whereas the polysaccharide was found ineffective in reducing the invasion potential of the melanoma cells (Figure 6.3)

Zymographic analysis

To determine the effect of test compounds on secreted gelatinases we used the zymogram analysis of culture supernatants of B16F10 melanoma, pre treated with non-toxic concentration of the extract as well as polysaccharide. Zymographic analysis of trypsin activated culture supernatant of untreated cells showed digested clear areas at 92KDa and 72KDa levels while the sample without trypsin activation did not show any clear areas indicating the inactive form of enzymes. Addition of 10mM EDTA to the incubation buffer also did not show any clear band indicating that the enzyme is a metalloproteanase. The conditioned media from Boerhaavia pretreated cells, which was then activated by trypsin, also did not- show any clear band indicating the inhibition of expression or activity of gelatinases by the treatment where as polysaccharide was ineffective in inhibiting the matrix metalloproteinase (Figure 6.4)

Discussion

In the present study, intra-peritoneal administration of the Boerhaavia extract as well as Pl resulted in marked reduction in the metastases by B16F10 melanoma. Maximum inhibition was obtained when the animals were treated prophylactically with the Boerhaavia extract (95% inhibition) and simultaneous administration could also reduce the tumour nodule formation by about 87%. Administration of the polysaccharide fraction Pl from Tinospora resulted in a 72% reduction in the metastatic colony formation of B16F10 melanoma cells. A significant inhibition on metastasis was also obtained in previous studies using the aqueous methanol extract of *T. cordifolia*. Maximum inhibition was obtained when the animals were pre-treated with the Boerhaavia extract prior to tumour induction.

The simultaneous administration could also reduce the tumour nodule formation by about 52% (Mathew S and Kuttan G 1998).

Estimation of hydroxyproline – the major building block of collagen – revealed the fibrous accumulation in the lungs of melanoma induced animals. Accumulation of extra cellular matrix (ECM) in the lung will reduce the pulmonary function. The elevated levels of hydroxyproline indicate the fibrosis due to high collagen level. The treated animals had much lower lung collagen hydroxyproline content indicating a reduced fibrosis and a smooth alveolar function. A reduction in the number of lung tumour nodules that are metastatic colonies of melanoma, correlates with these findings. Similar findings were observed in the histopathological analysis of lungs and all the treated animals could enhance the survival by more than double that of untreated control animals.

The acidic and basic modifications of monosaccharides yield uronic acids (glucuronic acid) and amino sugars (hexosamines). These form a vital part in many structural polysaccharides and glycosaminoglycans (GAG) found in the 'ground substance' of ECM. Tumour cells can induce the host stromal cells to supplement the matrix components necessary for the growing tumour (McKinnell et al, 1998). Hyaluronicacids (HA) is a GAG made of repeated disaccharide units of D-glucuronicacid and N-acetyl D-glucosamine (Tammi et al, 2002; Delpech et al, 1997). It is a well-known promoter of metastasis and elevated level is seen in several types of tumour regardless of tumour grade (Hautmann et al, 2002; Setala et al, 1994; Lipponen et al, 2001) and promotes metastasis by opening up spaces for tumour cells to migrate through ECM, by interacting with cell surface receptors for HA (Tammi et al, 2002; Delpech et al, 1997; Turley et al, 2002). But treatment

of Boerhaavia and Tinospora polysaccharide could reduce these levels significantly thereby indicating a decrease in the metastatic potential of B16F10 melanoma.

A close association between the metastatic ability of B16 melanoma sub lines and expression of membrane-associated γ-GT has been reported previously (Prezioso et al, 1993). Sialic acid, a family of acetylated derivatives of N-acetyl neuraminicacid, occurs as a terminal component of carbohydrate chain of glycoproteins and gangliosides. Earlier experiments have reported the secretion of glycoproteins (Bizik et al, 1985) and shedding of gangliosides (Portoukalian et al, 1978; Sela et al, 1989) in melanoma. A reduced level of these, by the treatment is indicative of a reduced tumour proliferation and metastasis in these animals.

Moreover the *in vitro* experiments shows that, even though the extract of Boerhaavia was reducing the proliferation of the tumour cell and inhibiting the invasion by interfering with the MMP activity, the polysaccharide fraction of Tinospora did not have any effect in these experimental setup. The *in vitro* experiments have also shown that the polysaccharide fraction was neither directly toxic to the tumour cells nor inhibited the proliferation as well. These results are indicative of the involvement of the immune system in the reduction of the metastatic potential of the drug treated animals. Since the polysaccharide fraction is a specific mitogen of B-cells the tumour reduction obtained may be mediated by B-cell or non-specific immune cells such as NK-cells. Further studies have to be conducted to find out the exact mechanism of action of the compound.

The aqueous methanol extract used in this study showed positive results for the presence of alkaloids by Dragondorffs reagent. The alcoholic and water extracts of *B. diffusa* is known to contain several bioactive molecules such as

reducing sugars starch and lignans liriodendrin and syringaresinol (Lami et al, 1991). Several Boeravionones (Boeravionones A,B,C,D,E,F etc) have also been isolated from *B. diffusa*. The pharmacological activity shown by the aqueous methanolic extract may be attributed to these diverse compounds. At present we do not know what all are the principal compounds in inhibiting the cascade of event of metastasis.

Table 6.1

Effect of *B.diffusa* and Tinospora polysaccharide (Pl) on lung colonization of B16F-10 melanoma cells in the animals.

Drug used	Dose	Mode	No. Tumour nodules Mean ± SD	% Inhibition compared to control
Control	-	-	250#	-
B.diffusa	0.5mg/dose	Prophylactic	13 ± 2.8*	95.0
		Simultaneous	32 ± 2.8*	87.0
Pl (Tinospora polysaccharide)	0.5mg/dose	Simultaneous	72 ± 10*	71.2

Values are mean ± SD

An arbitrary number of 250 are given for massive number of tumour nodules

* P< 0.001 (Values are compared with control)

The lungs were dissected out and observed for metastases on 21 day after induction of B16F-10 melanoma (10^5cells) through the lateral tail vein. Animals were treated with either Boerhaavia extract or Pl (10 doses at 24 hrs. interval, i.p)

Table 6.2

Effect of *B.diffusa* and Tinospora polysaccharide (PI) on the survival of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Survival Days	% ILS
Control	-	-	36.16	-
B.diffusa	0.5mg/dose	Prophylactic	90.5 ± 8.6	157
		Simultaneous	84.8 ± 7.7	140
PI - Tinospora	0.5mg/dose	Simultaneous	74.8 ± 6.7	111.4

Values are mean ± SD

Increase in life span (%ILS) = T-C/C × 100, where T and C are the number of days survived by the treated and control (vehicle treated) group of animals respectively.

Table 6.3

Effect of *B.diffusa* and Tinospora polysaccharide (Pl) on the Amount of Hydroxyproline of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Amount of OHproline μg/mg protein	% Inhibition compared to control
Normal level	-	-	0.9 ± 0.2	-
Control	-	-	23.5 ± 0.9	-
B.diffusa	0.5mg/dose	Prophylactic	4.8 ± 0.4*	79.5
		Simultaneous	5.4 ± 0.6*	77.0
Pl - Tinospora	0.5mg/dose	Simultaneous	8.3 ± 2*	64.6

After 21 days of B16F-10 melanoma cells (10^5 cell/50μl vol) implantation through the lateral tail vein, lungs were dissected out and assayed different biochemical parameters. The vehicle treated group is the control.

Values are mean ± SD

* P<0.001 (Values are compared with control)

Table 6.4

Effect of *B.diffusa* and Tinospora polysaccharide (PI) on the Amount of Hexosamine of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Amount of Hexosamine mg/100mg dry tissue	% Inhibition compared to control
Normal levels	-	-	0.4 ± 0.1	-
Control	-	-	4.85 ± 0.2	-
B.diffusa	0.5mg/dose	Prophylactic	0.41 ± 0.03*	90.3
		Simultaneous	0.46 ± 0.01*	90.5
PI - Tinospora	0.5mg/dose	Simultaneous	0.94 ± 0.02*	80.6

After 21 days of B16F-10 melanoma cells (10^5 cell/50µl vol) implantation through the lateral tail vein, lungs were dissected out and assayed different biochemical parameters. The vehicle treated group is the control.

Values are mean ± SD

* P<0.001 (Values are compared with control)

Table 6.5

Effect of *B.diffusa* and Tinospora polysaccharide (Pl) on the Amount of Uronic acids of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Amount of uronic acid μg/100mg wet tissue	% Inhibition compared to control
Normal levels	-	-	32.2 ± 2.0	-
Control	-	-	330.1 ± 23.7	-
B.diffusa	0.5mg/dose	Prophylactic	22.6 ± 7.8*	93.0
		Simultaneous	30.2 ± 5.1*	91.0
Pl - Tinospora	0.5mg/dose	Simultaneous	94.2 ± 17.4*	71.5

After 21 days of B16F-10 melanoma cells (10^5 cell/50μl vol) implantation through the lateral tail vein, lungs were dissected out and assayed different biochemical parameters. The vehicle treated group is the control.

Values are mean ± SD

* P<0.001 (Values are compared with control)

Table 6.6.

Effect of *B.diffusa* and Tinospora polysaccharide (PI) on the Amount of Sialic acids of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Amount of Sialic acids µg/ml serum	% Inhibition compared to control
Normal levels	-	-	21.3 ± 1.5	-
Control	-	-	102.2 ± 8.7	-
B.diffusa	0.5mg/dose	Prophylactic	41.9 ± 1.9*	58.9
		Simultaneous	56.0 ± 2.2*	45.0
PI - Tinospora	0.5mg/dose	Simultaneous	40.7 ± 10.0*	60.7

After 21 days of B16F-10 melanoma cells (10^5 cell/50µl vol) implantation through the lateral tail vein, lungs were dissected out and assayed different biochemical parameters. The vehicle treated group is the control.

Values are mean ± SD

* P<0.001 (Values are compared with control)

Table 6.7.

Effect of *B.diffusa* and Tinospora polysaccharide (Pl) on the Amount of γ-GT of B16F-10 melanoma carrying animals.

Drug used	Dose	Mode	Amount of γ-GT activity U/L	% Inhibition compared to control
Normal levels	-	-	8.2 ± 1.1	-
Control	-	-	35.3 ± 3.7	-
B.diffusa	0.5mg/dose	Prophylactic	5.4 ± 0.5*	85.2
		Simultaneous	10.6 ± 0.5*	77.1
Pl – Tinospora	0.5mg/dose	Simultaneous	12.5 ± 4.0*	77.1

After 21 days of B16F-10 melanoma cells (10^5 cell/50µl vol) implantation through the lateral tail vein, lungs were dissected out and assayed different biochemical parameters. The vehicle treated group is the control.

Values are mean ± SD

* P<0.001 (Values are compared with control)

Table 6.8

Cytotoxicity assay of *B.diffusa* and Tinospora polysaccharide (Pl) towards B16F-10 melanoma cells in culture.

Drug used	Concentration μg/ml	% Cytotoxicity
B.diffusa	1.0	0.0
	5.0	2.1
	25.0	12.4
	100.0	35.0
Pl – Tinospora	0.5	0.0
	2.5	0.0
	50.0	0.5
	100.0	5.0

The percentage dead cells was determined using the formula,

$$\%\ \text{Dead cells} = \frac{\text{Absorbance of treated}}{\text{Absorbance of Control}} \times 100$$

Figure 6.1. H&E stained sections of lung (×100) of metastases bearing animals

A) Normal lung

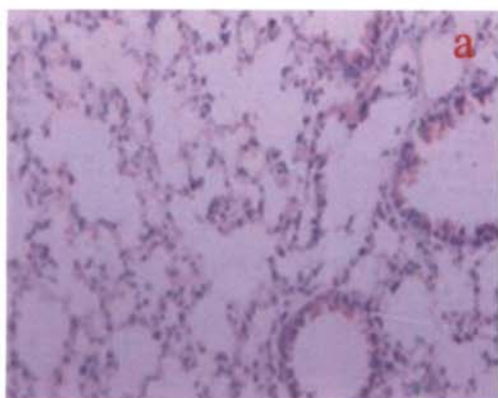
B) Control lung (vehicle treated)

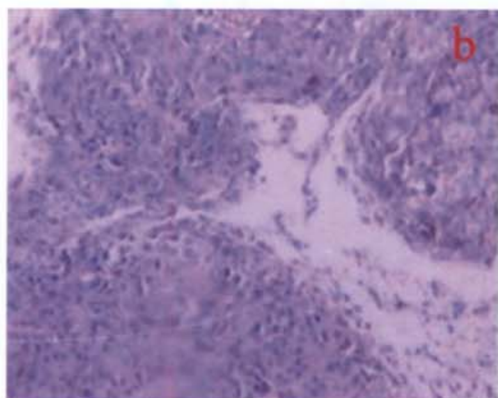
C) Tinospora PI treated simultaneously (10mg/Kg)

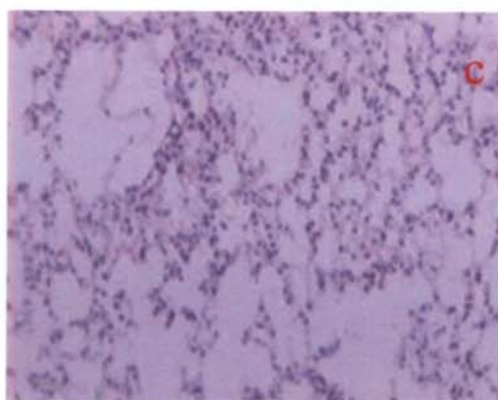
D) Boerhaavia Extract treated Prophylactically (25mg/Kg)

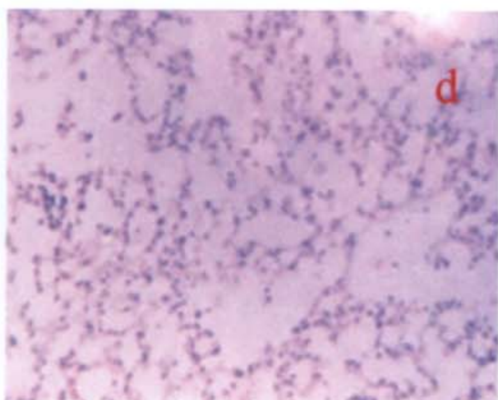
E) Boerhaavia Extract treated simultaneously (25mg/Kg)

Figure 6.1









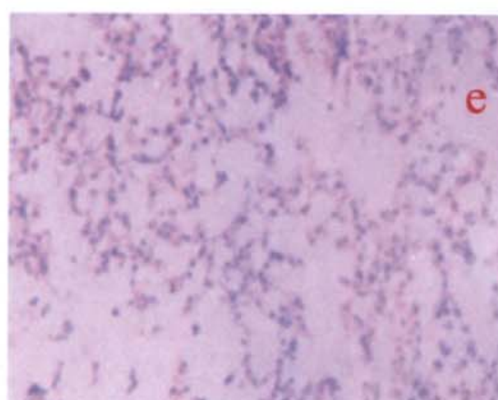


Figure 6.2 Effect of Boerhaavia and Tinospora PI on tumour cell proliferation

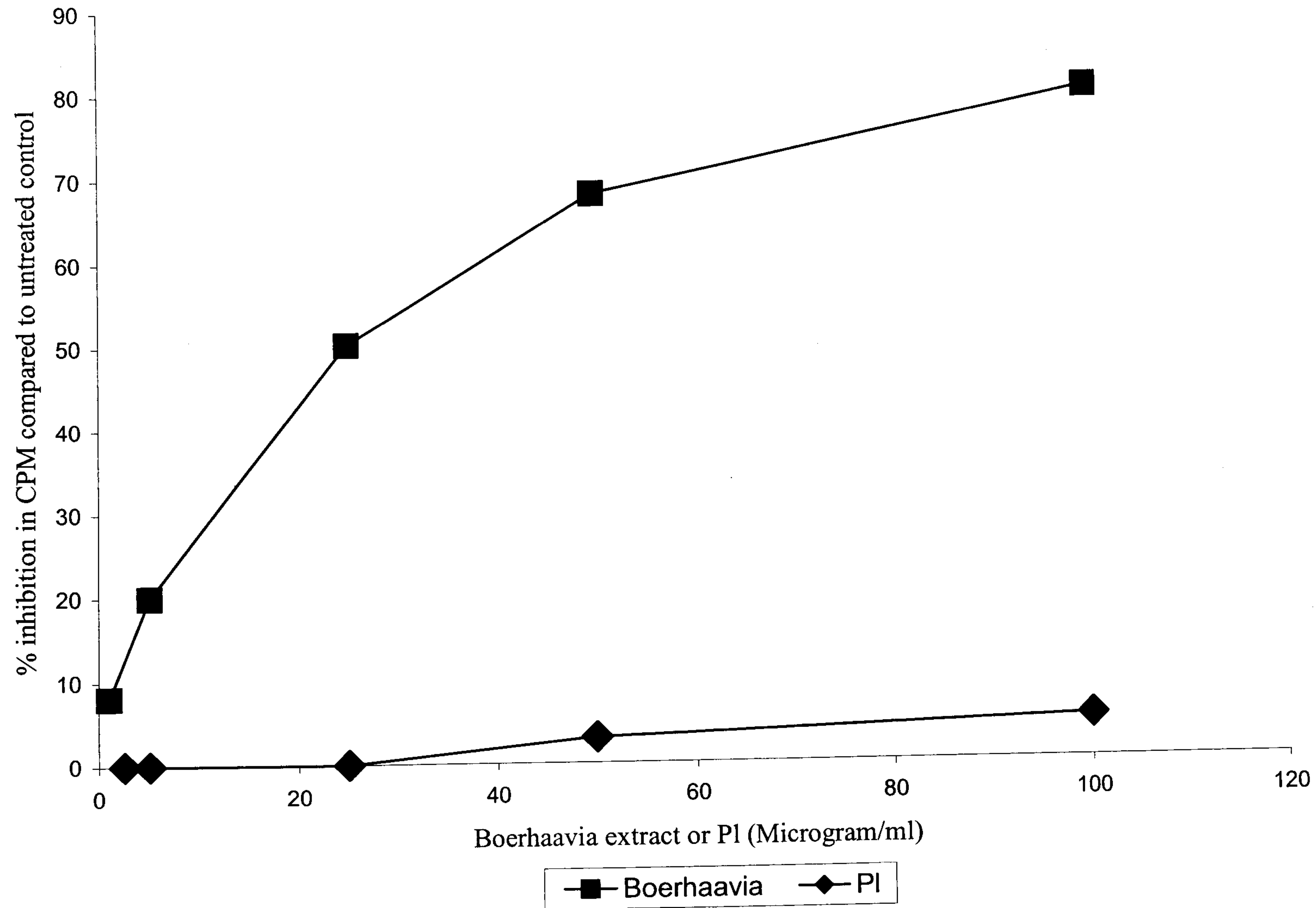
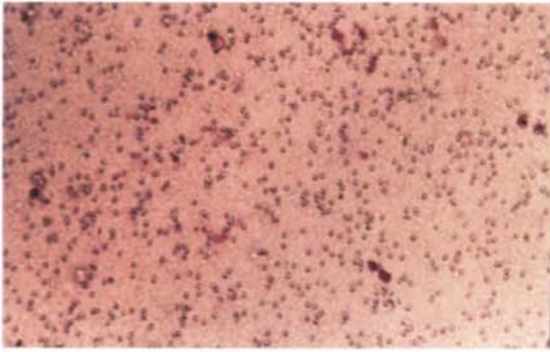


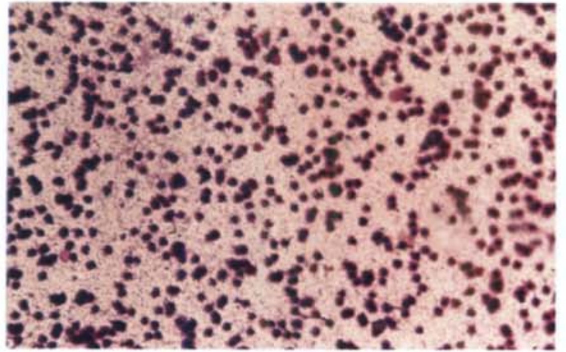
Figure 6.3 Inhibitory effects of *B. diffusa* and Tinospora polysaccharide on B16F10 melanoma invasion through collagen matrix.

A) Control '0' hour incubation
B) Control 24h incubation, with out any treatment
C) Tinospora PI (10.0µg/ml)
D) *B. diffusa* (5.0µg/ml)
E) *B. diffusa* (10.0µg/ml)

Figure 6.3



A



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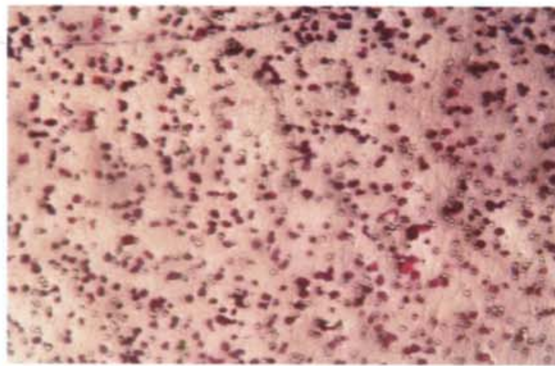

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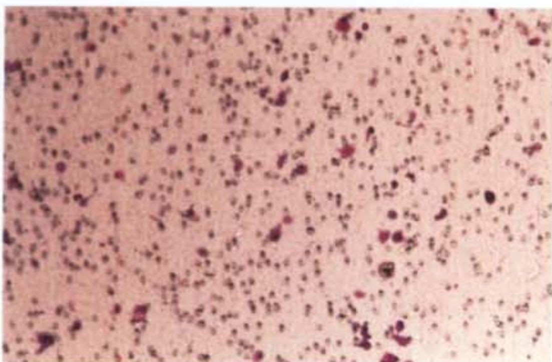

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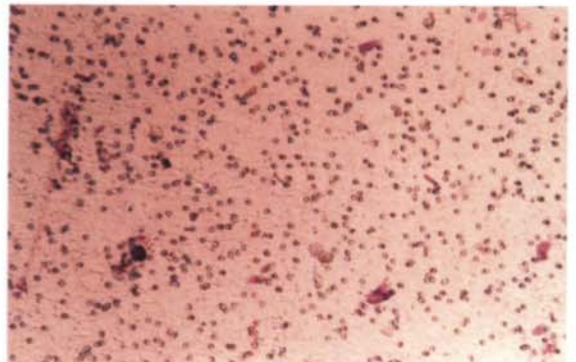


E

Figure. 6.4. Gelatin zymographic analysis

A. Without any trypsin activation prior to electrophoretic separation

B. Trypsin activation + EDTA in the substrate incubation buffer

C. Trypsin activated conditioned medium from untreated B16F10 melenoma

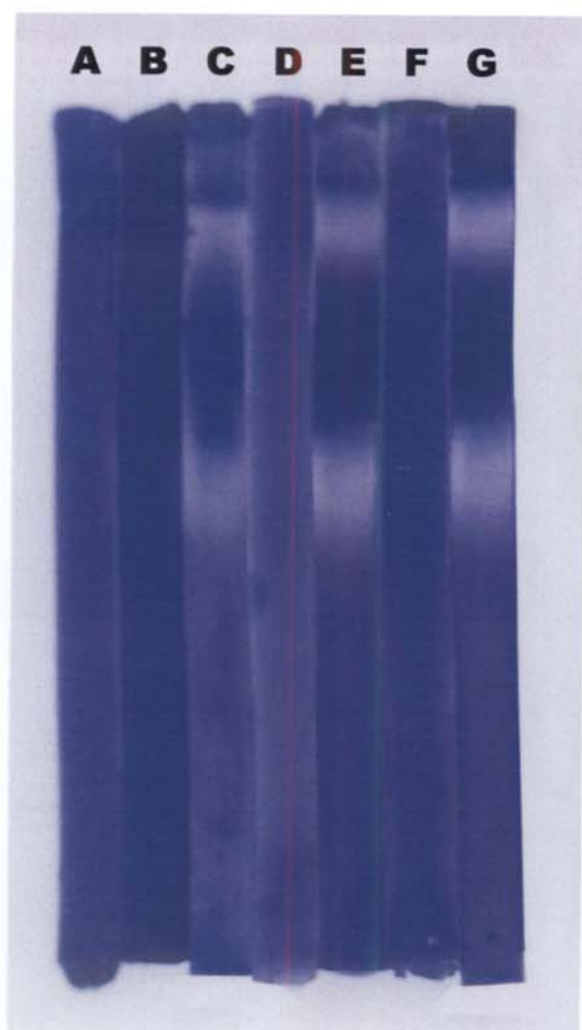
D. Trypsin activated conditioned medium from Boerhaavia extract treated (10μg/ml) B16F10 melanoma

E. Trypsin activated conditioned medium from Tinospora polysaccharide treated (10μg/ml) B16F10 melanoma

F. Trypsin activation + Boerhaavia extract (50μg/ml) in the substrate incubation buffer

G. Trypsin activation + Tinospora polysaccharide (50μg/ml) in the substrate incubation buffer

Figure 6.4



Summary and conclusion

Angiogenesis plays a key role in tumour progression leading to metastasis. Vascular remodeling in host tissues surrounding growing tumour is implicated in the successful development of tumour neovasculature (Zhang L et al 2003). The invasion, proliferation, migration and differentiation of endothelial cell leads to the formation of a new mature vessel. The onset of angiogenesis appears to be the result of an imbalance between stimulatory and inhibitory factors that leads to the activation of previously quiescent endothelial cells (Langley R et al 2003). Tumour cells produce several of these stimulatory factors and the inhibition of the production of these factors, and there by tumour-induced angiogenesis, will have greater therapeutic implication to arrest the tumour growth.

In the past ten years there have been tremendous increase in the knowledge of molecular mechanisms and pathophysiology of tumour angiogenesis. Many of these mechanisms have been exploited as new targets for drug development to arrest tûmour growth. More than 40 antiangiogenic agents are in clinical trials for their antitumour activity. Several pharmacologically active phytochemicals like alkaloids, flavanoids and polyphenols etc are shown to possess antiangiogenic activity. Several of the chemotherapeutic drugs that are derivatives of natural phytochemicals exert their antitumour activity via inhibiting tumour vessel formation. Plants, which are the sources of unique phytochemicals are known to possess diverse pharmacological activities. Plant extracts used this study (Boerhaavia and Tinospora) are traditionally used by Indian population for various ailments including cancer. Curcumin, the bioactive polyphenolic compound from *Curcuma longa* and many of its natural as well as synthetic derivatives that are included in this study are pharmacologically well known compounds. In our study both the plant extracts significantly inhibited the tumour angiogenesis in animals

and *in vitro*. Among the curcuminoids studied only some of them (CI, CIII and SC) had a significant inhibition in tumour angiogenesis. Those, which inhibited the *in vivo* angiogenesis, showed a correlating result in the *in vitro* systems.

The necessity of IL-1β for *in vivo* angiogenesis driven by B16 melanoma has clearly been documented. IL-1β knock out mice showed lesser degree of micro vessel formation compared to its wild type when induced with B16 melanoma (Voronov E,et al 2003) indicating the importance of host derived IL-1β in angiogenesis. The antitumour mechanism of thalidomide is found to be related to reduced serum level of TNF-α in myeloma (Li J, et al 2002) and it is known to destabilize the TNF-α mRNA thereby preventing angiogenesis (Marriott JB et al 1999). IL-6 indirectly promotes tumour angiogenesis through the up regulation of VEGF-A load in the platelet aggregates on tumour endothelium (Salgado R, et al 2002, 2003). VEGF is the most potent angiogenic factor, induces its effect directly on endothelial function. For GM-CSF, even though it did not modulate endothelial functions related to inflammation (Bussolino F et al 1991), it induces endothelial cells to proliferate and migrate. Elevation of the cytokines such as IL-1β, IL-6, TNF-α, GM-CSF and VEGF are strictly proangiogenic and treatment with the extracts of Boerhaavia and Tinospora and curcuminoids such as CI, CIII and SC regulated the levels and inhibited angiogenesis. Moreover the treatment was able to increase the serum level of antiangiogenic agents such as IL-2 and TIMP-1 and could successfully shift the equilibrium of proangiogenics and antiangiogenics in favour of an angiostatic condition.

Treatment with curcuminoids (CI, CIII and SC 1.0μg/ml) and extracts of Boerhaavia and Tinospora (10.0μg/ml) inhibited the micro vessel out growth from

the rat aorta ring induced in the conditioned medium from the B16F10 melanoma cells. Interestingly, aortic rings incubated with conditioned media from the pre-treated B16F10 cells also showed a marked reduction in vessel out growth when compared with rings incubated with conditioned medium from untreated B16F10 melanoma cells. This also correlates with the results of mRNA expression studies, where the treatment inhibited GM-CSF, IL-1β, IL-6, TNF-α and VEGF mRNA transcripts formation from B16F10 melanoma. These results indicate that the treatment effectively inhibited the proangiogenic factor from the melanoma cells without significantly affecting the viability of the cell. It clearly indicates the action is at a molecular level rather than the membrane permeability or direct cellular toxicity.

Curcumin (CI) has already proven to be antiangiogenic, and the mechanism suggested is by inhibiting the MMP activity (Taloor D et al 1998). Studies using B16F10 cells have shown that curcumin could inhibit the osteopontin induced pro-MMP-2 activation by modulating NFκB transcriptional pathway (Philip S et al 2001). Since the proinflammatory cytokines such as IL-1β, IL-6 and TNF-α, are NFκB dependant (Schwarz and Murphy 2001), the inhibitory effect in the elevation of serum level of these cytokines may be attributed to the inhibition of NFκB activation. VEGF, IL-8 and MMPs etc are also NFκB regulated genes (Pan et al 2002). Hence we anticipate that the decreased secretion of these proteins may be a direct consequence of the ability of curcumins/curcuminoids, Boerhaavia and Tinospora to down regulate/inhibit the transcription factor NFκB activity yet we don't have a direct evidence for it. Besides NFκB, AP-1 is another ubiquitous eukaryotic transcription factor that also is a target of cell signaling cascade (Surh YJ 2003). Like NFκB, AP-1 also has binding site in the promoter region of

inflammatory/angiogenesis mediators such as COX-2 and iNOS and the down regulation of these proteins is attributed to suppression of NFκB or c-Jun/AP-1 activation (Surh YJ et al 2001). Both of them may act independently or coordinately to regulate target gene expression and hence are prime targets of diverse classes of chemopreventive phytochemicals. Curcumin is proven to down regulate NFκB and AP-1 activation in several cancer cell lines and in HUVEC eventhough the intracellular pathway that leading to the activation of NFκB and AP-1 differ in different cell lines. Hence involvement of AP-1 in the regulation of angiogenic factor production by other agents used in this study such as CIII, SC, Boerhaavia and Tinospora cannot be ruled out without further study.

Human Umbilical Vein Endothelial Cell (HUVEC) has been widely accepted as a model of study of *in vitro* angiogenesis. In this study, we tried to evaluate the effect of *B. diffusa, T. cordifolia*, and the curcuminoid derivatives on the HUVEC proliferation, motility and invasion using nontoxic concentrations, in culture. Present study demonstrates that certain curcuminoids (CI, CIII, SC) and extracts of Tinospora and Boerhaavia inhibited the proliferation and migration of HUVECs induced by VEGF/bFGF in a dose dependent manner without significantly affecting the viability of the cell. Proteolytic degradation of the extra cellular matrix, the extend of tumour vasculature and metastasis correlate with the expression endopeptidases known as MMPs (Schnaper HW et al 1993) and our results also point that the curcuminoids and extracts that inhibited the MMP activity, as visualized from the zymogram analysis, had also inhibited the collagen matrix invasion by endothelial cell *in vitro*.

In short, the study materials that inhibited the proangiogenic factor secretion from the tumour cells also decreased their levels in the serum of tumour-angiogenesis induced animals. They also inhibited the biological activities of HUVEC such as invasion, motility and proliferation that are essential for neovessel formation. All these might be together contributing to the antiangiogenic activity shown by these agents.

Agent that showed most significant antiangigeic effect in this study – *Boerhaavia diffusa* was further evaluated for its antimetastatic activity in mice as well as in tissue culture systems. A polysaccharide fraction isolated from the stem of *Tinospora cordifolia* also evaluated in this manner. The *in vitro* experiments showed that, the extract of Boerhaavia was reducing the proliferation of the tumour cell and inhibiting the invasion by interfering with the MMP activity, but the polysaccharide fraction did not have any inhibitory effect in this experimental setup. But intra-peritoneal administration of the Boerhaavia extract as well as polysaccharide fraction resulted in marked reduction in the metastases by B16F10 melanoma in the mice and increased the life span compared to the untreated control. Treatment also reduced the hexosamine and uronic acids contents in the lungs. These two are the structural monomers of the disaccharide D-glucuronicacid-N-acetyl D-glucosamine. This disaccharide is liberated by the degradation of Hyaluronicacids by HAase (Hyaluronidase) and is a good promoter of angiogenesis (Hautmann et al, 2002). Reduced levels of its structural monosaccharides indicate a reduction in D-glucuronicacid-N-acetyl D-glucosamine level thereby negatively contributing for the success of tumour angiogenesis.

At present we do not know what are the principal compounds that inhibit the cascade of events of metastasis and angiogenesis, also the exact mechanism of action of curcuminoids at a molecular level or the structure activity relationship. The *in vitro* experiments have shown that the polysaccharide fraction from Tinospora was neither directly toxic to the tumour cells nor inhibited the proliferation. These results are indicative of the involvement of other mediators such as the immune system in the reduction of the metastatic potential. The alcoholic and water extracts of *B. diffusa* and *T. cordifolia* is known to contain several bioactive molecules such as reducing sugars, amino acids and alkaloids. Several Boeravionones (Boeravionones A,B,C,D,E,F etc) have also been isolated from *B. diffusa*. The stem of *T. cordifolia* contains some immunopotentiators and an arabinogalactan polysaccharide is characterized from it. The pharmacological activity shown by the aqueous methanol extract may be attributed to the presence of these diverse compounds. It is needed to proceed long to characterize the extract and to find out the exact mechanism for this biological activity.

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List of Abbreviations

Ang	: angiopoietin
AP-1	: activator protein-1
Ara	: arabinose
BM	: basement membrane
BsAb	: bispecific antibody
CAI	: carboxyamidotriazole
CAM	: chorioallantoic membrane
DS	: degree of sulphation
ECM	: extra cellular matrix
EMSA	: electrophoretic mobility shift assay
FBH	: foetal bovine heart endothelial cell
FGF	: fibroblast growth factor
FGF-1	: acidic fibroblast growth factor
FGF-2	: basic fibroblast growth factor
FGF-R	: FGF receptor
Flk-1	: VEGF-receptor 2
G-CSF	: granulocyte colony-stimulating factor
Glu	: glucose
GM-CSF	: granulocyte-macrophage colony-stimulating factor
HIF	: hypoxia inducible factor
HUVEC	: human umbilical vein endothelial cell
ICAM-1	: intercellular adhesion molocule-1
IFN	: interferon
IGF-1	: insulin-like growth factor 1
IL	: interleukin
IP-10	: inducible protein 10
MAPK	: mitogen - activated protein kinase
MEK	: MAPK kinase
MMP	: matrix metollo proteinase
MW	: molecular weight
NFAT	: nuclear factor of activated T cells

NFκB	: Nuclear Factor kappa B
NO	: nitric oxide
PA	: platelet activating factor
PDGF	: platelet derived growth factor
PF4	: platelet factor 4
PGE	: prostaglandin E
phVEGF$_{165}$	: plasmid-encoding human VEGF$_{165}$ isoform
PKC	: protein kinase C
PMA	: phorbol 12-myristate 13-acetate
R.T	: Room temperature
RIF	: radiation-induced fibro sarcoma
RT-PCR	: Reverse transcription –PCR
SPARC	: secreted protein acidic and rich in cysteine
TGF	: transforming growth factor
TNF	: tumor necrosis factor
TNP-470	: Takeda neoplastic product-470
t-PA/u-PA	: tissue type/urokinase plasminogen activator
TSP-1	: thrombospondin-1
tTF	: truncated tissue factor
UFH	: un fractionated heparin
VCAM-1	: vascular cell adhesion molecule-1
VEGF	: vascular endothelial growth factor
VEGF-R	: VEGF receptor
vWF	: von Willebrand factor/factor VIII-related antigen.

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