Chapter 9
Summary and conclusion
Cancer is a disturbance of growth characterized by excessive proliferation and spread of cells throughout the body without apparent relation to the physiological demands of the organs involved. Cancer arises from loss of a normal growth control. An increase in number of dividing cells creates a growing mass of tissue called a tumor or neoplasm. If the rate of cell division is relatively rapid and there are no suicide signals to trigger cell death, the tumor grows quickly in size, if the cells divide slowly, tumor growth is slower. Cancers are capable of spreading throughout the body by invasion and metastasis.

The process of metastasis is not random. Instead, it is a cascade of linked sequential steps that must be traversed by tumor cells if a metastasis is to develop (Fidler, 2003). Potentially metastatic cells have to exit the primary tumor site by loosening cell to cell contact, adhering to and degrading extracellular matrix (ECM), migrating through the subendothelial basement membrane of local post-capillary veins and lymphatic vessels and intravasating. Once in circulation, tumor cells face severe mechanical and immunosurveillance challenges. Surviving cells can arrest in the peripheral capillary bed of a distant organ, adhere to the subendothelial basement membrane, extravasate, adhere and migrate through the ECM, and form a colony at the new metastatic site. Further induction of neoangiogenesis must occur to assure continuous growth (Fidler, 2003). Interruption of the metastatic cascade at any of these steps can prevent the production of clinically symptomatic metastasis. The present study demonstrate Punarnavine could inhibit the metastatic nodule formation in lungs by inhibiting cellular proliferation, adhesion, migration and invasion of highly metastatic B16F-10 melanoma cells. For invading through the tissue, metastatic cells should penetrate the ECM and this is achieved by the release of a degradative enzyme called matrix metalloproteinases (MMPs) more specifically type IV MMPs, MMP-2 and MMP-9. Punarnavine also could inhibit the gene expression and release of MMP-2 and MMP-9 from B16F-10 melanoma cells showing its antimitastatic potential. The inhibited expression of Prolyl hydroxylase, Lysyl oxidase, Erk-1, Erk-2, VEGF, k-ras, proinflammatory cytokines such as TNF-α, IL-1β and IL-6 and upregulated expression of TIMP-1, TIMP-2 and nm23 also underline Punarnavine as a potent antimitastatic agent.
The field of ‘angiogenessis research’ began more than two decades ago as an inquiry into the mechanisms by which tumors induce a few blood supply. The field now expanded to include a diverse group of scientists who are addressing new central questions. However, the field of angiogenesis research was originally based on the hypothesis that ‘tumor growth is angiogenesis-dependent’. This hypothesis continues to be fruitful as it enlarges our understanding about tumor growth and metastasis.

Despite recent advances in our understanding of the molecular control of tumor growth and development, our knowledge about the mechanisms that mediate tumor metastasis remains rudimentary. Extensive laboratory data suggest that angiogenesis plays an essential role in cancer development, invasion and metastasis. Undoubtedly, the hypothesis that targeting angiogenesis could be a strategy for modern cancer therapies. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen \textit{in vitro} and an angiogenic inducer in a variety of \textit{in vivo} models. Hypoxia has been shown to be a major inducer of VEGF gene transcription. Importantly, recent data have shown that inhibiting VEGF results in a clinical benefit, including increased survival, in patients with advanced malignancies, providing the first clinical validation of the hypothesis that blocking angiogenesis is a strategy to treat cancer (Yang et al, 2003; Hurwitz et al, 2004). Here the treatment with natural products such as Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could inhibit the tumor directed capillary formation in C57BL/6 mice by inhibiting the VEGF production. Vascular relaxation, for example, mediated by nitric oxide (NO) is a prerequisite for endothelial cells to enter the angiogenic cascade. Several studies have pointed to the critical role of nitric oxide (NO) in VEGF-induced vascular permeability, as well as angiogenesis (Parenti et al, 1998; Ziche et al, 1996; Morbidelli et al, 1996). In all types of angiogenesis, either under physiologic or pathologic conditions, endothelial cell activation is the first process to take place. The level of NO was also elevated in angiogenesis induced animals, which may be the reason for increased tumor-directed capillary formation in them. Cancer cells can also stimulate angiogenesis directly by the secretion of some of the pro-inflammatory cytokines such as TNF and IL-1 (Powers et al, 2000.). TNF-\(\alpha\) might be directly involved in iNOS activation and acting as an additional signal for synergistic
induction of NO formation which induce the process of angiogenesis. These cytokines can also act as autocrine growth factors for tumor cells. Here the significant reduction in the level of serum NO, pro-inflammatory cytokines and elevated level of IL-2 in angiogenesis induced animals after the treatment with naturally occurring Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene show their anti-angiogenic potential.

The essential steps for a neovascular initiation include endothelial cell proliferation followed by migration, invasion and tube formation. The present study demonstrate nontoxic concentrations of Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could inhibit the HUVEC proliferation, migration, invasion and tube formation by dose dependent manner. Since angiogenesis involves migration/invasion of endothelial cells into surrounding stroma/tissues, proteases such as the matrix metalloproteinases (MMPs) are critically important. However, recently it has become clear that MMPs' role(s) in angiogenesis is more complex than simply degrading the extracellular matrix (ECM) to facilitate invading endothelial cells. MMP-2 and MMP-9 have been shown to be important in tumor invasion because of their ability to breakdown basement membrane. Gelatin zymography of HUVECs showed inhibition MMP-2 and MMP-9 production by the treatment with Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene. The elevated level of TIMP-1 in the serum of angiogenesis induced animals also support the successful shift of the equilibrium of pro and antiangiogenic factors in favour of antiangiogenic condition.

Nitric oxide (NO), a potent biological mediator, plays a key role in physiological as well as pathological processes. An area of great interest is the role of nitric oxide in the growth and metastasis of solid tumors, where it seems to have a complex action including both inhibitory and tumor-promoting activities. Modification of nitric oxide synthetase (NOS) activity in tumors, and hence NO biosynthesis, may be regarded as a promising means for selective tumor blood flow modification providing a novel approach for reducing tumor oxygenation, (Alexandrova et al, 2001). Here in the present study treatment with the naturally occurring compounds Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene significantly reduced the NO production in B16F-10 melanoma cells in a dose
dependent manner and downregulated expression of iNOS gene support the above observation.

While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, a large body of clinical and experimental data suggest a promoting role of NO in tumor progression and metastasis. Expression of NOS has been found to be correlated with the grade of some human tumors, such as breast, ovarian, cervix, gastric, head and neck cancer (Thomsen et al, 1994; Thomsen et al, 1995; Rosbe et al, 1995; Thomsen and Miles, 1998) and also associated with enhanced metastasis. The mouse iNOS promoter contains two NF-κB elements, which are critical for iNOS expression as site directed mutagenesis of either of these sites significantly reduces promoter activity, and chemical inhibitors of NF-κB prevent iNOS expression and NO production (Kim et al, 1997). Existence of both positive and negative regulatory AP-1 sites has been reported (Kizaki et al, 2001; Okada et al, 2003), but the composition of AP-1 which binds to these sites is not known. Overexpression of C-Fos, Fos-B and C-jun suppresses iNOS promoter activity (Okada et al, 2003; Yu et al, 2002; Chen et al, 2003; Gupta et al, 2002). The inhibited nuclear translocation of NF-κBp65, NF-κBp50, NF-κBc-Rel, c-Fos, ATF-2 and CREB-1 proteins by Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene treatment in B16F-10 cells showing a clear picture of their mechanism of action that cause downregulated expression of iNOS gene and inhibition of NO and VEGF mediated neovascularization and subsequent inhibition of metastatic progression.

Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation (Griffiths et al, 2002). They cannot detect the DNA damage, so unable to induce cell cycle arrest at DNA damage check points- one in late G1, which prevents entry into S phase, and one in late G2, which prevents entry into mitosis. In the present study, untreated control B16F-10 cells showed clear cell cycle progression and treatment of these cells with nontoxic concentrations of Punarnavine and Glycyrrhizic acid suppressed cell cycle progression by inducing DNA fragmentation while, Ursolic acid and Limonene delayed the cell cycling at
G0/G1 phase. Programmed cell death plays critical roles in a wide variety of physiological processes during fetal development and in adult tissues. In most cases, physiological cell death occurs by apoptosis as opposed to necrosis. Apoptosis is a morphological phenomenon. As viewed with the assistance of the light (or, preferably, the electron) microscope, the characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. The compounds Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene act as inducers of cell cycle delay and apoptosis in B16F-10 melanoma cells. B16F-10 cells treated with these compounds and after staining with hematoxylin and eosin method showed chromosomal breakage and membrane blebbing. The agarose gel electrophoresis pattern of DNA isolated from B16F-10 cells produced a characteristic ladder pattern. The TUNEL assay also confirmed the presence of apoptotic bodies by staining free 3'-OH termini enzymatically labeled with modified nucleotides of the Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene treated B16F-10 cells which support the above observation. The Reverse transcription-PCR analysis showed elevated expression of tumor suppressor gene p53 and downstream caspase, caspase-3. Punarnavine, Glycyrrhizic acid and ursolic acid could inhibit the expression of antiapoptotic gene Bcl-2. The inhibited nuclear translocation of NF-kB, C-Fos, CREB-1 and ATF-2 after the treatment with these compounds also support the apoptosis inducing potential of Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene.

The microarray analysis of B16F-10 cells treated with Punarnavine, showed an upregulation of 658 genes included in different categories, most importantly in cell cycle checkpoint and programmed cell death. The upregulation of genes involved in cell cycle checkpoint and programmed cell death support the pro-apoptotic role of Punarnavine. These observations support the G1 phase delay in cell cycle progression of B16F-10 cells by the treatment of Punarnavine. Punarnavine could upregulate GADD45β which is pro-apoptotic gene and tumor suppressor genes such as Retinoblastoma like 1 and SMAD. Punarnavine also could downregulate 366 genes included in important categories such as prostaglandin metabolism, prostaglandin transporter activity, bradykinin receptor activity. regulation of MAPK activity. The
important genes downregulated are Prostaglandin E synthase (PTGES), Prostaglandin-endoperoxidase synthase 1 (PTGS1), Phospholipase A2-group 5 (Pla2g5), Cytochrome P450, family 2, subfamily c, polypeptide 37 (Cyp2c37). The involvement of PGs in the pathogenesis and progression of cancer is now clearly known and any attempt to reduce its level or the inhibition of the expression of the genes involved in the prostaglandin metabolism will be appreciable in modern cancer research. Here the downregulated expression of several number of genes involved in PG metabolism support their anticancer and anti-metastatic potential.

Glycyrrhizic acid could upregulate 1736 genes involved in cell-cycle checkpoint and PCD in B16F-10 cells. It could also upregulate numerous pro-apoptotic genes and even the caspase cascade. The downregulation of several antiapoptotic and oncogenes like B-cell lymphoma 2 (Bcl2), B-cell lymphoma 9 like (Bcl9l), growth differentiation factor 5 (Gdf5), DNA-damage inducible transcript 3 (Ddit3), thymoma viral proto-oncogene 3 (Akt3), cyclin-dependent kinase 5, regulatory subunit 2 (p39) (Cdk5r2), transformed mouse 3T3 cell double minute 4 (Mdm4) and cyclin D3 (Ccnd3) by Glycyrrhizic acid treatment also support the apoptotic inducing and antiangiogeic potential of Glycyrrhizic acid.

In conclusion, the results obtained in the present study demonstrate the effectiveness of naturally occurring compounds such as Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene in the inhibition of metastasis, tumor specific angiogenesis and induction of apoptosis. Since NF-κB is a major transcription factor which activate the various genes involved in metastatic progression and cell survival, and iNOS is a major angiogenesis inducing and inflammatory gene, the effectiveness of these compounds in suppressing both NF-κB and iNOS make them promising agents for anticancer therapy. Punarnavine with its immunomodulatory and antimetastatic activities forms a new potential candidate for anticancer therapy. More studies should be conducted to trace the unknown molecular mechanisms, that involved in their action on cancer cells