Abstract
Cancer is a major public health problem in India and many other parts of the world. It is a class of diseases in which a group of cells display the ability to uncontrolled growth (division beyond the normal limits) and metastasis. Cancer may affect people at all ages, but risk tends to increase with age, due to the fact that DNA damage becomes more apparent in aging DNA.

It has been apparent that attacks of tumors by means of immune cells are efficient in circumventing the tumor progression. Punarnavine (40mg/kg body weight) was found to enhance the total WBC count, bone marrow cellularity and number of α-esterase positive cells which demonstrated activated immune system. The enhancement in the circulating antibody titer, the number of plaque forming cells (PFC) in the spleen and enhanced proliferation of splenocytes, thymocytes and bone marrow cells also support the above observation. Punarnavine also could significantly enhance the NK cell activity, antibody dependent cellular cytotoxicity (ADCC) and antibody dependent complement mediated cytotoxicity (ACC) in tumor-bearing as well as normal animals.

Metastasis is the hallmark of malignant transformation which is having a well coordinated sequential steps. Administration of Punarnavine (40mg/kg body weight) at different stages of tumor development such as prophylactically, simultaneously and 10 days after tumor inoculation could inhibit the metastatic colony formation of melanoma in lungs which cause increased survival of animals. The lung and serum biochemical parameters and histopathological analysis showed reduced fibrosis and metastasis. The levels of proinflammatory cytokines, VEGF, IL-2 and TIMP-1 also support the above observation. The down regulated expressions of MMP-2, MMP-9, ERK-1, ERK-2, k-ras, TNF-α, IL-1β, IL-6, Prolyl hydroxylase, lysyl oxidase, VEGF and upregulated expressions of TIMP-1, TIMP-2 and nm23 in the lung tissue of metastasis induced animals demonstrated the possible mechanism of action of Punarnavine.

Angiogenesis, the formation of new capillaries from preexisting vessels, is essential for tumor progression and metastasis. Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could inhibit angiogenesis in vitro and in vivo. The inhibited tumor associated capillary formation with the significant reduction of
elevated levels of serum VEGF, NO, proinflammatory cytokines and significant elevation of serum TIMP-1 and IL-2 levels in C57BL/6 mice by the treatment of the above compounds support their antiangiogenic potential. The dose depended inhibition of vessel growth from rat aortic ring as well as endothelial cell (HUVEC) proliferation, migration, invasion and tube formation with inhibition of protein expression MMP-2 and MMP-9 also demonstrate the inhibitory effect of Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene on different steps of neovessel formation.

Since negative regulation of cell cycle progression and induction of apoptosis are considered as one of the major mechanisms to anticancer and antimetastatic activity, the effect of Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene were tested for their effect on cell cycle and induction of apoptosis using B16F-10 melanoma cells. The compounds Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could act as inducers of cell cycle delay and apoptosis in B16F-10 melanoma cells. The Reverse transcription-PCR analysis showed the induction of tumor suppressor gene p53 and downstream caspase, caspase-3 in B16F-10 melanoma cells by the treatment with the above compounds. Punarnavine, Glycyrrhizic acid and Ursolic acid could inhibit expression of antiapoptotic gene Bcl-2.

NF-κB is a transcription factor which can induce the activation of numerous antiapoptotic and protooncogenes and is considered as a prime target for anticancer and antimetastatic therapies. Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could significantly inhibit the nuclear translocation of NF-κB subunits such as p65, p50, c-Rel as well as other transcription factors like c-Fos, ATF-2 and CREB-1 in B16F-10 melanoma cells. RT-PCR analysis of the genes of NF-κB p65, NF-κB p50, c-Fos, CREB-1 and ATF-2 showed downregulated expression after the treatment with these compounds. As cancer is regarded as an inflammatory disease, the major inflammatory agent Nitric oxide (NO) is also considered as a target for anticancer therapies. Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene could downregulate the level of NO in the supernatant of B16F-10 cells in a dose dependent manner. The RT-PCR analysis of iNOS gene showed downregulated
expression in B16F-10 cells treated with the above compounds compared to the untreated control cells. These data clearly demonstrate that the antimetastatic, antiangiogenic and apoptosis inducing activities of Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene are also due to the inhibition of NF-κB and iNOS mediated signaling.

A preliminary microarray analysis was performed to study the effect of Punarnavine and Glycyrrhizic acid on gene expression profile of several categories of genes included in metastatic and apoptotic signaling cascades. Microarray analysis of B16F-10 melanoma cells treated with Punarnavine showed upregulation of 658 genes and downregulation of 366 genes. Upregulated genes involved several cell cycle regulators and downregulated genes involved several genes in arachidonic acid metabolism. Glycyrrhizic acid could upregulate 1736 genes and many of them involved in apoptosis and cell cycle checkpoint. It could downregulate 1445 genes in B16F-10 cells and many of them are involved in cellular proliferation, cell-cell adhesion, cell-cell communication and negative regulation of caspase activation.

In summary, the present study demonstrates that Punarnavine the alkaloid from *Boerhaavia diffusa*, possess immunomodulatory activity and also exhibit significantly promising antimetastatic potential. Punarnavine and other natural compounds like Glycyrrhizic acid, Ursolic acid and Limonene could induce cell cycle delay and apoptosis in B16F-10 melanoma cells. Administration of these compounds effectively blocked the tumor specific angiogenesis in the *in vivo* and *in vitro* models. The inhibition of NF-κB mediated activation of oncogenic and antiapoptotic pathways and inflammatory responses would be one of the possible mechanism of action behind the antimetastatic, antiangiogenic and proapoptotic activities of the natural products Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene.

*Key words: Metastasis, Angiogenesis, Immunomodulation, Proinflammatory cytokines, NF-κB, iNOS, Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene.*