CHAPTER-8

SUMMARY AND CONCLUSION
Cancer is characterized by accelerated and uncontrolled growth, dysregulation of apoptosis, invasion and metastasis. While genetic and epigenetic mechanisms may underlie transformation, tumour microenvironment promotes the neoplastic process. In spite of major advancements in the field of medicine and technology, cancer still continues to be a threat to mankind. The therapeutic modalities available today, ranging from conventional to most modern targeted therapies, fail in several instances to eradicate cancer. Conventional therapies cause serious side effects and at best merely extend the patient's lifespan by a few years.

With the increasing level of the carcinogenic and mutagenic substances in the environment, the research to explore new anticancer compounds has become crucial day after day. Although many chemical anti-cancer agents are available, the wide spectrum side effects and emergence of chemotherapy resistant cancer cells among patients have made cancer research and discovery of new anti-cancer agents pivotal. Discovery of novel natural compounds with low toxicity to normal cells where as high specificity to cancer cells is an important area in cancer research. An agent that could efficiently inhibit the growth, migration and invasion of cancer cells would be a hopeful candidate to suppress cancer progression and metastasis and thereby could reduce mortality. Plants have been a prime source of several highly effective conventional drugs for the treatment of various forms of cancers. In many instances, the actual compound isolated from the plant may not serve as the drug, but leads to the development of potential novel agents. Use of plants or plant products as immunomodulators is getting more and more importance in the field of cancer research.

In the present study we have analyzed the inhibition of tumour progression by natural products in animal models and tried to elucidate possible mechanism of action in the molecular level. After screening several plants, we selected Ipomoea obscura for our study. We also selected a natural alkaloid Harmine having several pharmacological properties. I. obscura is a well-known medicinal plant belonging to the family Convolvulaceae used traditionally for various ailments. An indole alkaloid Ipobscurine is the major constituent of this plant. We have evaluated the inhibitory effect of Harmine, I. obscura and Ipobscurine on tumour progression and metastasis.

Inflammation can play a critical role in tumour suppression by stimulating an anti-tumour immune response, but more often it appears to stimulate tumour development. Epidemiologic and clinical researches indicate an increased risk of certain cancers in setting of chronic inflammation. Inflammation plays decisive roles at different stages of tumour development, including initiation, promotion,
malignant conversion, invasion, and metastasis. Inflammation also affects immune surveillance and responses to therapy.

Consequently we first studied the anti-inflammatory and anti-tumour activity of methanolic extract of *I. obscura* using carrageenan, dextran and formalin induced mouse inflammation models and mouse tumour models. Significant activity was found with *I. obscura* in both acute and chronic models and hence we evaluated the mechanism of its anti-inflammatory activity. We found that *I. obscura* could inhibit the various inflammatory mediators such as COX-2 and iNOS which are the key enzymes in the process of inflammation. We also found that the pro-inflammatory cytokines, nitric oxide and the acute phase protein, CRP are inhibited by the *I. obscura*. Since macrophages are one of the major sources of TNF-α and other pro-inflammatory cytokines during endotoxic shock, we investigated whether *I. obscura* affects LPS-induced TNF-α production both *in vivo* and *in vitro*. Treatment of LPS challenged mice with *I. obscura* caused a reduction in serum TNF-α level as evidenced from bioassay. There was a significant decrease in the levels of pro-inflammatory cytokine levels and CRP in LPS challenged mice after the treatment with *I. obscura*. To further explore the possible mechanism of these inhibitions by *I. obscura*, the mRNA expression levels of iNOS and COX-2 were also examined. A significant inhibition in the expression of iNOS and COX-2 gene by *I. obscura* in LPS stimulated macrophages was observed.

Anti-tumour studies revealed that *I. obscura* is cytotoxic towards various cell lines *in vitro* while nontoxic to normal human peripheral blood lymphocytes. These results indicate that cytotoxicity of *I. obscura* is tumour cell specific. Inhibition of DLA induced solid tumour development as well as increased life spans of EAC bearing animals by the administration of *I. obscura* strongly support the anti-tumour activity of *I. obscura* in *vivo*. Moreover, the extract significantly reduced tumour cell proliferation in a dose and time dependent manner. Inhibition in the tumour cell proliferation may be due to the involvement of *I. obscura* in metabolic events that leads to induction of apoptosis or cell cycle arrest.

Cancer cells also are resistant to many immunological barriers in the body. In order to achieve an effective anti-tumour immune response, appropriately activated immune cells should maintain their anti-tumour activity to overcome the immune suppressive tumour microenvironment. General or selective activation of various immunocompetent cells and their secretory function to maintain a healthy immune status may help in cancer prophylaxis as well as therapy. A significant enhancement in bone marrow cellularity and total WBC count by the administration of *I. obscura* as well as Ipobscurine clearly demonstrates its potentiating effect on haematopoiesis and cell mediated immune system. Moreover
an increased presence of α-esterase positive bone marrow cells indicate that these compounds could also enhance the differentiation and proliferation of stem cells. At the same time there was no decrease in the body weight of the animals during the experiment indicating non toxicity of the test compounds. The cell-mediated components of the immune system are equipped with multiple effector mechanisms capable of eradicating tumours. It is generally held that CD8+ cytotoxic T lymphocytes (CTL) and NK cells are the most potent anti-tumour effectors and natural products could modulate these cells thereby decreasing the incidence of tumour. Enhanced activity of NK cells, ADCC and ACC after the administration of I. obscura and Ipobscurine represent the augmentation of cell-mediated immune response which may also have strong positive correlation with the observed anti-tumour activity. Furthermore I. obscura and Ipobscurine effectively enhance CTL activity and increase the survival of experimental animals through the enhanced production of IL-2 and IFN-γ.

Both I. obscura and Ipobscurine stimulated the humoral response, as there was a significant enhancement in the circulating antibody titre as well as number of antibody producing cell present in the spleen. The compounds were found to stimulate the lymphoid organ weight demonstrating that the compounds enhanced the production of immune cells. More over both I. obscura and Ipobscurine significantly promoted the proliferation of spleen, thymus and bone marrow cells as evident from blastogenesis assay. The above results suggest that I. obscura and Ipobscurine stimulate cell mediated as well as humoral arms of immune system and can be deemed as anti-tumour agent with immunomodulatory activity.

One of the major causes of death in cancer patients is due to the ability of tumor cells to metastasize. Metastasis is a complex process that requires malignant cells to leave the primary tumor and proliferate at a distant site. Several plants and phytochemicals were identified as nontoxic chemopreventive agents especially during metastasis. These compounds act as anti-cancer agents by regulating various cellular and molecular events leading to tumour progression. We also evaluated the effect of Harmine, I. obscura and Ipobscurine on the metastasis using experimental animal model. Metastasis was induced by injecting highly metastatic B16F-10 melanoma cell line through lateral tail vein, which can induce colonies of tumour nodules in the lungs that promote collagen deposition leading to lung fibrosis. But the formation of tumour nodules was significantly decreased by the treatment with Harmine, I. obscura and Ipobscurine especially when they were administered simultaneously with the tumour cells. This was further confirmed by analyzing various lung and serum biochemical parameters and the histopathological analysis that showed comparatively similar pattern to normal after treatment.
To understand the possible molecular mechanism underlying the anti-metastatic effect of these compounds, the effects of these compounds on various inflammatory mediators as well as expression levels of various metastatic genes were evaluated. Interestingly, we found that serum levels of several pro-inflammatory cytokines in metastasis induced animals were inhibited on treating with these compounds. Recent reports on similar studies support our findings that pro-inflammatory cytokines have potent tumour-promoting activity by inducing tumour angiogenesis, synthesis of MMPs, or by directly supporting tumour cell growth. The results of the study also clearly showed that these compounds significantly inhibited iNOS and COX-2 giving further support for anti-inflammatory mediated tumour regression by test compounds.

Down regulation in the expression of pro-metastatic genes such as MMPs, ERK 1/2, prolyl hydroxylase and lysyl oxidase in metastasis induced lungs after administration of test compounds give further evidence for their anti-metastatic activity. It is possible that one of the major steps by which Harmine exhibits an anti-metastatic effect in melanoma cells is through inhibition of MMP expression by negatively regulating ERK activation to suppress tumour formation and metastasis whereas *I. obscura* and Ipobscurine shows anti-metastatic activity by specifically inhibiting inflammatory mediators involved in metastasis. Metalloproteinases have been implicated in the denaturation of the basement membrane during the metastatic invasion of tumour cells. These compounds also inhibited MMP production and expression in metastatic condition. Moreover Harmine, *I. obscura* and Ipobscurine could down regulate the expression of genes involved in collagen synthesis and its metabolism which are highly expressed in tumour bearing lungs such as lysyl oxidase and prolyl hydroxylase. There was a significant up-regulation in the expression of endogenous MMP inhibitor TIMP-1, TIMP-2 and anti metastatic gene nm23 with the treatment with these test compounds showing inhibitory role of these compounds on tumour metastasis.

*In vitro* studies were also showed a significant inhibitory effect in the various steps of metastasis. Zymographic analysis and *in vitro* expression analysis showed that test compounds inhibited the production of matrix metalloproteinases thereby diminished invasiveness potential of B16F-10 cells.

Cell mediated immune system is responsible for the early detection and elimination of tumour cells. Hence immunotherapeutic approaches aiming to enhance the cell mediated immune responses is of high value towards the prevention of metastasis. Administration of Ipobscurine enhanced the NK cell activity, ADCC and ACC in metastasis bearing animals. Immunopotentiating
cytokine: IL-2 and INF-γ also showed an enhancement after the treatment with Ipobscurine.

In tumour growth and angiogenesis, process of formation of new blood vessels from pre-existing ones is uncontrolled and unlimited. Since angiogenesis is pre-requisite for the growth of solid tumours, vascular targeting has been explored as a potential strategy to suppress tumour growth and metastasis. Several phytochemicals were also been evaluated for inhibiting various components associated with tumour angiogenesis. It was found that tumour directed capillary formation was increased as tumour progress, indicating a direct correlation between the tumour survival and vessel count. Administration of Harmine, *I. obscura* or Ipobscurine significantly inhibited the tumour specific new blood vessel formation in C57BL/6 mice when angiogenesis was induced with B16F-10 melanoma cells. VEGF, the most important mitogenic and survival factor that contributes in enhanced tumour growth and metastasis was significantly inhibited by the compounds. The significant reduction in VEGF after the treatment may be the reason for the inhibition in tumour directed capillary formation.

Inflammatory signals also regulate the expression and secretion of various angiogenic factors and the process of neo-vascularization. Our study showed that there was an elevated level of pro-inflammatory cytokines in the serum of untreated control animals after tumour induction. But treatment with test compounds inhibited the production of these cytokines and this might be the cause for the reduction of serum VEGF levels of treated animals, since most of these pro-inflammatory cytokines are good inducers of VEGF. Administration of these compounds also significantly reduced the elevated level a pro-angiogenic nitrite level after tumour challenge. Furthermore our study also showed a significant decrease in expression of endothelial cell marker CD31 after treatment with test compounds.

As in the case of metastasis experiment, here also TIMP level was increased in animals treated with the compounds suggesting that increased endogenous TIMP could inhibit MMP activity and consequently inhibit ECM remodeling. The level of IL-2 was also increased after the treatment since IL-2 have been shown to stimulate the immune system against the tumour growth by VEGF induced angiogenesis.

Various *in vitro* models for the evaluation of anti-angiogenic activity also clearly demonstrated that the test compounds can inhibit different steps in tumour angiogenesis such as proliferation, migration, tube formation and vessel spouting from rat aortic ring. MTT assay results clearly suggested that these activities were not due to direct toxic effect of test compounds whereas these compounds impart their role in various signaling pathways of angiogenesis. Treatment with test
compounds significantly inhibited invasion of endothelial cells through collagen coated membrane. This may be due to the inhibition in proliferation of HUVECS and the release or activation of MMP as evident from the zymogram. Rat aortic ring assay also showed the inhibitory role of test compounds in VEGF induced new vessel formation from pre existing blood vessels in vitro.

The anti-angiogenic activity of these compounds at molecular level also studied and found that the test compounds inhibit pro-angiogenic gene expressions in tumour cells when they are stimulated with growth factors or hypoxic stimulus. Here also, COX-2 and iNOS were the important targets of test compounds as observed in previous experiments. The inhibitory action of these compounds on COX-2 and iNOS also adds further support for the inhibitory action of these compounds on tumour progression by restricting new blood vessel formation towards growing tumour. Additionally these compounds down modulate the expression of VEGF and restrict further growth of tumour. Remodeling of ECM by MMPs is a crucial step in angiogenic cascade as it promotes endothelial cell migration, proliferation and tube formation. MMPs facilitate endothelial cell sprouting by liberating matrix bound pro-angiogenic factors such as VEGF, FGF, and TGF-β. In our study, zymographic analysis revealed that the test compounds could inhibit the production of MMPs by endothelial cells.

The most significant regulator of VEGF production is hypoxia. As a tumour increases in mass and becomes hypoxic, VEGF is induced and stimulates growth of new vessels. Transcription of VEGF mRNA is up-regulated in hypoxia through HIF-1 that bind to the VEGF promoter. Interestingly we found that Harmine have the capacity to inhibit HIF expression under hypoxic condition. This inhibition also reflected in the VEGF release under hypoxic condition. However, in I. obscura or Ipobscurine treated cells there was no significant inhibition in the expression of HIF, which indicate that the reduced expression of VEGF is not HIF mediated.

We further evaluated the mechanism of tumour regression by Harmine, I. obscura and Ipobscurine in B16F-10 melanoma cells. Inhibiting cell proliferation and increasing apoptosis in tumours are effective ways to prevent tumour growth, and eliminate cancers. Recently many anti-cancer studies are focused on the manipulation of the apoptotic process for the treatment and prevention of cancer. Apoptosis or programmed cell death occur under genetic control by set of genes and has become one of the major intervening targets in cancer chemoprevention. In consequence the compounds that can influence apoptosis without producing toxic side effects are of great significance today.

In this experiment treatment of melanoma cells with Harmine, I. obscura or Ipobscurine induced morphological changes that included condensation of nuclear
chromatin, formation of apoptotic bodies and blebbing of the cell membrane which are biochemical hallmark of apoptosis. DNA from treated melanoma cells also showed characteristic ladder pattern of discontinuous DNA fragments. More over presence of pyknotic nuclei that are characteristics of cells undergoing apoptosis were confirmed by tunnel assay in test compounds treated cells.

To reveal the precise molecular mechanism of Harmine, *I. obscura* and Ipobscurine induced apoptosis in B16F-10 melanoma cells, we analyzed the expression various apoptotic regulator genes at the mRNA level. Expression of pro-apoptotic genes such as P53, Caspase-3, 9 and Bax were significantly induced at the earlier phase of treatment suggesting that it was an initiator or inducer of the apoptotic mechanism by the test compounds. In addition Harmine could also enhance the activation of pro-apoptotic genes such as caspase 8 and Bid which are involved in extrinsic pathway. But treatment with *I. obscura* or Ipobscurine could not enhance Caspase-8 and Bid expressions. Harmine and *I. obscura* or Ipobscurine could down-regulate the expressions of Bcl-2 in B16F-10 melanoma cells. Activation of Caspase-8 and Bid along with other caspsases indicates the involvement of Harmine in both extrinsic and intrinsic pathways of apoptosis. But *I. obscura* and Ipobscurine activates only the intrinsic pathway of apoptosis. Our study also found that these compounds cause cell cycle arrest in Go/G1 phase and showed an evident apoptotic sub-G0/G1 peak in B16F10 melanoma cells.

We have also checked the inhibitory potential of these compounds on the activation and nuclear translocation of transcription factors. Transcription factors participate in the regulation of diverse biological processes such as immune and inflammatory responses, cell growth and apoptosis of cells. Constitutive activation of NF-κB and chronic inflammation has a major role in tumour development and are seen in most tumour types and inhibition of NF-κB will lead to down-regulation of the NF-κB regulated anti-apoptotic proteins and other pro-inflammatory cytokines thereby promoting apoptotic cell death. Transcription factor NF-κB and AP-1 regulates the expression of various molecules such as MMPs, COX-2, iNOS and pro-inflammatory cytokines, all of which promote tumour cell invasion and angiogenesis. We could find that Harmine, *I. obscura* and Ipobscurine could inhibit the nuclear translocation of sub units of NF-κB such as p65, p50, c-Rel and subunits of AP-1 such as c-Fos and ATF-2 and CREB in B16F-10 melanoma cells. NF-κB and AP-1 have selectively regulated the expression of pro-inflammatory cytokines such as IL-1, IL-6, GM-CSF and TNF-α. The anti-metastatic and anti-angiogenic activity described in previous chapter may be related to the induction of apoptosis by these compounds in the cancer cells.
To conclude, we tried to explore the anticancer property of some natural products such as Harmine, *I. obscura* and Ipobscurine which are found in the indigenous system of medicine. The result not only indicated the effectiveness of these compounds in the inhibition of metastasis, angiogenesis and the promotion of apoptosis, it also revealed the mechanisms by which these compounds inhibit metastasis, angiogenesis and promote apoptosis. Since chronic inflammation is the cause of most cancers, the anti-inflammatory activity of *I. obscura* and Ipobscurine and the effectiveness of Harmine in suppressing various genes involved in the tumour progression make them promising agents for anticancer therapy. So the present study clearly demonstrate that these compounds are promising anticancer agents which can be used alone or along with other cancer therapies as it can inhibit tumour cell progression by restricting neo-vessel formation towards tumours and also has the ability to affect several other targets of tumour progression.