CHAPTER 8
SUMMARY AND CONCLUSION
Cancer is a major health problem and is the second leading cause of death in the world. Globally, 9 million deaths result from cancer each year, a figure that WHO anticipates will rise to 20 million by 2020, again due to increased life expectancy and lifestyle changes (Jemal et al., 2007). Based on the National Cancer Registry (India) reports there are nearly 800,000 new cancers cases in India every year. Invasion and metastasis are the greatest obstacles to the successful tumour treatment. Metastasis is a multi-stage process involving adhesion of the cancer cells to the basement membrane, invasion through the basement membrane, cancer cell motility, intravasation, transit in the blood or lymph, extravasation and growth at a new site (Fidler, 1990). Any drug, which can inhibit one of the steps in the cascade, will be useful in the inhibition of tumour metastasis. Death from cancer is most often due to metastases that are resistant to conventional therapies.

The metastatic process is comprised of multiple events involving cell invasion, cell motility, surface adhesion properties, and degradation of extracellular matrix (ECM). Thus, degradation of the ECM and components of the basement membrane by a concerted action of proteinases, such as matrix metalloproteinases (MMPs) play a critical role in tumour invasion and metastasis (Westermarck and Kahari, 1999; Yoon et al., 2003); MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are type IV collagenases that degrade basement membrane collagen (Salo et al., 1985). These two MMPs are also unique in their ability to degrade denatured interstitial collagen or gelatin. Both gelatinases are expressed in many different types of cancer cells; however, they are predominately produced in stromal cells located adjacent to the tumours (Heppner et al., 1996). In human malignancies, increased MMP-2 and MMP-9 expression and activity correlate with poor prognosis and decreased survival (Kallakury et al., 2001; Yoshizaki et al., 2001). Nowadays MMPs have been considered as a promising target for anti-cancer drugs, and a number of synthetic matrix metalloproteinase inhibitors have been developed and tested (Nelson et al., 2000). In the present study, incubation of B16F-10 melanoma cells with \textit{V.cinerea} and Vernolide-A down regulated the MMP-2 and MMP-9 gene expression and production. Tissue Inhibitors Matrix Metalloproteinases (TIMP), which are endogenous inhibitors of MMP, directly suppresses the MMP activity, thereby inhibiting ECM remodeling. The significant enhancement in the level and
expression of TIMP-1 in *V. cinerea* and Vernolide-A treated mice compared to the non-treated control in metastatic condition, also support the role of *V. cinerea* and Vernolide-A in stimulation of immunity against highly metastatic tumour in C57BL/6 mice. 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 nm23 also underline the antimetastatic potential of *V. cinerea* and Vernolide-A.

One of the major drawbacks of current cancer therapeutic practices, such as chemotherapy and radiotherapy, is immunosuppression (Santin et al., 2000). The immune system is able to identify and destroy altered cells due to altered tumour surface antigens. Dysfunctions of the immune system by tumour induced/derived factors make immune cells helpless against the development of cancer. Immunity has been shown to be suppressed in cancer. Tumour development, outgrowth, and metastasis are under the surveillance of the immune system. Animal studies demonstrated a key role for cell mediated immunity (e.g., NK, macrophages and cytotoxic T lymphocyte activity) in controlling metastasis (Brittenden et al., 1996). Tumour cells evade host defenses by the production of soluble factors that downregulate the function of lymphocytes, macrophages, and NK cells (Nelson and Nelson, 1987). In addition, the production of factors in abnormal amounts by tumour-bearing hosts may alter normal cytokine network and cause a deleterious imbalance of the immune system (Handel-Fernandez et al., 1997). NK cells are a subpopulation of lymphocytes that spontaneously recognize and kill virally infected cells as well as tumour cells during the metastatic process (Chomczynski and Sacchi, 1987). In our study, administration of *V. cinerea* and Vernolide-A could enhance the NK cell activity in C57BL/6 mice during metastatic condition.

Immunomodulation through natural substances may be considered as an alternative for the prevention and cure of neoplastic diseases. Immunomodulators have shown to augment specific cellular and humoral immune response (Duke, 1985). They may activate cytotoxic effector cells, such as cytotoxic T lymphocytes, natural killer (NK) lymphocytes, macrophages, and activated neutrophils (Fidler and Poste, 1985). Immunomodulators can regulate the cytokine production such as tumour necrosis factor, interleukins and interferons and these cytokines may, in turn
activate T-cells or Natural killer cells (NK cells). A number of plants used in traditional medicines have been shown to stimulate immune responses, and several active principles have been isolated and characterized from plants (Agarwal and Singh, 1999). Some of the plants with known immunomodulatory activity are *Viscum album* (Kuttan and Kuttan, 1992), *Tinospora cordifolia* (Mathew and Kuttan, 1999), *Withania somnifera* (Davis and Kuttan, 2000) and *Piper longum* (Sunila and Kuttan, 2004). *Vernonia cinerea* L. (Asteraceae) has many therapeutic uses in different traditional medicine of the world. Different parts of the plant are of different therapeutic values. Sesquiterpene lactones (SLs) are the active constituents of a variety of medicinal plants used in traditional medicine for the treatment of inflammatory diseases. Various SLs have been demonstrated to execute their anticancer capability via inhibition of inflammatory responses, prevention of metastasis and induction of apoptosis. All SLs contain a common functional structure, an α-methylene-γ-lactone group, and this important chemical characteristic means that the thiol-reactivity of SLs is an underlying mechanism responsible for their bioactivities (Zhang et al., 2005). Vernolide-A is a sesquiterpene lactone present in the plant *Vernonia cinerea* L. Administration of *V. cinerea* and Vernolide-A was found to increase the circulating antibody titer and antibody forming cells indicating its stimulatory effect on the humoral arm of immune system. 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 *V. cinerea* and Vernolide-A enhance the NK cell activity, ADCC and ACC in EAC and B16F-10 melanoma bearing animals.

Angiogenesis, the development of new blood vessels from the endothelium of a pre-existing vasculature, is a critical process required by most solid tumours to support their localized growth and metastatic dissemination within the host (Griffioen and Molema, 2000; Carmeliet and Jain, 2000). The process of angiogenesis consists of several steps, which include the stimulation of endothelial cells by growth factors such as VEGF, the subsequent degradation of the ECM by proteolytic enzymes such as MMPs, followed by invasion through ECM, migration and proliferation of endothelial cells and finally the formation of new capillary tubes (Carmeliet, 2000). VEGF, a survival factor for endothelial cells by inhibiting
apoptosis, is a key angiogenic factor frequently used by tumours and tissues to
switch on their angiogenic phenotypes. This potent and unique angiogenic protein
stimulates capillary formation, endothelial cell migration and proliferation as well as
increases vascular permeability (Ferrara, 1999). It was previously shown that VEGF
gene expression level is positively correlated with those of MMP-2 and MMP-9
(Munaut et al., 2003). Our studies demonstrated that the treatment with V.cinerea,
Vernolide-A, Perillic acid, Nomilin and Oleanolic acid significantly downregulated
both the production and expression of MMPs and VEGF.

The proinflammatory cytokines such as IL-1β, IL-6, TNF-α and GM-CSF act
as autocrine growth factors for tumour angiogenesis. These cytokines could be
prometastatic or proangiogenic and their deregulated expression directly correlate
with the metastatic potential of several human carcinomas (Isner and Asahara, 1993).
Moreover, altered levels of proinflammatory and proangiogenic factors are observed
in various forms of cancer (Chen et al., 1999). In this study, V.cinerea, Vernolide-A,
Perillic acid, Nomilin and Oleanolic acid treatment significantly reduced the elevated
levels of proinflammatory cytokines. IL-2 was the first cytokine used clinically for
treating cancer (Neville et al., 2001). It has been reported that IL-2 augment innate
immunity by stimulating natural killer cells and also promotes proliferation and
differentiation of helper T-cells, cytotoxic T-cells and B-cells (Caligiuri et al., 1993).
Administration of V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid
drastically enhanced the serum IL-2 level in angiogenesis induced animals,
compared to untreated control animals.

A decreased aerobic (hypoxic) condition is present in the majority of tumours
and is associated with poor prognosis due to the protection it affords to radiotherapy
and chemotherapy. Hypoxia in tumours induces the release of cytokines that promote
vascularization and thereby enhance tumour growth and metastasis. Recent major
advances have provided insight into the role hypoxia plays in cancer biology
(Brahimi-Horn et al., 2001). Hypoxia-inducible factor 1 (HIF-1) controls oxygen
delivery (via angiogenesis) and metabolic adaptation to hypoxia (via glycolysis).
HIF-1 consists of a constitutively expressed HIF-1β subunit and an oxygen- and
growth-factor-regulated HIF-1α subunit (Semenza, 2002). HIF-1α was found to be
present at high levels in human tumour specimens, and its levels were found to be
positively related to tumour progression, metastasis, and resistance to chemo/radiotherapy (Zhong et al., 1999; Birner et al., 2000). In addition, the effects of HIF-1α on tumour growth and angiogenesis have been demonstrated in xenograft tumour models (Ryan et al., 2000). Gene expression of HIF-1α was found to be downregulated by the treatment of vernolode-A.

Cancer is a disorder characterized by uncontrolled proliferation and reduced apoptosis. Apoptosis is a highly regulated and active form of cell death that is used to eliminate excess damaged or cancerous cells throughout life in a variety of organisms, thus maintaining normal development, tissue remodeling, and homeostasis. It is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). Two major pathways, extrinsic and intrinsic, have been identified for the induction of apoptosis (Sprick and Walczak, 2004). In the present study, the morphological analysis of B16F-10 melanoma cells treated with nontoxic concentrations of V. cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid showed the presence of apoptotic bodies, cytoplasmic shrinkage and nuclear condensation in a dose dependent manner.

The tumour suppressor gene, p53 was initially identified as the ‘guardian of the genome’ based on its ability to induce apoptosis. It is a key mediator of cell response to a variety of stresses, including DNA damage and abnormal growth regulation. Activated p53 initiates a cascade of events that result in either growth arrest at one of the cell cycle checkpoints or apoptosis leading to the elimination of genetically altered cells, thus exerting its tumour suppressor function (Fridman and Lowe, 2003). The Bcl-2 protein family is an important regulator of apoptosis, which consists of anti-apoptotic (such as Bcl-2) and pro-apoptotic members (such as Bax) (Farrow and Brown, 1996). The Bcl-2 family has been shown to be a p53 target. Bax, the pro-apoptotic member, is up-regulated in a number of systems during p53-mediated apoptosis (Martin and Elkon, 2004). Caspases are a family of cysteine proteases that play important roles in regulating apoptosis (Kohler et al., 2002). Activation of caspase-dependent signaling pathways are via cleavage of the procaspase-9 or -8 results in the downstream activation of caspase-3, finally leading to apoptosis (Slee et al., 1999). The expression patterns of p53, Bax and Bcl-2 were
studied and found that tumour suppressor, p53 and proapoptotic gene Bax were upregulated whereas antiapoptotic gene, Bcl-2 was downregulated in B16F-10 melanoma cells treated with *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid.

Caspases are cysteine proteases, plays a central role in the initiation and execution phases of apoptosis. Activation of caspases is recognized as a key element in the apoptotic process (Lorenzo and Susin, 2004). Upon activation, these enzymes cleave specific substrates and thereby mediate many of the typical biochemical and morphological changes in apoptotic cells, such as cell shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Kohler et al., 2002). Caspase-3 is the execution caspase in the DNA fragmentation process and other morphological changes associated with apoptosis (Cohen, 1997). Caspase-9 and caspase-8 are upstream caspases (initiator) whereas caspase -3 is downstream caspase (effector). In this study, we got clear upregulation of caspase-9 and caspase-3 expression in B16F-10 cells treated with *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid. Caspase- 8 did not show any expression in treated cells. From the above data it was clear that *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid induce apoptosis in B16F-10 melanoma cells via p53 activated Bax induced caspase-9 mediated intrinsic pathway not through caspase-8 mediated extrinsic pathway.

NF-κB is an important transcription factor in cell survival which plays a pivotal role in regulating inflammatory responses, cell growth/ differentiation and apoptosis and often found in various cancer cells. Pro-inflammatory cytokines such as TNF-α and IL-1β can induce the NF-κB cell survival signaling pathway by the phosphorylation and degradation of IκB followed by nuclear translocation of NF-κB and target gene expression (Kutuk and Basaga, 2004). Activation and nuclear translocation of transcription factors such as c-fos, ATF-2 and CREB-1 has been reported in many cancers (Mori et al., 1999; Huang et al., 1998; Angel and Karin, 1991). In the present study, we found that *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid could inhibit the activation or nuclear translocation of transcription factors such as NF-κB p65, NF-κB p50, NF-κB c-Rel, c-Fos, ATF-2, and CREB.
In conclusion, the results obtained in our study indicate the immunomodulatory and antimetastatic potential of \textit{V.cinerea} and Vernolide-A. Natural products such as \textit{V.cinerea}, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid inhibited tumour angiogenesis and also induced apoptosis in B16F-10 melanoma cells. Further studies should be conducted to trace the unknown molecular mechanisms involved in their action on cancer cells.