ABSTRACT
Cancer, one of the leading causes of death world-wide is a genetic disease that usually arises from the accumulation of several mutations. Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation. It is estimated that about 9 million new cancer cases are diagnosed every year and over 4.5 million people die from cancer each year in the world. Cancer rates in India are considerably lower than those in more developed countries such as the United States. Data from population based cancer registries in India show that the most frequently reported cancer sites in males are lung, oesophagus, stomach, and larynx. In females, cancers of the cervix, breast, ovary, and oesophagus are the most commonly encountered (WHO). The estimated number of new cancers in India per year is about 7 lakhs and over 3.5 lakhs people die of cancer each year.

The immune system is known to be involved in the etiology as well as pathophysiolgic mechanism of many diseases. Modulation of immune response is highly relevant in tumour cell destruction. One of the main reasons for the rapid progression of human cancers is the ability of tumour cells to escape from the immune surveillance mechanism of the body. Immunosuppression is one of the major obstacles in the conventional cancer treatment such as chemo and radiotherapy. The effect of _Vernonia cinerea_ L. and Vernolide-A on the immune system were studied using BALB/c mice. Intraperitoneal administration of _V.cinerea_ and Vernolide-A were found to enhance the total WBC count, bone marrow cellularity and number of α - esterase positive cells. Treatment of _V.cinerea_ and Vernolide-A was found to increase the circulating antibody titer and antibody forming cells indicating their stimulatory effect on the humoral arm of immune system. However, increased antibody titre remained several days thereafter indicating that the immunological activity could be sustained for several days. Moreover, _V.cinerea_ and Vernolide-A were found to stimulate the weight of lymphoid organs such as spleen and thymus indicating that _V.cinerea_ and Vernolide-A stimulated the production of immune cells. Mitogens are known to stimulate lymphocytes and to cause blastogenic transformation leading to their proliferation. Poke Weed mitogen (PWM) has been found to induce killer cells in human peripheral blood mononuclear cells, and its intraperitoneal injection prolongs the survival of tumour-bearing mice. Radioactive (³H)-thymidine incorporation assay clearly show a significantly enhanced proliferation of both thymocytes and splenocytes in the presence and
absence of specific mitogens *in vitro* and *in vivo*. All these observations clearly demonstrate the stimulatory effect of *V. cinerea* and Vernolide-A on immune system.

Cell mediated immune response plays a critical role in the elimination of tumours. Effect of *V. cinerea* and Vernolide-A on cell-mediated immune (CMI) response was studied in normal as well as tumour-bearing BALB/c mice. Administration of *V. cinerea* and Vernolide-A significantly enhanced natural killer (NK) cell activity in both normal as well as tumour-bearing animals, and it was observed earlier than in tumour-bearing control animals. Antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent complement-mediated cytotoxicity (ACC) were also enhanced significantly in both normal as well as tumour-bearing animals after *V. cinerea* and Vernolide-A administration compared with untreated control tumour-bearing animals. The level of cytokines such as Interleukin (IL)-2 and Interferon (IFN)-γ were also enhanced by the treatment of *V. cinerea* and Vernolide-A in both normal as well as tumour-bearing animals. This study demonstrated that *V. cinerea* and Vernolide-A stimulate the cell mediated immune responses such as Natural Killer Cell, Antibody-Dependent Cellular Cytotoxicity, and Antibody-Dependent Complement-Mediated Cytotoxicity through enhanced secretion of IL-2 and IFN-γ.

Metastasis is the major problem of treatment failure in cancer patients. Metastasis may also be controlled by immunosurveillance, and the process only succeeds when immune defenses were partially or completely abrogated. Metastatic cells have to perform a series of events to reach a distant site for establishing a new colony. If any agent can interrupt any of the steps involved in the metastatic cascade, the probability for its clinical trials will be promising. The effect of *V. cinerea* and Vernolide-A on the inhibition of lung metastasis induced by B16F-10 melanoma cells was studied in C57BL/6 mice. *V. cinerea* and Vernolide-A significantly inhibited lung tumour formation and significantly increased the life span. Moreover, lung collagen hydroxyproline, uronic acid, and hexosamine and also serum sialic acid, gamma GT, and VEGF levels were found to be significantly lower in treated animals compared to the untreated controls. Histopathological analysis of the lung tissues also correlated with these findings. *V. cinerea* and Vernolide-A treatment significantly inhibited the invasion of B16F-10 melanoma cells across the collagen matrix coated polycarbonate membrane placed in the Boyden chamber. *V. cinerea*
and Vernolide-A down regulated the production and expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and GM-CSF. *V.cinerea* and Vernolide-A administration could suppress or down regulate the expression of MMP-2, MMP-9, lysyl oxidase, prolyl hydroxylase, K-ras, ERK-1, ERK-2, and VEGF and also up regulate the expression of nm-23, TIMP-1 and TIMP-2 in the lung tissue of metastasis-induced animals. It also inhibited the protein expression of MMP-2 and MMP-9 in gelatin zymographic analysis of B16F-10 cells. These results indicate that *V.cinerea* and Vernolide-A could inhibit the metastatic progression of B16F-10 melanoma cells in C57BL/6 mice by regulating MMPs, VEGF, prolyl hydroxylase, lysyl oxidase, ERK-1, ERK-2, TIMPs, nm-23 and proinflammatory cytokine gene expression in metastatic lung tissue.

One of the major reasons for the rapid progression of cancers is the ability of tumour cells to escape from the immune surveillance mechanism of the body. Modulation of immune responses is highly relevant in tumour cell destruction. Effect of *V.cinerea* and Vernolide-A on the cell mediated immune (CMI) response in metastatic condition was studied using C57BL/6 mice model. Administration of *V.cinerea* and Vernolide-A enhanced natural killer (NK) cell activity, antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent complement mediated cytotoxicity (ACC) and the activity was observed in treated group much earlier compared to the metastatic tumour bearing control. Administration of *V.cinerea* and Vernolide-A significantly enhanced the production of IL-2 and TIMP-1 in metastatic tumour-bearing animals. In addition, *V.cinerea* and Vernolide-A significantly downregulated the serum levels of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and GM-CSF during metastasis. All these results demonstrate that *V.cinerea* and Vernolide-A could enhance the immune response against metastatic progression of B16F-10 melanoma cells in mice.

Angiogenesis is the development of new blood vessels from the pre-existing vascular beds, plays a pivotal role in tumour growth, invasion and metastasis. New blood vessel formation by angiogenesis involves the degradation of extracellular matrix combined with sprouting and migration of endothelial cells from preexisting capillaries. Inhibition of angiogenesis is currently perceived as one of the promising strategies in the treatment of cancer. We studied the antiangiogenic activity of *V.cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid using *in vivo* as
well as in vitro models. *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid significantly inhibited tumour directed capillary formation. The level of serum proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and GM-CSF, and also level of serum VEGF, a proangiogenic factor were found to be elevated in angiogenesis induced animals which were significantly reduced by the treatment of *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid in C57BL/6 mice. Administration of *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid significantly enhanced the production of antiangiogenic factors such as IL-2 and TIMP-1. In vitro studies using rat aortic ring assay showed that *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid at non-toxic concentrations significantly inhibited microvessel sprouting and also exhibited a significant inhibition in the proliferation, migration, and tube formation of endothelial cells, which are key events in the process of angiogenesis. *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid significantly inhibited the invasion of the collagen matrix by HUVECs in a dose dependent manner and also showed a inhibition in the activation of procollagenase to active collagenase of metalloproteinases. Taken together, these results demonstrate that *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid inhibits tumour-specific angiogenesis by downregulating the production of pro-angiogenic factors like pro-inflammatory cytokines, VEGF, and MMPs and also by upregulating the anti-angiogenic factors such as IL-2 and TIMP-1.

Radiation therapy is a widely used conventional treatment modality for local control of solid tumours. Hypoxia is an important phenomenon in the tumour microenvironment which plays a critical role in malignant tumour progression and treatment resistance. Hypoxic cancer cells are highly resistant to radiotherapy which is one of the major reasons for the treatment failure. The cellular response to hypoxia is mediated through the hypoxia inducible transcription factor-1 (HIF-1). HIF-1 is critically important for tumour progression and angiogenesis. The effect of Vernolide-A on the inhibition of radiation induced tumour angiogenesis was studied in C57BL/6 mice. Vernolide-A administration significantly reduced the tumour volume of radiation exposed mice. Serum VEGF level was drastically elevated during tumour progression and irradiation, which were significantly reduced by the treatment of Vernolide-A. Immunohistochemical analysis exhibited a reduced
vascular density by the treatment of Vernolide-A. ³H-thymidine incorporation assay and soft agar assay showed that Vernolide-A could inhibit the proliferation of B16F-10 melanoma cells in vitro along with radiation. Vernolide-A showed a significant inhibition in the invasion of irradiated B16F-10 melanoma cells across the collagen matrix. Vernolide-A inhibited the radiation induced gene expression of HIF-1α and VEGF in B16F-10 cells. It inhibited the VEGF receptor (Flk-1) expression in HUVECs. Gelatin zymographic analysis showed that Vernolide-A could inhibit the radiation induced activation of matrix metalloproteinases. Taken together, these results indicate that Vernolide-A inhibits radiation induced tumour angiogenesis by regulating HIF-1α, MMP-2, MMP-9 and VEGF.

Apoptosis, or programmed cell death, is an essential event that plays an important role in organism development and homeostasis. A tumour is a disease state characterized by proliferation disorder or apoptosis obstacle. Inducing apoptosis is an efficient method of treating cancers. There is abundant evidence that administration of naturally occurring compounds can induce apoptosis in various cancer cells. In this study we investigated the effect of V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid on the induction of apoptosis as well as its regulatory effect on the activation of transcription factors in B16F-10 melanoma cells. Treatment of B16F-10 cells with nontoxic concentration of V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid showed the presence of apoptotic bodies and induced DNA fragmentation in a dose depended manner. Cell cycle analysis and TUNEL assays also confirmed the observation. The proapoptotic genes p53, Bax, caspase-9 and caspase-3 found upregulated in V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid treated cells, whereas the antiapoptotic gene Bcl-2 was downregulated. The study also reveals that V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid treatment could inhibit the activation and nuclear translocation of p65, p50, and c-Rel subunits of nuclear factor-κB, and other transcription factors such as c-fos, activated transcription factor-2, and cyclic adenosine monophosphate response element-binding protein in B16F-10 melanoma cells. These results suggest that V.cinerea, Vernolide-A, Perillic acid, Nomilin and Oleanolic acid induces apoptosis via activation of p53 induced caspase-3 mediated pro-apoptotic signaling and suppression of NF-κB induced bcl-2 mediated survival signaling.
In summary, the present study demonstrates the immunomodulatory and antimetastatic potential of *Vernonia cinerea* and Vernolide-A. Vernolide-A is a potent sesquiterpene lactone from *Vernonia cinerea*. Natural products such as *V. cinerea*, Vernolide-A, Perillic acid, Nomilin and Oleanolic could induce cell cycle delay and apoptosis in B16F-10 melanoma cells. Administration of these compounds effectively blocked the tumour specific angiogenesis in the *in vivo* and *in vitro* models. The inhibition of NF-κB mediated activation of oncogenic and antiapoptotic pathways and regulation of MMP activation would be one of the possible mechanisms of action behind the antimetastatic, antiangiogenic and proapoptotic activities of these natural products.

*Key words: Immunomodulation, Metastasis, Angiogenesis, Hypoxia, Apoptosis, Proinflammatory cytokines, NF-κB, Vernonia cinerea L., Vernolide-A, Perillic acid, Nomilin and Oleanolic acid.*