Cancer is a group of diseases of higher multicellular organisms characterized by alterations in the expression of multiple genes, leading to deregulation of normal cellular program for cell division and differentiation. This results in an imbalance of cell replication and cell death that favours growth of a tumour cell population. Cancer incidence data generated by population-based cancer registries in India predicts that total cancer cases are likely to be 979,786 in the year 2010 (Takiar et al., 2010). The link with chronic inflammation and cancer has been recognized for certain cancers several decades earlier.

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases, including cancer (Gokhale et al., 2003). The principal function of the immune system is to provide protection against foreign invading pathogens. Targeted immune response is generated by the combined interaction of the cellular and humoral responses. Tumour growth can lead to the suppression of immunity; both systemically and in the microenvironment of the tumour.

Despite improvements in diagnosis, surgical techniques, local and systemic adjuvant therapies, most deaths from cancer result from the progressive growth of metastases that are resistant to conventional therapies. Metastasis is a multistep process in which cancer cells derived from the primary tumour migrate to regional or distant sites where they reinitiate their development (Chiang and Massague, 2008). The tumour cells along with adjacent stromal tissue secrete and activate various proteinases to mediate ECM degradation. The invasive and metastatic potential of cancer has been correlated in a number of studies with the activity of various proteinases.

Angiogenesis is a physiological process by which capillaries sprout from preexisting blood vessels. Neovascularisation is a requirement for solid tumour growth beyond 1–2 mm in diameter and play an important role by supplying nutrients and allowing metastatic spread (Cox et al., 2000). The angiogenic process commences when endothelial cells in the vessel wall are exposed to angiogenic factors from the surrounding tissue (Rak et al., 1993). The microvessel density within the tumour mass confers a poor prognosis in many solid tumours.
Important safeguards like DNA repair mechanisms and check-points exist within the cells to prevent replication of cells bearing mutations. As the final safeguard, there is apoptosis, causing death of defective cells and thus prevent mutations that cause cancer (Sellers and Fisher, 1999). Apoptosis is a terminal cell fate, a highly regulated “suicide” process that is distinct from necrosis. It is programmed, active, highly selective mechanism of cell death (Best et al., 1999). Apoptosis is performed by proteases; caspases and nucleases, activated by a family that includes positively acting Bax and negatively acting Bcl-2 proteins.

In current clinical scenario, radiation therapy alone is often used with curative intent for localized tumours, and is synergistic with chemotherapy in susceptible cancers. Unfortunately, this therapy is not tumour specific and has other serious side effects, most important of which is myelosuppression and there is probability of developing metastatic disease in distant organ sites (Lawrence et al., 2008). Chemotherapy is the most effective modality of managing metastasis of cancer cells. It is also a non specific mode of treatment with serious adverse effects like bone marrow suppression, mucositis and hair loss. Bone marrow myeloid cells are the first to be adversely affected by chemotherapy, leading to decline in the number of peripheral blood cells (DeVita, 2008). So the development of a new category of drugs that can inhibit the process of metastasis without much side effects, is a key issue because of the reality that cancer cells, which are resistant to current therapies will eventually dominate the cell population and cause the mortality.

Natural plant products have played a pivotal role in the health care of many cultures, both ancient and modern. According to WHO, 75% of people in developing countries still rely on plant-based traditional medicines for primary health care. Immunostimulation via natural substances is considered to be a most promising way for the prevention and cure of neoplastic diseases. *Aerva lanata* is an important medicinal plant belonging to the family *Amaranthaceae*. 10-Methoxycanthine-6-one (10-MC) is a β carboline alkaloid from the plant *Aerva lanata*. Thujone, a monoterpenoid ketone, occurs in nature as a mixture of α/β diastereoisomers, in the essential oils of Artemisia, Salvia, Thuja and Juniperus species.
OBJECTIVES OF THE PRESENT STUDY

1) To determine the anti-inflammatory and anti-tumour activities of *A. lanata*, 10-methoxycanthin-6-one and Thujone using *in vivo* and *in vitro* systems.

2) To determine the immunomodulatory effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone.

3) To determine the effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone on the inhibition of metastasis and to study the mechanism of its action using *in vivo* and *in vitro* systems.

4) To determine effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone on the regulation of cell cycling and inducing apoptosis in B16F-10 melanoma cells.

5) To determine the effect of the isolated compound, 10-methoxycanthin-6-one on the reversal of toxicity of current therapies – Radiation and chemo therapy.

METHODS AND RESULTS

The plant and plant products analysed in this study is given below. *Aerva lanata*, a common medicinal herb belonging to the family *Amaranthaceae*, was obtained from Amala Ayurveda Pharmacy, Thrissur, India. 10-Methoxycanthine-6-one (MC) is a β carboline alkaloid from the plant *Aerva lanata*. Thujone, a monoterpenoid ketone, was obtained from Sigma Aldrich (Bangalore, India)

*In vivo* toxicity of *A. lanata* ethanolic extract and its isolated compound 10-methoxycanthin-6-one and Thujone were analysed by 14-day short-term toxicity study using BALB/c mice. The non-toxic concentrations of *A. lanata*, 10-MC and Thujone were found to be 10mg/Kg body weight, 0.5mg/Kg body weight and 1mg/Kg body weight respectively and these non-toxic doses were used for further studies.

Anti-tumour activity of *A. lanata*, 10-MC and Thujone was assessed using *in vivo* and *in vitro* models. *In vivo* anti-tumour study was determined by studying the effect on tumour development in DLA induced solid tumour model and life span of EAC induced ascites tumour bearing mice. Animals treated with test compounds had significant reduction in tumour volume when compare to untreated control animals. TUNEL assay analysis of paraffin sections of solid tumour revealed that test compound treated animals have more apoptotic cells when compared to control
animals. The life span of ascites tumour bearing mice was also increased when they were treated with *A. lanata*, 10-MC and Thujone. The effect of test compounds on the viability of various cancer cells (L929, HeLa, KB, Jurkat, EL-4, K-562) and normal cells was assessed by MTT assay. The effect of non-toxic concentrations of *A. lanata* (5, 10 and 25 µg/ml), 10-MC (0.5, 1 and 2 µg/ml) and Thujone (2, 5 and 10 µg/ml) on tumour cell proliferation was analysed by ³H-thymidine incorporation assay. There was significant inhibition in the incorporation of radioactive thymidine to DNA of proliferating tumour cells, indicating reduction in the rate of proliferation.

The anti inflammatory activity of *A. lanata*, 10-MC and Thujone was studied using acute (carrageenan and dextran models) and chronic (formalin model) inflammation models by analysing paw thickness at various time points and serum levels of pro inflammatory cytokines. 10-MC produced maximum inhibition in both acute and chronic inflammation models followed by *A. lanata* and Thujone. Increase in the production of pro inflammatory cytokines (TNF-α, IL-1β and IL-6) and nitric oxide (NO) by lipopolysaccharide stimulated macrophages was normalized when they were treated with test compounds.

The immunomodulatory activity of *A. lanata*, 10-MC and Thujone was analysed using BALB/c mice. Treatment of animals with *A. lanata* (10mg/Kg body weight), 10-MC (0.5mg/Kg body weight) and Thujone (1mg/Kg body weight) was found to enhance the total WBC count, relative weight and cellularity of lymphoid organs, bone marrow cellularity and the number of α-esterase positive cells. Proliferation of spleenocytes, thymocytes and bone marrow cells from *A. lanata*, 10-MC and Thujone treated animals in the presence and absence of various mitogens were also studied. The effect of test compounds on the circulating antibody titre by haemagglutination method and number of antibody producing cells in spleen by Jern’s plaque assay were checked in BALB/c mice injected with SRBC as the antigen. There was significant enhancement in the levels of circulating antibody and the number of plaque forming cells in the spleen of test compound treated animals.

Effect of *A. lanata*, 10-MC and Thujone on the cell mediated immune response in normal and tumour bearing animals were analysed by determining the natural killer cell activity, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (ACC) and the generation of cytotoxic T lymphocytes (CTL). Administration of *A. lanata*, 10-MC and Thujone significantly
enhanced the NK cell activity, ADCC and ACC in normal and tumour-bearing animals. There was also a significant enhancement in the generation of CTL, which is evident by increase in life span of animals received tumour cells after Winn’s neutralization with the CTL generated in presence of test compounds.

Anti metastatic effect of A. lanata, 10-MC and Thujone was analysed using in vitro as well as in vivo model. For in vivo studies, lung metastasis was induced in C57BL/6 mice by injecting B16F-10 melanoma cells through lateral tail vein (Fidler, 1978; Liotta, 1986). Mice were sacrificed on 21st day after induction of metastasis. Treatment of metastatic tumour bearing animals with A. lanata, 10-MC and Thujone significantly reduced the number of lung tumour nodules and increased the life span. Moreover control animals had elevated levels of lung collagen hydroxyproline, uronic acid and hexosamine. The serum levels of sialic acid, gamma GT, and VEGF were found to be significantly lowered in test compound treated animals. A. lanata, 10-MC and Thujone also down regulated the production of pro inflammatory cytokines such as TNF-α, IL-1β, IL-6 and GM-CSF. Histopathological analysis of the lung tissues also correlated with these findings.

The gene expression profile of MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF, ERK-1, ERK-2, COX-2, iNOS, nm23, lysyl oxidase and prolyl hydroxylase were carried out by RT-PCR to investigate the molecular mechanism of anti metastatic activity of A. lanata, 10-MC and Thujone. Mice treated with test compounds had suppression or down regulation in the expression of lysyl oxidase, prolyl hydroxylase, iNOS, COX-2, MMP-2, MMP-9, ERK-1, ERK-2, and VEGF. There was up regulation in the expression of nm23 and TIMP in the lung tissue of A. lanata, 10-MC and Thujone treated mice.

Administration of 10-MC enhanced NK cell activity, ADCC and ACC in metastatic tumour bearing animal and the maximum activity was observed much earlier compared to the tumour bearing control. Administration of 10-MC also enhanced the production of IL-2 and IFN-γ in metastatic tumour- bearing animals.

In vitro anti metastatic activity was determined using B16F-10 melanoma cells. The effect of test compounds on the rate of proliferation of B16F-10 cells was analysed by 3H-thymidine incorporation assay. Inhibition of tumour cell motility, tumour cell adhesion to collagen matrix and invasion of Type I collagen coated polycarbonate membrane were determined using modified Boyden chamber. The effect of test compounds on the production of matrix metalloproteinase (MMP-2 and
MMP-9) was evaluated using conditioned medium from *A. lanata* (5, 10 and 25 μg/ml), 10-MC (0.5, 1 and 2 μg/ml) and Thujone (1, 2 and 5 μg/ml) treated B16F-10 melanoma cells by gelatin zymography (Billings *et al.*, 1991). Ethanolic extract of *A. lanata*, 10-MC and Thujone significantly inhibited tumour cell proliferation as well adhesion to collagen matrix. Treatment with test compounds significantly inhibited the migration and invasion of B16F-10 melanoma cells. There was a reduction in the protein expression of MMP-2 and MMP-9 in B16F-10 cells as evident by gelatin zymography.

Antiangiogenic activity of *A. lanata*, 10-MC and Thujone were analysed using *in vivo* and *in vitro* models. Treatment with test compounds was found to inhibit the tumour associated capillary formation in C57BL/6 mice induced by highly metastatic B16F-10 melanoma cells. The levels of serum NO, VEGF, TIMP-1, TNF-α, IL-1β, IL-2 and IL-6 were estimated using specific ELISA kits. Control group of animals had elevated levels of serum VEGF, NO and pro inflammatory cytokines, which were significantly reduced in test compound treated animals. The treatment with *A. lanata*, 10-MC and Thujone resulted in enhancement in serum TIMP-1 and IL-2 levels when compared to control group. *In vivo* vascular density during angiogenesis was determined by matrigel plug assay using anti-CD31 antibody (Akhtar *et al.*, 2002). Mean vascular density in *A. lanata*, 10-MC and Thujone treated animals was much lower than the control group.

The *in vitro* antiangiogenic activity of *A. lanata*, 10-MC and Thujone was determined by analysing microvessel outgrowth from rat aortic ring (Ng *et al.*, 2003) and experiments using isolated human umbilical vein endothelial cells (HUVECs). The effect of test compounds on the proliferation of HUVEC by ³H-thymidine assay and invasion of HUVEC using modified Boyden chamber were studied. The wound healing assay was conducted to study the effect of these compounds on inhibition of migration of HUVECs. The effect of *A. lanata*, 10-MC and Thujone on the tube formation (Gupta *et al.*, 2002) of HUVECs was also studied. The effect of test compound on the production of MMP-2 and MMP-9 was analysed by gelatin zymography.

Nontoxic concentrations of *A. lanata*, 10-MC and Thujone was found to inhibit the microvessel growth from rat aortic ring. HUVEC treated with test compounds was found to have significant inhibition in proliferation, migration, invasion and tube
formation in dose dependent manner. Gelatin zymographic analysis showed inhibition in MMP-2 and MMP-9 activity in cells treated with test compounds.

Effect of A. lanata, 10-MC and Thujone on induction of apoptosis in B16F-10 melanoma cell line was studied by analysing morphology of cells, DNA laddering pattern, cell cycling pattern and TUNEL assay. Treatment of B16F-10 cells with A. lanata (5, 10 and 25 µg/ml), 10-MC (0.5, 1 and 2 µg/ml) and Thujone (2, 5 and 10 µg/ml) resulted in the formation of apoptotic bodies and DNA fragmentation with a ladder pattern in agarose gel. TUNEL assay confirms that B16F-10 cells undergo apoptosis when treated with the test compounds. Cell cycle analysis reveals an increase in the sub-G0 population of cells with a decrease in G2-M phase cells in a time dependent manner, which confirmed the above said observation.

The expression of genes involved in regulation of cell cycle and apoptosis in B16F-10 melanoma cell was evaluated by RT-PCR method. The expression of pro apoptotic genes; p53, bax, caspase-9 and caspase-3 were found to be upregulated in A. lanata, 10-MC and Thujone treated cells, whereas the anti apoptotic gene Bcl-2 was downregulated. A. lanata, 10-MC and Thujone treatment showed downregulation of cyclin D1 gene expression and upregulated p21 and p27 gene expression in B16F-10 melanoma cells.; the genes which are involved in cell cycle regulation.

The effect of 10-MC and Thujone on the activation of NF-κB in B16F-10 melanoma cells was analysed using BD Mercury TransFactor kit. Treatment of tumour cells with 10-MC and Thujone was found to inhibit the activation and nuclear translocation of transcription factors such as NF-κB (p65, p50, and c-Rel subunits), c-fos, ATF-2 and CREB.

The effect of 10-MC on immunosuppression during radiation therapy and chemotherapy was analysed using in vivo system. Mice were treated with gamma radiation/cyclophosphamide along with the administration of 10-MC. Haematological parameters, relative weight of lymphoid organs, bone marrow cellularity and serum cytokine levels were monitored. Tumour bearing mice receiving gamma radiation/cyclophosphamide was found to have suppression of immune system indicated by reduction in total WBS count, relative weight and cellularity of thymus and spleen, bone marrow cellularity and the serum IL-2 levels. Treatment of mice with 10-MC was found to bring back these immune parameters to normal levels.
Augmentation of therapeutic benefits of radiation by 10-MC was studied using B16F-10 melanoma induced solid tumour model. There was significant reduction in tumour volume in 10-MC treated mice receiving a lower dose of radiation when compared to mice treated with 6G radiation alone. The elevated level of serum VEGF in 6G radiation-exposed mice was significantly lowered when animals were treated with 10-MC. The expression of VEGF, HIF-1α and COX-2 genes were found modulated by 10-MC treatment. Rate of proliferation and colony forming ability of B16F-10 melanoma cells treated with different doses of radiation was significantly lowered when they were treated with 10-MC.

In summary, the present study reveals the immunomodulatory activity of A. lanata, 10-Methoxyxanthin-6-one and Thujone; augmenting both humoral and cell mediated immune responses and also curtail the inflammatory responses. The test compounds also exhibited a significant anti metastatic potential in both in vivo and in vitro systems. A. lanata, 10-MC and Thujone could induce cell cycle delay and apoptosis in B16F-10 melanoma cells through p53 dependent mechanism; both may contribute to anti tumour and anti metastatic activities. Treatment with these compounds was found to effectively block the tumour specific angiogenesis; in vivo and in vitro. 10-MC was found to be having more immunomodulatory and anti metastatic potential. 10-MC treatment was found to reverse radiation therapy and chemotherapy induced immunosuppression and augment therapeutic efficacy of lower dose radiation therapy.
The thesis has been divided into 9 chapters as follows:

Chapter 1: Review of literature
Chapter 2: Material and methods
Chapter 3: Anti tumour and anti inflammatory activities of *Aerva lanata*, 10-Methoxycanthin-6-one and Thujone
Chapter 4: Effect of *Aerva lanata*, 10-Methoxycanthin-6-one and Thujone on immune system
Chapter 5: Anti metastatic activity of *Aerva lanata*, 10-Methoxycanthin-6-one and Thujone
Chapter 6: Effect of *Aerva lanata*, 10-Methoxycanthin-6-one and Thujone on tumour specific angiogenesis
Chapter 7: Effect of *Aerva lanata*, 10-Methoxycanthin-6-one and Thujone on the induction of apoptosis in tumour cells
Chapter 8: Effect of 10-Methoxycanthin-6-one on the reversal of toxicity of current therapies – Radiation therapy and Chemotherapy
Chapter 9: Summary and conclusion

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