CHAPTER 4
ANTIMETASTATIC ACTIVITY OF VERNONIA CINerea AND VERNOLIDE-A
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**4.4. DISCUSSION**
4.1. INTRODUCTION

Cancer is recognized worldwide to be a major health problem. Metastasis, a hallmark of cancer, is the primary cause of death for most cancer patients. It is the single most important obstacle to curative cancer therapy. Although there are several drugs available to encounter cancer growth in humans, there are no drugs presently available to specifically inhibit the metastasis of cancer cells. There is a cascade of events leading to the metastasis of tumours, where tumour cells disseminate from the primary tumour, migrate through the basement membrane, survive in the circulatory system, invade into a secondary site, and start to proliferate (Stafford et al., 2008; Steeg, 2003). Any drug which can inhibit one of the steps in the cascade will be useful in the inhibition of tumour metastasis. Cell mediated immunity (CMI) represents a major component of the host response against both intracellular pathogens and tumour cells (Burleson et al., 2010). Decreased function or dysfunction of innate immune cells, especially cytotoxic T lymphocytes and NK cells, in cancer patients receiving chemotherapy and radiotherapy are responsible for their reduced immunity (Tsavaris et al., 2002). Hence, activation of immune responses in cancer patients is a vital step in countering the threat imposed by conventional therapies as well as by the tumour itself.

The metastatic process is comprised of multiple events involving cell motility, cell invasion, surface adhesion properties, and degradation of extracellular matrix (ECM) (Stetler-Stevenson et al., 1993). Thus, degradation of the ECM and components of the basement membrane caused by a concerted action of proteinases, such as matrix metalloproteinases (MMPs) play a critical role in tumour invasion and metastasis (Westermark and Kahari, 1999). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are type IV collagenases that degrade basement membrane collagen (Salo et al., 1985). MMP activity is usually closely regulated by endogenous inhibitors. The best characterized of these are the tissue inhibitors of metalloproteinases or TIMPs. Their inhibitory activity results from the formation of a non-covalent complex of the TIMPs to the C-terminal domain of the pro MMPs (Kolkenbrock et al., 1991; Makimura et al., 1993). MMPs are also necessary for releasing proangiogenic factors like vascular endothelial growth factor (VEGF) (Rundhaug, 2005). VEGF is overexpressed by tumour cells and plays an important role in the process of neovascularization and metastasis (Carmeliet and Jain,
VEGF acts on endothelial cells to induce cell migration and proliferation (Li et al., 2003; Conway et al., 2001). Compounds that block or suppress the metastatic cascade have potential as anticancer agents. Therefore, it is essential to search for novel antimetastatic agents that are efficient and have minimum side effects.

Several inflammatory cytokines have been linked with carcinogenesis and tumour initiation, promotion, and metastasis, which suggests that inflammation, is associated with cancer development (Alex et al., 2002). Numerous studies have indicated that tumour cell exhibit an elevation in constitutive production of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and GM-CSF (Lazar-Moinar et al., 2000; Dong et al., 1999; Naldini and Carraro, 2005; Sparano et al., 2004). The secretion of lysyl oxidase and prolyl hydroxylase, two enzymes which favor collagen biosynthesis has been shown in various cancer cell lines (Takahashi et al., 2000; Kirschmann et al., 2002). The increased expression of lysyl oxidase and prolyl hydroxylase suffices to induce collagen accumulation and fibrosis (Akiri et al., 2003; Myllyharju, 2003).

Medicinal plants have a long history of use in the treatment of cancer (Hartwell, 1982). We have already reported the anti-metastatic activities of several herbal preparations (Leyon and Kuttan, 2004; Leyon et al., 2005; Thejass and Kuttan, 2005; Guruvayoorappan and Kuttan, 2008). Vernonia cinerea Less. (Asteraceae) has many therapeutic uses in different traditional medicines all over the world. Different parts of the plant are of different therapeutic values. Vernolide-A (C_{21}H_{28}O_{7}) is a sesquiterpene lactone present in the plant Vernonia cinerea L., (Asteraceae). Biological evaluation showed that Vernolide-A has potent cytotoxicity against human KB, DLD-1, NCI-661, and Hela tumour cell lines (Kuo et al., 2003). In the present study, the effect of V. cinerea and Vernolide-A on the inhibition of pulmonary metastasis induced by B16F-10 melanoma cells in C57BL/6 mice was evaluated with special emphasis on the mechanism of action using in vitro models.

4.2. MATERIALS AND METHODS

4.2.1. Animals

6-8 week old male C57BL/6 mice
4.2.2. Chemicals
DMEM with 10% FBS, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Hydroxyproline, Glucuronic acid lactone, N-acetyl neuraminic acid, Radioactive thymidine $[^3]$H, Highly specific quantitative sandwich ELISA kit for mouse VEGF were used for this study.

4.2.3. Cell lines
B16F-10 melanoma cells and K-562 cells.

4.2.4. Induction of metastasis
Metastasis was induced to animals by injecting B16F-10 melanoma cells ($10^6$ cells/animal) via the lateral tail vein (Fidler, 1978).

4.2.5. Administration of *V.cinerea* and Vernolide-A
*V.cinerea* and Vernolide-A were dissolved in minimum volume of ethanol and resuspended in 1% gum acacia and was given to animals intraperitoneally (i.p) at a concentration of 20mg/kg body weight and 0.5mg/kg body weight respectively. For *in vitro* studies 0.5, 1, and 2 μg/ml of *V.cinerea* and 0.01, 0.05, and 0.1 μg/ml of Vernolide-A were used.

4.2.6. Determination of the effect of *V.cinerea* and Vernolide-A on lung tumour nodule formation and survival rate of tumour-bearing animals
Metastasis was induced in three groups (14 animals/group) of C57BL/6 mice as described in chapter 2. Group 1 was kept as metastatic tumour bearing control. Group 2 and 3 were treated with *V.cinerea* and Vernolide-A respectively and continued for 10 consecutive days, starting from the same day of tumour inoculation. Eight animals from each group were sacrificed on the 21st day after tumour challenge, lungs were excised and blood was collected. Lungs were used for morphological examinations of metastatic tumour nodules and for the estimation of collagen hydroxyproline (Bergman and Loxley, 1940), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) contents. Serum was separated from the blood and used for determining the sialic acid (Skoza and Mohos, 1976), and γ-glutamyl transpeptidase (GGT) levels (Szasz, 1976). Serum VEGF was estimated by using highly specific ELISA kit purchased from R&D system, USA as per manufacturer’s protocol. A portion of the lung was used for histopathological
analysis. The rest of the six animals in each group were observed for their survival. The mortality of the animals was observed and the percentage increase in life span (%ILS) was calculated using the formula \(\%\text{ILS}=\frac{T-C}{C} \times 100\), where \(T\) represents the number of survival days of treated animals and \(C\) represents the number of survival days of control animals.

Histopathological analysis was carried out by fixing the whole lungs of both treated as well as untreated control animals in formaldehyde (10%), dehydrated using various concentrations of alcohol and embedded in paraffin wax. Sections (4µm) were stained with haematoxylin and eosin.

4.2.7. Determination of the effect of *V.cinerea* and Vernolide-A on serum cytokine and TIMP-1 production in metastatic tumour bearing animals

A similar set of experiments were set up as mentioned above and blood was collected from each animal on 21st day after B16F-10 transplantation. Serum was separated and used for the estimation of various cytokines such as IL-1β, IL-6, TNF-α, GM-CSF, IL-2 and TIMP-1 were assayed using respective ELISA kits as per manufacturer’s protocol.

4.2.8. Determination of the effect of *V.cinerea* and Vernolide-A on MMP-2, MMP-9, VEGF, Erk-1, Erk-2, k-ras, prolyl hydroxylase, lysyl oxidase, TNF-α, IL-1β, IL-6, TIMP-1, TIMP-2 and nm-23 gene expression profile in metastatic tumour bearing animals

Total RNA was isolated from the lungs, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Amplification was performed using specific primers of MMP-2, MMP-9, VEGF, Erk-1, Erk-2, k-ras, prolyl hydroxylase, lysyl oxidase, TNF-α, IL-1β, IL-6, TIMP-1, TIMP-2 and nm-23. The amplified products were electrophoresed on a 1.8% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

4.2.9. Determination of the effect of *V.cinerea* and Vernolide-A on cell mediated immune response in metastatic tumour bearing animals

Male C57BL/6 mice were divided into three groups (12 animals/group). Group I were kept as metastatic tumour bearing untreated control. Group II and III were treated with *V.cinerea* and Vernolide-A respectively for 5 consecutive days. Metastasis was induced to all the animals on 5th day of drug administration. Animals
were sacrificed at various time intervals, spleen and blood was collected and processed. Spleen cells were used as effector cells for assaying the NK-cell activity and ADCC by 4 h $^{51}$Cr-release assay as explained in chapter 2. Serum was used for assaying ACC by trypan blue exclusion method as described in chapter 2.

**IN VITRO ANTIMETASTATIC STUDIES**

4.2.10. Determination of cell viability by MTT assay

B16F-10 melanoma cells were seeded (5000 cells/well) in 96-well flat bottomed titre plate and incubated for 24 h at 37 °C in 5% CO$_2$ atmosphere. Different concentrations of *V. cinerea* (0.5–100 μg/ml) and Vernolide-A (0.01–10 μg/ml) were added and incubated further for 48 h. The cell viability was assessed by the MTT assay and percentage viability was calculated (Chapter 2).

4.2.11. Tumour cell proliferation assay

B16F-10 melanoma cells (5000 cells/well) were plated in a 96-well culture plate and incubated at 37 °C in 5% CO$_2$ atmosphere. After 24 h, various concentrations of *V. cinerea* (0.5, 1, and 2 μg/ml) and Vernolide-A (0.01, 0.05, 0.1 μg/ml) were added and further incubated for 48 h. $^3$H-thymidine was added to each well (1 μCi/well) and incubation was continued for additional 18 h. After completing incubation, the plates were centrifuged and the culture supernatant was removed, the cells were washed three times with PBS and then treated with ice cold PCA for 15 min. The resulting precipitate was dissolved in 0.5 N NaOH and was added to the scintillation fluid and kept overnight in the dark. The radioactivity was counted using a Rack Beta liquid scintillation counter.

4.2.12. Tumour cell adhesion assay

Tumour cell adhesion assay was carried out as explained in chapter 2. Briefly, B16F-10 melanoma cells were seeded on to Type I collagen coated wells of flat-bottomed titre plates, in the absence and presence of *V. cinerea* (0.5, 1, and 2 μg/ml) and Vernolide-A (0.01, 0.05, and 0.1 μg/ml) and incubated at 37°C for 5h. After incubation cells were washed, the adhering cells were fixed, stained and counted under microscope. Each experiment was done in triplicate.
4.2.13. Collagen matrix invasion assay

Tumour cell invasion assay was carried out in modified Boyden Chamber as described in chapter 2. Briefly, the lower compartment of the chamber was filled with serum free DMEM and polycarbonate filter of 8µm pore size was placed above this. Each filter was coated with 25µl of Type I collagen to form a thin continuous film on the top of the filter. B16F-10 melanoma cells (10⁵/150µl DMEM) were added to upper chamber and incubated at 37°C in 5% CO₂ for 10 h in the presence and absence of different concentrations of *V. cinerea* (0.5, 1, and 2 µg/ml) and Vernolide-A (0.01, 0.05, and 0.1 µg/ml). After incubation the cells on the lower surface of the membrane filter were fixed, stained and counted. The results are presented as percentage inhibition of invasion.

4.2.14. Tumour cell motility assay

Tumour cell motility assay was performed in the same manner as the invasion assay except that polycarbonate filters were of collagen free. *V. cinerea* (0.5, 1, and 2 µg/ml) and Vernolide-A (0.01, 0.05, and 0.1 µg/ml) were added along with B16F-10 melanoma cells to the upper compartment of the Boyden chamber. After incubation at 37°C for 24 h, the number of cells migrating to the lower chamber was determined using a haemocytometer. The results are expressed as percentage motility.

4.2.15. Gelatin zymography

Gelatin zymography was performed according to the procedure described in Chapter 2. After determining the protein concentration, supernatant containing the proteases from *V. cinerea* (0.5, 1, and 2 µg/ml) and Vernolide-A (0.01, 0.05, and 0.1 µg/ml) treated and untreated melanoma cells were subjected to zymographic analysis with or without trypsin activation. Gels were stained with Gelcode Blue stain reagent and clear digested area was visualized against the dark background.

**Statistical analysis**

Values were expressed as mean ± S.D. The statistical analysis was done by using one-way ANOVA followed by Dennett’s test.
4.3 RESULTS

4.3.1. Effect of *V. cinerea* and Vernolide-A on lung tumour nodule formation and survival rate of tumour-bearing animals

There was a significant reduction in lung tumour nodule formation when the animals were administered with *V. cinerea* or Vernolide-A compared to the untreated metastatic tumour bearing control animals (Fig 4.1). Metastatic tumour bearing untreated control animals developed massive number of lung nodules and assigned an arbitrary number of 250 (Liotta, 1986). When *V. cinerea* and Vernolide-A were administered simultaneously with metastatic induction, the number of metastatic tumour nodules was significantly inhibited by 78.8% and 89.39%, respectively (Table 4.1). The survival rate of the animals was also increased by the treatment of *V. cinerea* (72.5%) and Vernolide-A (88.51%)

4.3.2. Effect of *V. cinerea* and Vernolide-A on lung collagen hydroxyproline content

Effect of *V. cinerea* and Vernolide-A on lung collagen hydroxyproline content is shown in Table 4.2. In metastatic tumour bearing untreated control animals the lung hydroxyproline content was drastically elevated to 22.56 ± 2.41 μg/mg protein compared to normal level (1.42 ± 0.22 μg/mg protein). This elevated level was significantly reduced to 10.53 ± 1.4 μg/mg protein in *V. cinerea* treated animals and to 6.62 ± 0.82 μg/mg protein in Vernolide-A treated animals.

4.3.3. Effect of *V. cinerea* and Vernolide-A on lung uronic acid level

Effect of *V. cinerea* and Vernolide-A on lung uronic acid levels is shown in Table 4.2. The uronic acid level in the metastatic tumour bearing control animals was found to be 324.81 ± 12.8 μg/100mg tissue, whereas treatment with *V. cinerea* and Vernolide-A could significantly reduce the level to 112.61 ± 10.2 μg/100mg tissue and 88.56 ± 6.53 μg/100mg tissue respectively.

4.3.4. Effect of *V. cinerea* and Vernolide-A on lung hexosamine content

The effect of *V. cinerea* and Vernolide-A on lung hexosamine content is shown in Table 4.2. The hexosamine content in the normal animals was found to be 0.52 ± 0.14 mg/100mg lyophilized tissue. The level of hexosamine was highly elevated in untreated metastatic tumour bearing control animals (4.26 ± 0.49 mg/100mg.
Figure 4.1

Morphology of lungs of metastasis induced animals

(a) Normal
(b) Metastatic tumour bearing untreated control animal
(c) Metastatic tumour bearing animal treated with *V.cinerea* (20mg/kg body weight) simultaneously
(d) Metastatic tumour bearing animal treated with Vernolide-A (0.5mg/kg body weight) simultaneously
Table 4.1. Effect of *V. cinerea* and Vernolide-A on lung tumour nodule formation and survival rate of metastatic tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of tumour nodules</th>
<th>% inhibition of nodule formation</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour control</td>
<td>250#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>V. cinerea</em></td>
<td>52.82±4.5*</td>
<td>78.8</td>
<td>72.5</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td>26.51±4.5*</td>
<td>89.39</td>
<td>88.51</td>
</tr>
</tbody>
</table>

Metastasis was induced by injecting B16F-10 melanoma cells (1 x 10^6 cells) to all animals via lateral tail vein. *V. cinerea* and Vernolide-A were administered at a concentrations of 20mg/kg body weight and 0.5mg/kg body weight, respectively for 10 consecutive days. The control group was kept without any treatment. After 21 days, the lungs were dissected out and observed for metastatic tumour nodules. Values are expressed as mean ± SD.

#An arbitrary number of 250 are given for massive number of tumour nodules.

*p*<0.05
Table 4.2. Effect of *V. cinerea* and Vernolide-A on lung collagen hydroxyproline, lung uronic acid and lung hexosamine levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lung Collagen Hydroxyproline (μg/mg protein)</th>
<th>Lung Uronic acid (μg/100 mg tissue)</th>
<th>Lung Hexosamine (mg/100 mg lyophilized tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.42±0.22</td>
<td>36.60±4.82</td>
<td>0.52±0.14</td>
</tr>
<tr>
<td>Metastatic control</td>
<td>22.56±2.41</td>
<td>324.81±12.8</td>
<td>4.26±0.49</td>
</tr>
<tr>
<td><em>V. cinerea</em></td>
<td>10.53±1.4 *</td>
<td>112.61±10.2 *</td>
<td>1.43±0.38 *</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td>6.62±0.82 *</td>
<td>88.56±6.53 *</td>
<td>1.21±0.25 *</td>
</tr>
</tbody>
</table>

Metastasis was induced by injecting B16F-10 melanoma cells (1 x 10^6 cells) to C57BL/6 mice via lateral tail vein. *V. cinerea* and Vernolide-A were administered at a concentrations of 20mg/kg body weight and 0.5mg/kg body weight, respectively for 10 consecutive days. The control group was kept without any treatment. After 21 days, the lungs were dissected out and assayed different biochemical parameters. Values are expressed as mean ± SD. *p<0.05.
lyophilized tissue) which was reduced to $1.43 \pm 0.38 \text{ mg/100mg lyophilized tissue}$ and $1.21 \pm 0.25 \text{ mg/100mg lyophilized tissue}$ by treatment with *V.cinerea* and Vernolide-A respectively.

### 4.3.5. Effect of *V.cinerea* and Vernolide-A on serum sialic acid level

Metastatic tumour bearing control animals showed drastically elevated level of serum sialic acid ($112.52 \pm 8.31 \mu\text{g/ml serum}$) compared to normal ($24.83 \pm 2.64 \mu\text{g/ml serum}$). This was reduced to $41.26 \pm 3.51 \mu\text{g/ml serum}$ and $34.62 \pm 2.85 \mu\text{g/ml serum}$ by *V.cinerea* and Vernolide-A treatment (Table 4.3).

### 4.3.6. Effect of *V.cinerea* and Vernolide-A on serum γ-glutamyl transpeptidase (γ-GT)

The serum level of γ-GT in normal animals was found to be $26.43 \pm 2.38 \text{ nmol p-nitroaniline/ml}$, whereas the level was found to be increased in metastatic tumour bearing control animals ($115.61 \pm 6.14 \text{ nmol p-nitroaniline/ml}$). Administration of *V.cinerea* and Vernolide-A could reduce these elevated levels to $46.82 \pm 4.38 \text{ nmol p-nitroaniline/ml}$ and $38.62 \pm 2.54 \text{ nmol p-nitroaniline/ml}$ respectively (Table 4.3).

### 4.3.7. Effect of *V.cinerea* and Vernolide-A on serum VEGF level

Serum VEGF level is shown in the Table 4.3. The normal serum VEGF level was only $16.8 \pm 2.4 \text{ pg/ml}$. This was increased to $154.2 \pm 16.2 \text{ pg/ml}$ in metastatic tumour-bearing mice. This elevated level of serum VEGF was reduced significantly (p<0.05) in *V. cinerea* (81.92 ± 8.3 pg/ml) and Vernolide-A (75.3 ± 6.5 pg/ml) treated animals.

### 4.3.8. Effect of *V.cinerea* and Vernolide-A on proinflammatory cytokine production (IL-1β, IL-6, TNF-α and GM-CSF) in metastatic tumour bearing animals

Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in the serum of metastasis-induced animals showed a varying pattern during the period of study (Table 4.4.). The normal level of IL-1β was found to be $21.8 \pm 2.4 \text{ pg/ml}$. In metastatic tumour bearing control animals, the level of IL-1β was found to be elevated to $61.5 \pm 5.2 \text{ pg/ml}$ on the 21st day after tumour challenge. Administration of *V.cinerea* and Vernolide-A could significantly lower this level to $41.6 \pm 4.3 \text{ pg/ml}$ and $35.2 \pm 5.1 \text{ pg/ml}$ respectively. In control animals, the TNF-α level was
Table 4.3. Effect of *V. cinerea* and Vernolide-A on serum sialic acid, γ-glutamyl transpeptidase (γ-GT) and serum vascular endothelial growth factor (VEGF) level of metastatic tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum sialic acid (μg/ml serum)</th>
<th>Serum γ-GT (nmol p-nitroaniline/ml serum)</th>
<th>VEGF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24.83±2.64</td>
<td>26.43±2.38</td>
<td>16.8 ± 2.4</td>
</tr>
<tr>
<td>Metastatic control</td>
<td>112.52±8.31</td>
<td>115.61±6.14</td>
<td>154.2 ± 16.2</td>
</tr>
<tr>
<td><em>V. cinerea</em></td>
<td>41.26±3.51*</td>
<td>46.82±4.38*</td>
<td>81.92 ± 8.3*</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td>34.62±2.85*</td>
<td>38.62±2.54*</td>
<td>75.3 ± 6.5*</td>
</tr>
</tbody>
</table>

Metastasis was induced by injecting B16F-10 melanoma cells (1 x 10^6 cells) to C57BL/6 mice via lateral tail vein. *V. cinerea* and Vernolide-A were administered at a concentrations of 20mg/kg body weight and 0.5mg/kg body weight, respectively for 10 consecutive days. The control group was kept without any treatment. After 21 days, the animals were euthanized by decapitation, blood was collected and serum biochemical parameters were assessed. Values are expressed as mean ± SD. *p<0.05
Table 4.4. Effect of *V. cinerea* and Vernolide-A on proinflammatory cytokine production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokines (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1β</td>
</tr>
<tr>
<td>Normal</td>
<td>21.8 ± 2.4</td>
</tr>
<tr>
<td>Control</td>
<td>61.5 ± 5.2</td>
</tr>
<tr>
<td><em>V. cinerea</em></td>
<td>41.6 ± 4.3*</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td>35.2 ± 5.1*</td>
</tr>
</tbody>
</table>

Metastasis was induced by injecting B16F-10 melanoma cells (1 x 10⁶ cells) to C57BL/6 mice via lateral tail vein. *V. cinerea* and Vernolide-A were administered at a concentrations of 20mg/kg body weight and 0.5mg/kg body weight, respectively for 10 consecutive days. The control group was kept without any treatment. After 21 days, the animals were euthanized by decapitation, blood was collected and serum separated and used for the estimation of cytokine levels. Values are expressed as mean ± SD. *p<0.05
drastically elevated to 312 ± 32.5 pg/ml on the 21st day after tumour challenge when compared to normal level (22.6 ± 4.1 pg/ml). Administration of *V. cinerea* and Vernolide-A was found to effectively lower the elevated TNF-α level to 186 ± 21.3 pg/ml and 164 ± 26.6 pg/ml respectively.

Serum IL-6 level was found to be highly elevated to 481 ± 24.5 pg/ml in metastatic untreated control animals, which was significantly reduced to 264 ± 18.7 pg/ml and 241 ± 27.4 pg/ml in the *V. cinerea* and Vernolide-A-treated animals respectively (Table 4.4). Serum GM-CSF level showed an elevation in control animals (38.5 ± 3.6 pg/ml) from the normal value of 18.4 ± 2.3 pg/ml. Administration of *V. cinerea* and Vernolide-A was found to significantly reduce the elevated level of GM-CSF to 27.8 ± 3.2 pg/ml and 22.8 ± 4.3 pg/ml.

### 4.3.9. Effect of *V. cinerea* and Vernolide-A on serum IL-2 and Tissue inhibitors of metalloproteinase-1 (TIMP-1) level in metastatic tumour bearing animals

The effect of *V. cinerea* and Vernolide-A on serum IL-2 and TIMP-1 levels are shown in Table 4.5. The IL-2 levels in the serum after treatment with *V. cinerea* and Vernolide-A was found to be significantly enhanced to 34.62 ± 2.4 pg/ml and 42.53 ± 2.5 pg/ml compared to metastatic control animals (9.77 ± 1.1 pg/ml). Similarly the reduced level of serum TIMP-1 (463.71 ± 25 pg/ml) was found to be significantly increased after the administration of *V. cinerea* and Vernolide-A to 645.3 ± 43 pg/ml and 673.8 ± 48 pg/ml respectively.

### 4.3.10. Histopathological analysis of lung

The hematoxylin and eosin (H&E)-stained sections of lung tissues are shown in Fig 4.2 (100 X). In the control animals massive tumour growth and fibrosis reduced the alveolar space thereby reducing the vital capacity of the lung. Same areas were characterized by necrosis around the alveolar passages and bronchioles. Simultaneous administration of the *V. cinerea* and Vernolide-A showed a reduction in tumour mass around alveoli and pleura.

### 4.3.11. Effect of *V. cinerea* and Vernolide-A on MMP-2, MMP-9, VEGF, Erk-1, Erk-2, k-ras, prolyl hydroxylase, lysyl oxidase, TNF-α, IL-1β, IL-6, TIMP-1, TIMP-2 and nm-23 gene expression profile in metastatic lungs

The mRNA expression of prometastatic genes such as MMP-2, MMP-9, VEGF, Erk-1, Erk-2, k-ras, prolyl hydroxylase, lysyl oxidase, TNF-α, IL-1β and IL-6 genes were
Table 4.5. Effect of *V.cinerea* and Vernolide-A on serum IL-2 and Tissue inhibitors of metalloproteinase-1 (TIMP-1) level in metastatic tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (pg/ml)</th>
<th>TIMP-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>22.2±2.3</td>
<td>596±38</td>
</tr>
<tr>
<td>Control</td>
<td>9.77±1.1</td>
<td>463.7±25</td>
</tr>
<tr>
<td><em>V.cinerea</em></td>
<td>34.62±2.4*</td>
<td>645.3±43*</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td>42.53±2.5*</td>
<td>673.8±48*</td>
</tr>
</tbody>
</table>

Metastasis was induced by injecting B16F-10 melanoma cells (1 x 10⁶ cells) to C57BL/6 mice via lateral tail vein. *V.cinerea* and Vernolide-A were administered at a concentrations of 20mg/kg body weight and 0.5mg/kg body weight, respectively for 10 consecutive days. The control group was kept without any treatment. After 21 days, the animals were euthanized by decapitation, blood was collected and serum separated and the levels of IL-2 and TIMP-1 were estimated by ELISA method. All the values are mean ±SD. *p<0.05
**Figure 4.2**

**Histopathological analysis of lung of metastatic tumour bearing animals**

(a) Normal

(b) Metastatic tumour bearing untreated control animal

(c) Metastatic tumour bearing animal treated with *V.cinerea* (20mg/kg body weight) simultaneously

(d) Metastatic tumour bearing animal treated with Vernolide-A (0.5mg/kg body weight) simultaneously
Figure 4.2
found to be upregulated and also antimetastatic genes such as TIMP-1, TIMP-2 and nm-23 were found to be downregulated in the lung tissue of metastatic tumour control animals. Interestingly, the expressions of prometastatic genes were downregulated or inhibited and also the antimetastatic genes were upregulated in the lung tissue of mice treated with *V.cinerea* and Vernolide-A (Fig 4.3).

4.3.12. Effect of *V.cinerea* and Vernolide-A on cell mediated immune responses in metastatic tumour bearing animals

**Natural killer cell activity**

As shown in Fig.4.4 treatment with *V. cinerea* as well as Vernolide-A significantly enhanced the NK cell activity and it was observed much earlier compared to untreated metastatic tumour bearing control animals. Maximum NK cell mediated target cell lysis was observed on 5th day by the administration of *V. cinerea* (32.52% cell lysis) and Vernolide-A (38.42% cell lysis). While in metastatic tumour bearing untreated control animals the maximum NK cell activity was observed only on day 9 and it was 10.41% cell lysis.

**Antibody-dependent cellular cytotoxicity**

Administration of *V. cinerea* and Vernolide-A produced significant enhancement in the ADCC activity. *V. cinerea* and Vernolide-A treated animals showed maximum ADCC on 9th day with maximum target cell lysis of 32.21% and 37.62% respectively, whereas in untreated metastatic tumour bearing control animals maximum ADCC was observed only on 15th day (13.23% cell lysis) (Fig.4.5).

**Antibody-dependent complement-mediated cytotoxicity**

Significant enhancement in ACC was observed in *V. cinerea* and Vernolide-A treated metastatic tumour bearing animals. Maximum ACC was observed in *V. cinerea* (21% cell lysis) and Vernolide-A (23% cell lysis) treated animals on day 15, while in untreated metastatic tumour bearing control animals maximum ACC activity was 14% cell lysis which was on day 17 (Fig.4.6).

**IN VITRO ANTIMETASTATIC STUDIES**

4.3.13. Effect of *V.cinerea* and Vernolide-A on the cell viability by MTT assay

Cytotoxicity of *V. cinerea* and Vernolide-A towards B16F-10 melanoma cells in culture is shown in Table 4.6. *V. cinerea* and Vernolide-A at concentrations of 0.5–2
Figure 4.3

Effect of *V. cinerea* and Vernolide-A on gene expression in metastatic tumour bearing animals

(a) MMP-2:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(b) MMP-9:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(c) VEGF:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(d) Erk-1:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(e) Erk-2:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(f) k-ras:  Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V.cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH
Figure 4.3

(g) Prolyl hydroxylase:
Lane 1: Molecular weight marker
Lane 2: Untreated metastatic tumour bearing control
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)
Lane 5: GAPDH

(h) Lysyl oxidase:
Lane 1: Molecular weight marker
Lane 2: Untreated metastatic tumour bearing control
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)
Lane 5: GAPDH

(i) TNF-α:
Lane 1: Molecular weight marker
Lane 2: Untreated metastatic tumour bearing control
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)
Lane 5: GAPDH

(j) IL-1β:
Lane 1: Molecular weight marker
Lane 2: Untreated metastatic tumour bearing control
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)
Lane 5: GAPDH

(k) IL-6:
Lane 1: Molecular weight marker
Lane 2: Untreated metastatic tumour bearing control
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)
Lane 5: GAPDH
Figure 4.3

(l) TIMP-1  
Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(m) TIMP-2  
Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH

(n) nm-23  
Lane 1: Molecular weight marker  
Lane 2: Untreated metastatic tumour bearing control  
Lane 3: Treated with *V. cinerea* (20mg/kg body weight)  
Lane 4: Treated with Vernolide-A (0.5mg/ kg body weight)  
Lane 5: GAPDH
Figure 4.3

l) 527bp 414bp
m) 527bp 525bp
n) 527bp 310bp
Figure 4.4

Effect of *V. cinerea* and Vernolide-A on NK cell activity

Male C57BL/6 mice were divided into three groups (12 animals/group). Group I were kept as metastatic tumour bearing untreated control. Group II and III were treated with *V.cinerea* and Vernolide-A respectively for 5 consecutive days. Metastasis was induced to all the animals on 5th day of drug administration. Animals were sacrificed at different time points, Spleen was processed to get single cell suspension and was used as effector cells to determine NK cell activity by $^{51}$Cr-release assay.
Figure 4.4.
Figure 4.5

Effect of *V. cinerea* and Vernolide-A on ADCC

Male C57BL/6 mice were divided into three groups (12 animals/group). Group I were kept as metastatic tumour bearing untreated control. Group II and III were treated with *V. cinerea* and Vernolide-A respectively for 5 consecutive days. Metastasis was induced to all the animals on 5th day of drug administration. Animals were sacrificed at different time points, Spleen was processed to get single cell suspension and was used as effector cells to determine ADCC by $^{51}$Cr-release assay.
Figure 4.5

Graph showing the percentage of cell lysis over the number of days for three different conditions: Metastatic Control, Vernolide-A, and V.cinerea. The graph indicates a peak in cell lysis at around day 9 for the Metastatic Control and day 11 for Vernolide-A and V.cinerea, respectively.
Effect of *V. cinerea* and Vernolide-A on ACC

Male C57BL/6 mice were divided into three groups (12 animals/group). Group I were kept as metastatic tumour bearing untreated control. Group II and III were treated with *V. cinerea* and Vernolide-A respectively for 5 consecutive days. Metastasis was induced to all the animals on 5th day of drug administration. Animals were sacrificed at different time points, blood was collected; serum was separated and incubated with B16F-10 cells (1x10^4 cells/100μl) and complement (50 μl) at 37°C for 3 hr. The cytotoxicity was assessed by trypan blue exclusion method.
Figure 4.6

The chart shows the percentage of cell lysis over the number of days for different treatments:

- **Metastatic Control**
- **Vernolide-A**
- **V. cinerea**

The y-axis represents the percentage of cell lysis, ranging from 0% to 30%. The x-axis represents the number of days, ranging from 1 to 21. The graph indicates that Vernolide-A and V. cinerea show a higher percentage of cell lysis compared to the Metastatic Control.
Table 4.6. Effect of *V.cinerea* and Vernolide-A on induction of cytotoxicity in B16F-10 melanoma cells

<table>
<thead>
<tr>
<th>Concentrations (μg/ml)</th>
<th>% of viable cells</th>
<th>% of dead cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V.cinerea</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>18</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>61</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td><strong>Vernolide-A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>0.5</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

B16-F10 melanoma cells (5000 cells/well) were plated in a 96 well titer plate with different concentrations of *V.cinerea* and Vernolide-A and incubated for 48 h. Percentage of viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay).
μg/ml and 0.01–0.1 μg/ml were found to be non-toxic to B16F-10 melanoma cells respectively, and these concentrations were used for further in vitro experiments.

### 4.3.14. Effect of *V. cinerea* and Vernolide-A on tumour cell proliferation

The effect on the proliferation of B16F-10 melanoma cells by *V. cinerea* and Vernolide-A showed a dose dependent inhibition in the cell proliferation which is presented in Table 4.7. B16F-10 melanoma cells when grown alone, it incorporate more ³H-thymidine and had very high cpm (4652.6 ± 262) while in the presence of *V. cinerea* at a concentration of 0.5μg/ml, 1μg/ml and 2μg/ml produced 3495.7 ± 236 cpm (24.86%), 2561.8 ± 206 cpm (44.93%) and 1884.7 ± 216 cpm (59.49%), respectively. Similarly, presence of Vernolide-A at a concentration of 0.1μg/ml significantly reduces the proliferation of B16F-10 melanoma cells by 64.84% (1635.4 ± 184 cpm). Again, the lower concentrations of Vernolide-A, 0.05μg/ml (2475.3 ± 175 cpm, 46.79%) and 0.01μg/ml (3528.4 ± 218 cpm, 24.16%) also showed considerable inhibition in the B16F-10 cell proliferation.

### 4.3.15. Effect of *V. cinerea* and Vernolide-A on the adhesion of B16F-10 melanoma cells to the collagen matrix

The effect of *V. cinerea* and Vernolide-A on the adhesion of B16F-10 melanoma cells to collagen matrix is given in Table 4.8. There was a dose dependent inhibition of tumour cell adhesion. *V. cinerea* at concentration of 0.5μg/ml, 1μg/ml and 2μg/ml could inhibit the tumour cell adhesion by 8.2%, 18.5% and 31.5% respectively. For Vernolide-A the percentage inhibition of tumour cell adhesion was 12.3%, 21.4% and 34.8% at a concentration of 0.01μg/ml, 0.05μg/ml and 0.1μg/ml respectively.

### 4.3.16. Effect of *V. cinerea* and Vernolide-A on the invasion of B16F-10 melanoma cells to the collagen matrix

Invasion of B16F-10 melanoma cells through collagen matrix was significantly inhibited by *V. cinerea* and Vernolide-A in a dose dependent manner (Fig.4.7). Large number of tumour cells on the lower side of the membrane indicates high invasive property of untreated B16F-10 cells (Fig.4.7a). The number of invaded cells was reduced in the presence of *V. cinerea* and Vernolide-A. *V. cinerea* at a concentration of 2μg/ml significantly inhibited the tumour cell invasion by 82.52% (Fig.4.7d) where as 1μg/ml and 0.5μg/ml produced an inhibition of 68.61% (Fig.4.7c) and 26.83% (Fig.4.7b), respectively. B16F-10 melanoma cells showed 88.62%, 72.93%
Table 4.7. Effect of *V. cinerea* and Vernolide-A on proliferation of B16F-10 melanoma cells by $^3$H-thymidine uptake assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactive count/minute (CPM)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16F-10 alone</td>
<td>4652.6±262</td>
<td>–</td>
</tr>
<tr>
<td><em>V. cinerea</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 µg</td>
<td>3495.7±236*</td>
<td>24.86</td>
</tr>
<tr>
<td>1 µg</td>
<td>2561.8±206*</td>
<td>44.93</td>
</tr>
<tr>
<td>2 µg</td>
<td>1884.7±216*</td>
<td>59.49</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01 µg</td>
<td>3528.4±218*</td>
<td>24.16</td>
</tr>
<tr>
<td>0.05 µg</td>
<td>2475.3±175*</td>
<td>46.79</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>1635.4±184*</td>
<td>64.84</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells ($5 \times 10^3$ cells/well) were grown in 96 well flat bottom plate. After 24 h various concentrations of *V. cinerea* and Vernolide-A were added and incubation was continued for 48 h. After incubation, $^3$H-thymidine was added to each well (1 µCi/well) and incubation was continued for 18 h. Cells were lysed and radioactivity was counted by using Rack Beta liquid scintillation counter. Values are mean±S.D. *p<0.05.
Table 4.8. Effect of *V.cinerea* and Vernolide-A on the adhesion of B16F-10 melanoma cells to the collagen matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition of adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V.cinerea</em></td>
<td></td>
</tr>
<tr>
<td>0.5 μg</td>
<td>8.2</td>
</tr>
<tr>
<td>1 μg</td>
<td>18.5</td>
</tr>
<tr>
<td>2 μg</td>
<td>31.5</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td></td>
</tr>
<tr>
<td>0.01 μg</td>
<td>12.3</td>
</tr>
<tr>
<td>0.05 μg</td>
<td>21.4</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>34.8</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1x10^5 cells /ml) were seeded into collagen Type-I coated wells of flat bottomed titre plates and incubated in presence of different concentrations of *V.cinerea* and Vernolide-A for 4 h at 37°C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.
Figure 4.7

Effect of *V. cinerea* and Vernolide-A on the collagen matrix invasion

(a) Untreated B16F-10 cells

(b) B16F-10 cells treated with *V. cinerea* (0.5 μg/ml)

(c) B16F-10 cells treated with *V. cinerea* (1 μg/ml)

(d) B16F-10 cells treated with *V. cinerea* (2 μg/ml)

(e) B16F-10 cells treated with Vernolide-A (0.01 μg/ml)

(f) B16F-10 cells treated with Vernolide-A (0.05 μg/ml)

(g) B16F-10 cells treated with Vernolide-A (0.1 μg/ml)
and 31.42% inhibition of invasion when they were treated with Vernolide-A at 0.1μg/ml (Fig.4.7g), 0.05μg/ml (Fig.4.7f) and 0.01μg/ml (Fig.4.7e) concentrations, respectively (Table 4.9).

4.3.17. Effect of V. cinerea and Vernolide-A on the motility of B16F-10 melanoma cells to the collagen matrix

V. cinerea and Vernolide-A inhibited the motility of tumour cells across the polycarbonate filters in a dose dependent manner and the results are presented in Table 4.10. In the presence of 2μg/ml, 1μg/ml and 0.5μg/ml of V. cinerea, B16F-10 melanoma cells showed 65.4%, 37.9% and 18.2% inhibition on tumour cell motility whereas Vernolide-A could inhibit the tumour cell motility by 72.6%, 48.2% and 21.5% at concentrations of 0.1μg/ml, 0.05μg/ml and 0.01μg/ml respectively.

4.3.18. Gelatin zymographic analysis

Gelatin zymographic analysis shows that V. cinerea and Vernolide-A inhibited the gelatinase, MMP-2 and MMP-9 production in B16F-10 melanoma cells (Fig.4.8). Trypsin activated culture supernatant of untreated B16F-10 melanoma cell, after zymography showed clear area of digestion at the region of MMP-2 and MMP-9 (92kD and 72kD). When the same sample loaded gel was incubated in 10mM EDTA, did not show any clear digested area confirming that the enzyme responsible for the digestion of gelatin in the gel is a metalloproteinase. Untreated B16F-10 culture supernatant without trypsin activation, also did not show clear digested area indicating that the enzyme is produced as zymogen. The conditioned medium of V. cinerea (2μg/ml and 1μg/ml) and Vernolide-A (0.1 μg/ml and 0.05μg/ml) pretreated B16F-10 melanoma cells, which was then activated with trypsin, did not show any clear area of digestion indicating that V. cinerea and Vernolide-A inhibited the production of MMP-2 and MMP-9 in B16F-10 melanoma cells.

4.4 DISCUSSION

The essential characteristics of cancer cells are the ability to invade surrounding tissues and metastasize to regional and remote sites. Metastases are the cause of 90% of human cancer deaths. It is not a passive process but involves strict up and down regulations in the expression of specific genes. Metastasis is an exceedingly complex process, which occurs through a series of sequential steps that include the invasion of adjacent tissues, intravasation, transport through the circulatory system, and arrest at
Table 4.9. Effect of *V. cinerea* and Vernolide-A on invasion of B16F-10 melanoma cells through the collagen matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition of invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cinerea</em></td>
<td></td>
</tr>
<tr>
<td>0.5 μg</td>
<td>26.83</td>
</tr>
<tr>
<td>1 μg</td>
<td>68.61</td>
</tr>
<tr>
<td>2 μg</td>
<td>82.52</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td></td>
</tr>
<tr>
<td>0.01 μg</td>
<td>31.42</td>
</tr>
<tr>
<td>0.05 μg</td>
<td>72.93</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>88.62</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1x10^5 cells /150μl DMEM) were seeded collagen Type-I coated polycarbonate membrane on the upper compartment of the chamber. The lower compartment was filled with serum free DMEM. Cells were incubated in presence of different concentrations of *V. cinerea* and Vernolide-A for 10 h at 37°C. Membranes were removed, fixed, stained and the cells that had migrated in the test and control were counted.
Table 4.10. Effect of *V.cinerea* and Vernolide-A on the motility of B16F-10 melanoma cells to the collagen matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition of motility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V.cinerea</em></td>
<td></td>
</tr>
<tr>
<td>0.5 μg</td>
<td>18.2</td>
</tr>
<tr>
<td>1 μg</td>
<td>37.9</td>
</tr>
<tr>
<td>2 μg</td>
<td>65.4</td>
</tr>
<tr>
<td>Vernolide-A</td>
<td></td>
</tr>
<tr>
<td>0.01 μg</td>
<td>21.5</td>
</tr>
<tr>
<td>0.05 μg</td>
<td>48.2</td>
</tr>
<tr>
<td>0.1 μg</td>
<td>72.6</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1x10^5 cells /ml) were seeded into Collagen Type-I coated wells of flat bottomed titre plates and incubated in presence of different concentrations of *V.cinerea* and Vernolide-A for 4h at 37°C. Adhering cells were fixed with 5% formaldehyde, stained with crystal violet and counted.
Figure 4.8

Effect of *V. cinerea* and Vernolide-A on MMP-2 and MMP-9 production by B16F-10 melanoma cells

(1) Condition medium from untreated B16F-10 melanoma cells without trypsin activation.
(2) Condition medium from untreated B16F-10 melanoma cells after trypsin activation.
(3) Condition medium from untreated B16F-10 melanoma cells after trypsin activation+EDTA.
(4) Condition medium from pretreated B16F-10 melanoma cells (2µg/ml *V.cinerea*) after trypsin activation.
(5) Condition medium from pretreated B16F-10 melanoma cells (1µg/ml *V.cinerea*) after trypsin activation.
(6) Condition medium from pretreated B16F-10 melanoma cells (0.1µg/ml Vernolide-A) after trypsin activation.
(7) Condition medium from pretreated B16F-10 melanoma cells (0.05µg/ml Vernolide-A) after trypsin activation.
Figure 4.8
a secondary site, extravasation and growth in a secondary organ (Mehlen and Puisieux, 2006).

Therapeutic treatment modalities of chemotherapy, radiotherapy and surgery, while effective for some patients with solid malignancies, cannot be successfully used for others with progressive outgrowth of metastasis (Jain, 1991). Highly malignant tumour cell subpopulations are often resistant to therapy with chemotherapeutic, cytoreductive anticancer drugs (Heppner, 1986; Poste, 1986). Cisplatin and other drugs used in conventional cancer chemotherapy are limited by their serious side effects of nephrotoxicity and peripheral neuropathy. The key factor for toxicities is immune suppression. Due to these acute toxicities it has become necessary to develop second-generation drugs, which are equally effective but less toxic (Bruce, 1993). The use of natural products against metastasis is less toxic and more effective. Natural herbal medicines have been used clinically to treat cancer patients, or they have been used as adjuvant therapy, because they have minimal side effects and can also decrease the side effects of chemotherapy and/or radiotherapy (Zee-Cheng, 1992).

In the present study, we analyzed the anti-metastatic activity of *V. cinerea* and Vernolide-A and their mechanism of action. B16F-10 melanoma cells are highly metastatic and form colonies of tumour nodules in the lungs when administered through tail vein, which in turn promote lung fibrosis and collagen deposition. The methanolic extract of *V. cinerea* and its isolated compound, Vernolide-A has been found to have an effective inhibition on tumour nodule formation. Tumour nodules are the metastatic colonies of B16F-10 melanoma cells, and they promote the lung fibrosis and collagen deposition in the lung. This inhibition of tumour nodules correlates with an increase in the life span of the metastatic tumour-bearing animals.

The state of lung fibrosis was evaluated by estimating the lung collagen hydroxyproline content, because during lung fibrosis collagen is deposited massively in the alveolus of lungs. A part of collagen (15-30%) is hydroxyproline (Voet and Voet, 1995) which results in the reduction of pulmonary function. In the *V. cinerea* and Vernolide-A treated groups there were a significant reduction in the lung collagen hydroxyproline content than the control tumour-bearing animals. In the tumour cells, oxidation of the primary alcohol group of aldoses of sugar derivatives occur, yielding uronic acid. This uronic acid leads to the formation of glucuronic
acid lactone, which is an esterified form of uronic acid. In the presence of glucuronic acid lactone, propyl hydroxylase enzyme converts the prohydroxyproline to hydroxyproline. Glucuronic acid lactone also activates fiber formation from collagen during fibrosis. Hexosamine is a significant compound in lung tumour cells. It plays an important role in the synthesis of N-acetyl neuraminic acid (sialic acid), which is a component of glycolipids present on the surface of tumour cells (Voet and Voet, 1995). Treatment with *V. cinerea* and Vernolide-A could reduce the amount of uronic acid, hexosamine and sialic acid in tumour bearing animals indicating its inhibitory effect on metastasis.

Metastatic property of tumour cells highly upregulated with the elevated levels of sialic acid content (Fernandez et al., 1991). GGT a marker of cellular proliferation was increased in the serum of tumour-bearing animals compared with its level in normal animals. GSH is synthesized intracellularly and provides energy to the tumour cells by GGT. GGT catalyzes this GSH breakdown (Clapper and Siarka, 1998). Both sialic acid and GGT are direct markers of proliferative, metastatic cells. The treatment of *V. cinerea* and Vernolide-A showed significant reduction in serum sialic acid and γ-glutamyl transpeptidase content indicating decreased incidence of tumour cell proliferation and metastasis.

The antibody-dependent cellular cytotoxicity (ADCC) is a well-recognized immune effector mechanism in which antigen-specific antibodies direct immune effector cells of the innate immunity to the killing of the antigen expressing cancer cells (Ahmad and Menezes, 1996). NK cells are a type of lymphocytes and are part of the first line of innate defense against cancer cells and virus-infected cells (Moretta et al., 2001). They are large granular lymphocytes, distinguishable from T or B lymphocytes by their surface phenotype, cytokine profile, and the ability to mediate spontaneous cytotoxicity against a broad range of targets (Trinchieri, 1989). NK cells circulate in the blood, where they account for about 5–15% of circulating lymphocytes (Whiteside and Herberman, 1994). NK cells constitute the principal ADCC effector cells. They can react against and destroy target cell without prior sensitization to it. Lytic activity of NK cells is enhanced by interferons and interleukin 2 (IL-2) (Moore, 1985). Our study shows that administration of *V. cinerea* and Vernolide-A could upregulate the IL-2 production as well as stimulate NK cell activity resulting enhancement of ADCC against B16F-10 melanoma cells.
in mice. Complement is a system of plasma proteins that can be activated by antibody leading to a cascade of reactions that occurs on the surface of pathogens and generates active components with various effector functions. Complement proteins are responsible for cell lysis and mediation of inflammation and enhanced phagocytosis. The enhancement of ACC activity in *V. cinerea* and Vernolide-A treated C57BL/6 mice compared to the control during metastatic condition shows the effect of *V. cinerea* and Vernolide-A on the stimulation of complement proteins.

Cytokines are large family of soluble proteins, which serve as mediators of immune responses participating in the communication and regulation of inflammatory responses (Cerami, 1992). Several inflammatory cytokines have been linked with tumourigenesis, which suggests that inflammation is associated with cancer development (Lazar-Moinar et al., 2000). Numerous studies have indicated that tumour cells exhibit an elevation in constitutive production of the proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and GM-CSF. More recently, some cytokines have also been implicated as putative mediators of some of metastasis (Andela et al., 2000; Zerbini et al., 2003). The basis for constitutive expression of these cytokines during tumour progression is unknown. Treatment with *V. cinerea* and Vernolide-A significantly down regulated the production and expression of these proinflammatory cytokines. Interleukin-2 (IL-2) has many immunopotentiating effects, such as proliferation of T cells, B cells, NK cells and monocytes, augmentation of cytotoxicities of T cells and NK cells which exhibit high cytolytic activities against tumour cells. Although the immunomodulating property of IL-2 promoted their use in the treatment of cancer patients, their diverse side effects, made limitations in their use (Ognibene et al., 1988; Rosenberg et al., 1994). Administration of *V. cinerea* and Vernolide-A enhanced the production of serum IL-2 in metastatic tumour bearing mice compared to untreated metastatic control.

Matrix metalloproteinases are a family of zinc dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix and thereby up regulates invasion and metastasis (Stetler-stevenson et al., 1996; Chambers and Matrisian, 1997). Among the MMPs reported earlier, MMP-2 and MMP-9 are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Zucker et al., 1993; Bernhard et al., 1994). Several experiments also proved that MMPs not only break down the physical
barrier of extracellular matrix but also modulates the growth factors and cytokines stored in the extracellular matrix, which may promote neoplastic progression (Voet and Voet, 1995). The result of zymographic analysis indicates that administration of *V. cinerea* and Vernolide-A inhibited the activation of matrix metalloproteinases; thereby inhibited the invasion of B16F-10 melanoma cells through the collagen matrix. Tissue Inhibitor of Metalloprotease (TIMP) inhibits MMP activity; thereby suppressing tumour invasion and metastasis (Chambers and Matrisian, 1997). Studies in experimental mouse models have revealed that enhanced TIMP expression can decrease metastasis and inhibit angiogenesis (Bloomston et al., 2002). MMPs are also inhibited by common chelators (Woessner Jr. and Nagase H, 2000). Mouse studies indicate that, when MMP2 is lacking or TIMP-1 is overproduced, formation of new tumours decreases (Itoh et al., 1998). In the present study, *V. cinerea* and Vernolide-A treatment had down regulated the expression of MMP-2 and -9 as well as upregulated the expression of TIMP-1 and -2 indicating its regulatory effect on the inhibition of matrix metalloproteinase.

The nm 23 gene was first identified as a gene whose expression was reduced in highly metastatic rodent tumours relative to poorly metastatic tumour cells (Steeg et al., 1998). Altered expression of this putative metastatic suppressor gene nm23 (Steeg et al., 1998; Hartsough et al., 2001), is considered to play an important role during the acquisition of metastatic ability. The altered expression of prolyl hydroxylase and lysyl oxidase, the enzymes which catalyze the cross linking in extracellular matrix has been identified in various tumours (Takahashi et al., 2000; Kirschmann et al., 2002). *V. cinerea* and Vernolide-A treatment down regulated the expression of prolyl hydroxylase and lysyl oxidase and up regulated the expression of metastatic suppressor gene, nm23 in tumour bearing animals.

The ERK signaling pathway, also known as mitogen-activated protein kinase (MAPK) pathway, is a major determinant in the control of cell growth, cell differentiation, and cell survival. This pathway, which operates downstream of Ras, is often upregulated in tumours and represents an attractive target for anticancer therapy (Lewis et al., 1998; Pearson et al., 2001). Inhibition of the ERK pathway is expected to result in antimetastatic as well as antiangiogenic effects (Kohno and Pouyssegur, 2003). VEGF, a proangiogenic factor, is regarded as one of the earliest and important signals to regulate multistep cascade of tumour angiogenesis and
preferably stimulates the proliferation and migration of endothelial cells and induces the expression of metalloproteinases (Coultas et al., 2005). Overexpression of VEGF in tumour cells enhances tumour growth and metastasis in several animal models by stimulating vascularization. In this study, the expression of k-ras, ERK-1, ERK-2 and VEGF have been down regulated by the treatment of *V. cinerea* and Vernolide-A. These results show that *V. cinerea* and Vernolide-A could inhibit all the pathways which link the MMPs and VEGF to tumour survival, proliferation and invasion.

The above experimental evidences strongly suggest the antimetastatic potential of *V. cinerea* and Vernolide-A. *V. cinerea* and Vernolide-A inhibited tumour cell invasion and metastasis through stimulating CMI and also by regulating MMPs, VEGF, prolyl hydroxylase, lysyl oxidase, ERK-1, ERK-2, TIMPs, nm23 and proinflammatory cytokine gene expression in metastatic lung tissue.