CHAPTER 5

ANTI METASTATIC ACTIVITY OF 
AERVA LANATA,
10-METHOXYCANTHIN-6-ONE AND 
THUJONE
## TABLE OF CONTENTS

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS
5.2.1. Animals
5.2.2. Cell lines
5.2.3. Chemicals
5.2.4. Administration of *A. lanata*, 10-MC and Thujone
5.2.5. Determination of the anti metastatic activity of *A. lanata*, 10-MC and Thujone (*in vivo*)
   a) Pulmonary colonization assay
   b) Histopathological analysis of lungs
   c) Expression of various pro and anti metastatic genes
5.2.6. Cell-mediated immune response in metastatic tumour bearing animals
5.2.7. IL-2 and IFN-γ production in metastatic tumour bearing animals
5.2.8. Determination of the anti metastatic activity of *A. lanata*, 10-MC and Thujone (*in vitro*)
   a) Tumour cell adhesion assay
   b) Migration assay
   c) Collagen matrix invasion assay
   d) Gelatin zymography

5.3. RESULTS
5.3.1. *In vivo* anti metastatic activity of *A. lanata*, 10-MC and Thujone
   a) Effect on the inhibition of lung metastasis and survival.
   b) Effect lung and serum biochemical parameters of metastatic tumour bearing animals
   c) Effect on serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 level in metastases bearing animals
d) Histopathological analysis of lungs

e) Effect on the expression of various pro and anti metastatic genes

5.3.3. Effect of 10-MC on cell mediated immune responses in metastatic tumour bearing animals

a) Effect on NK cell mediated lysis

b) Effect on antibody-dependent cellular cytotoxicity

c) Effect on antibody-dependent complement-mediated cytotoxicity

5.3.4. Effect of 10-MC on IL-2 and IFN-γ production in metastatic tumour bearing animals

5.3.5. Anti metastatic activity of A. lanata, 10-MC and Thujone in the in vitro system

a) Adhesion of B16F-10 melanoma cells to collagen matrix

b) Migration of B16F-10 melanoma cells across polycarbonate membrane

c) Collagen matrix invasion of B16F-10 melanoma cells

d) Gelatin zymographic analysis of MMP production

5.4. DISCUSSION
5.1. INTRODUCTION

The most obvious mark of aggressive cancer is metastasis which is associated with nearly every type of cancer. Transformed cells without the ability to grow destructively would form only benign tumours, which in most cases are not much a serious health concern. Mortality of cancer is largely caused by locally and distally invasive growth. For metastasis to occur cancer cells must migrate from a primary site to other sites through invasion of surrounding tissues, penetration of the basement membrane of blood vessels, entrance into circulation, reattachment to the wound layer of vascular epithelial cells, and ultimately emigration to specific organs (Deng et al., 2010). Despite significant advances in the treatment of primary tumours, the development of metastasis presents a continuing therapeutic challenge.

The first step in metastasis is the detachment of tumour cells from the primary tumour mass that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix (Sato et al., 2005). Tissue degradative enzymes such as MMP-2 and 9 play critical roles in the invasion. MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer, suppresses tumour cell apoptosis, and enhances angiogenesis as well as impairs the host immunological surveillance (Iiizumi et al., 2008).

Over 60% of the world’s population, 80% in case of developing countries depends directly on medicinal plants for their therapeutic purposes. Furthermore, herbal medicines and isolated bioactive microchemicals as alternative cancer therapy has great potential in cancer treatment due to their low toxicity (Kang et al., 2005). The present study has been designed to evaluate the anti metastatic activity of A. lanata ethanolic extract, 10-Methoxycanthin-6-one and Thujone using both in vivo and in vitro systems.
5.2. MATERIALS AND METHODS

5.2.1. Animals

Male C57BL/6 mice (4-6 weeks old) were used for this study.

5.2.2. Cell lines

B16F-10 melanoma cells, a highly metastatic cell line, EL-4 and K-562 were used for the study.

5.2.3. Chemicals

Hydroxy proline, N-acetyl neuraminic acid (NANA), Glucosamine hydrochloride and glucuronic acid lactone, Glycyl glycine, γ-glutamyl-4-nitroanilide, ECM, haematoxylin and eosin. All other chemicals and reagents were of analytical grade.

5.2.4. Administration of A. lanata, 10-MC and Thujone

A. lanata extract and 10-MC were dissolved in minimum volume of ethanol and suspended in 1% gum acacia. A. lanata was administered at a dose of 10mg/Kg body weight and 10-MC at 0.5mg/Kg body weight, intraperitoneally. Thujone was dissolved in paraffin oil for in vivo administration, at a dose of 1mg/Kg body weight, intraperitoneally. Control group of animals were given 1% gum acacia intraperitoneally. A. lanata extract and Thujone were administered by three different modalities.

1. Prophylactically with tumour induction (P): animals were treated with 10 consecutive doses of A. lanata/Thujone prior to tumour inoculation.
2. Simultaneously with tumour (S): A. lanata/Thujone was given to animals simultaneously with metastatic tumour cells for 10 consecutive days.
3. After tumour development (D): 7 days after tumour inoculation, A. lanata/Thujone was administered for 10 consecutive days.

10-MC was given simultaneously with tumour induction for 10 consecutive days. For in vitro studies, the test materials was dissolved in DMSO and resuspended in medium to obtain required concentrations with less than 0.1% DMSO content. A. lanata was used at 25, 10 and 5 μg/ml concentrations. 10-MC was used at 2, 1 and 0.5 μg/ml concentrations. Thujone was used at 10, 5 and 2μg/ml concentrations.
5.2.5. Determination of the anti metastatic activity of *A. lanata*, 10-MC and Thujone (*in vivo*)

**a) Pulmonary colonization assay**

C57BL/6 mice were divided into 8 groups (N = 16/group). All the animals were induced metastasis by injecting B16F-10 melanoma cells (10^6 cells/animal) through lateral tail vein. Group I animals were kept as untreated metastatic tumour bearing control. To three groups of animals *A. lanata* was administered intraperitoneally in three different modalities; Prophylactic to tumour administration (Group II), Simultaneously with tumour administration (Group III) and administration after tumour development (Group IV). Group V, group VI and group VII animals received Thujone in prophylactic, simultaneous and developed modalities. Group VIII animals were treated with 10-MC simultaneous to tumour administration. 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, 1970), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) contents. Serum was separated from the blood and used for the estimation of sialic acid oza (Skoza and Mohos, 1976) and γ-glutamyl transpeptidase (GGT) levels (Szasz, 1976) as explained in Chapter 2. Serum IL-1β, IL-2, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 was estimated using ELISA kit as per manufacture’s protocol. Lung from one animal was used for histopathological analysis and another animal for total RNA isolation. 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.

**b) Histopathological analysis of lungs**

Lung tissues were fixed in 10% formalin, dehydrated in different concentrations of alcohol and embedded in paraffin wax. Sections (4μm) were stained with eosin and hematoxylin.

**c) Expression of various pro and anti metastatic genes**

Total RNA was isolated from the lungs of animals from simultaneous modality *A. lanata* and 10-MC and prophylactic modality of Thujone
(Chomczynski and Sacchi, 1987), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Amplification was performed using specific primers for prolyl hydroxylase and lysyl oxidase, iNOS, COX-2, MMP-2, MMP-9, TIMP-1, TIMP-2, ERK-1, ERK-2, VEGF, nm23 and the housekeeping gene, GAPDH (Chapter 2). The cycling conditions used were as follows; 1 min at 94°C, 1 min at 58°C and 1 min at 72°C for 40 cycles, followed by a 10-minute extension at 72°C. The amplified products were electrophoresed on agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

5.2.6. Cell-mediated immune response in metastatic tumour bearing animals

C57BL/6 mice were divided into 2 groups (n=36/group). Group I animals received B16F-10 cells through lateral tail vein (1×10⁶ cells/animal) and kept as metastatic tumour control. Group II animals received 5 doses 10-MC and B16F-10 cells (1×10⁶ cells/animal) after the last dose of drug treatment. Animals in all of the above mentioned 3 groups were then sacrificed at different time intervals (n=3/time point/group), beginning at 24h post-tumour induction. 6 mice without any treatment were sacrificed along with other groups of animals on first time point to check the cell mediated immune responses in naïve mice. Spleen and blood were collected; spleen cells was processed to yield single cell suspensions using standard protocols and used as effector cells for NK mediated target cell lysis and antibody-dependent cell-mediated cytotoxicity assays using radioactive chromium (Gupta and Bhattacharya, 1983) as explained in Chapter 2. Serum was isolated from the blood and used for the estimation of antibody-dependent complement-mediated cytotoxicity by trypan blue exclusion method as described in Chapter 2.

5.2.7. IL-2 and IFN-γ production in metastatic tumour bearing animals

Blood was collected from above animals by tail vein bleeding on 7th and 21st days after tumour inoculation; blood collected and serum used for the estimation of IL-2 and IFN-γ as per ELISA kit manufacturer's protocol.
5.2.8. Determination of the anti metastatic activity of *A. lanata*, 10-MC and Thujone (*in vitro*)

The following parameters were used to assess the anti metastatic mechanism of action using *in vitro* system.

**a) Tumour cell adhesion assay**

Tumour cell adhesion assay was carried out by seeding B16F-10 melanoma cells (5000 cells/well) into Type I collagen coated wells of flat-bottomed titre plates, in the absence and presence of *A. lanata*, 10-MC and Thujone 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.

**b) Migration assay**

The effect of *A. lanata*, 10-MC and Thujone on the migration of the tumour cells was carried out using the Boyden chamber. The polycarbonate filters were placed in Boyden chambers. The lower compartment of the chamber was filled with serum-free DMEM, and B16F-10 cells (10⁵ cells/chamber) suspended in DMEM were then seeded on to the upper chamber in the presence and absence of test compounds and incubated for 10 hours at 37°C in 5% CO₂. The number of migrated cells to the lower compartment was determined by hemocytometer counting and results expressed as percentage inhibition.

**c) Collagen matrix invasion assay**

The invasion assay was carried out in modified Boyden chambers as described by Albini *et al.*, (1987) The lower compartment of the chamber was filled with serum free DMEM and a polycarbonate filter coated with 25 μg Type I collagen was placed above this. B16F-10 melanoma cells (10⁵ cells/150μl DMEM) were then seeded on to the upper chamber in the presence and absence of *A. lanata*, 10-MC and Thujone and incubated at 37°C in 5% CO₂ atmosphere for 10h. After incubation, the filters were removed, fixed with methanol and stained with crystal violet. Cells migrating to the lower surface of the polycarbonate filters were counted under a microscope. The results are expressed as percentage inhibition of invasion.
d) Gelatin zymography

SDS-PAGE was performed with 5% gelatin incorporated in the separating gel (Billings et al., 1991) as described in Chapter 2. Sub-confluent cultures of B16F-10 melanoma cells were incubated with serum free medium for 24h in the presence and absence of test compounds. 50μl conditioned medium (equivalent to 100μg protein) was subjected to zymographic analysis. Gels were fixed, stained and clear bands were visualized against a dark background.

5.3. RESULTS

5.3.1. In vivo anti metastatic activity of A. lanata, 10-MC and Thujone

a) Effect on the inhibition of lung metastasis and survival.

Metastatic tumour bearing animals treated with A. lanata, 10-MC and Thujone showed significant reduction in tumour nodule formation (Figure 5.1). Metastatic control animals had massive growth of tumour and were given an arbitrary-maximum countable number of 250 (Table 5.1) as described by several other authors (Hill et al., 1994). The three different test compound administration were found to be significantly (p<0.05) effective in inhibiting lung metastasis. Of these, Simultaneous administration of 10-MC (0.5mg/Kg body weight, i.p.) produced maximum inhibition of lung nodules (73.71%) followed by simultaneous mode of administration (70.53%) of A. lanata (25mg/Kg body weight, i.p.). Treatment of animals with Thujone prophylactically, simultaneously and after tumour development produced 59.45%, 57.64% and 29.89% inhibition of lung tumour nodule formation.

Administration of A. lanata, 10-MC and Thujone resulted in significant enhancement in the life span of metastatic tumour bearing animals (Table 5.1). Maximum increase in survival rate of 67.83% was observed when 10-MC was administered simultaneously, followed by 65.3% increase in simultaneous treatment with A. lanata, and 48.2% increase when A. lanata administered prophylactically to tumour induction. Simultaneous and prophylactic treatment of tumour bearing animals with Thujone produced 32.16% and 33.67% increase in life span of the animals when compared to tumour control group of animals.
Figure 5.1

Effect on lung tumour nodule formation and histopathology

a) Metastatic tumour control
b) Tumour + A. lanata simultaneously
c) Tumour + 10-MC simultaneously
d) Tumour + Thujone prophylactically
Table 5.1

Effect on lung tumour colony formation and survival rate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of lung tumour nodules</th>
<th>% inhibition on lung colony formation</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour control</td>
<td>250#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. lanata</em> (Prophylactically)</td>
<td>94.13 ± 6.60*</td>
<td>62.36</td>
<td>48.2</td>
</tr>
<tr>
<td><em>A. lanata</em> (Simultaneously)</td>
<td>73.57 ± 6.75*</td>
<td>70.53</td>
<td>65.3</td>
</tr>
<tr>
<td><em>A. lanata</em> (After tumour development)</td>
<td>153.71 ± 10.09*</td>
<td>38.51</td>
<td>27.1</td>
</tr>
<tr>
<td>10-MC (Simultaneously)</td>
<td>65.71 ± 3.77*</td>
<td>73.71</td>
<td>67.83</td>
</tr>
<tr>
<td>Thujone (Prophylactically)</td>
<td>101.38 ± 3.66*</td>
<td>59.45</td>
<td>33.67</td>
</tr>
<tr>
<td>Thujone (Simultaneously)</td>
<td>106.14 ± 2.34*</td>
<td>57.64</td>
<td>32.16</td>
</tr>
<tr>
<td>Thujone (After tumour development)</td>
<td>175.29 ± 4.23*</td>
<td>29.89</td>
<td>16.08</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1 × 10⁶) were injected into each animal via the lateral tail vein and treated with respective test compound in different modalities. Animals were sacrificed on the 21st day and lung tumour nodules counted. For the survival study, death caused by tumour burden was recorded, and the life span was calculated. Values are mean ± SD. *P < 0.05. # - An arbitrary-maximum countable number.
b) Effect lung and serum biochemical parameters of metastatic tumour bearing animals.

The effect of *A. lanata*, 10-MC and Thujone on lung collagen hydroxyproline, hexosamine and uronic acid content is shown in Table 5.2. Tumour control animals showed an increased level of lung collagen hydroxyproline (21.25 ± 0.94 μg/mg protein) which was significantly (p<0.05) reduced in animals treated with simultaneous modality of 10-MC (6.41 ± 0.29 μg/mg protein) and *A. lanata* (8.41 ± 0.52 μg/mg protein). Administration of Thujone prophylactically with tumour induction (14.73 ± 0.95 μg/mg protein), simultaneously with tumour induction (15.26 ± 1.25 μg/mg protein) and Thujone treatment after development (17.54 ± 1.18 μg/mg protein) also reduced lung collagen hydroxyproline levels.

Tumour-bearing control animals had a high level of lung uronic acid content (316.72 ± 17.29 μg/100 mg tissue wet wt) (p<0.05) compared to normal animals (25.74 ± 1.83 μg/100 mg tissue wet wt). Maximum reduction of uronic acid levels was found when animals were treated simultaneously with 10-MC (128.32 ± 6.74 μg/100 mg tissue wet wt) and *A. lanata* (158.30 ± 9.86 μg/100 mg tissue wet wt), and Thujone prophylactically to tumour induction (174.15 ± 9.46 μg/100 mg tissue wet wt).

Hexosamine content was also high in the lungs of tumour control (3.39 ± 0.18 mg/100 mg tissue dry wt.) compared to normal animals (0.42 ± 0.01 mg/100 mg tissue dry wt.). Simultaneous treatment of 10-MC (1.04 ± 0.06 mg/100 mg tissue dry wt.) significantly (p<0.05) reduced the lung hexosamine content. In both simultaneous administration of *A. lanata* (1.28 ± 0.07 mg/100 mg tissue dry wt.) and prophylactic administration of Thujone (1.97 ± 0.08 mg/100 mg tissue dry wt.) also significantly reduced lung hexosamine in metastatic animals.

The effect of *A. lanata*, 10-MC and Thujone on serum biochemical parameters is presented in Table 5.3. The serum sialic acid level of control metastatic tumour bearing animals was highly increased (134.76 ± 8.39 μg/ml serum) as compared to normal (22.64 ± 1.03 μg/ml serum). Here also the simultaneous administration of 10-MC (50.47 ± 3.64 μg/ml) and *A. lanata* (62.86
Table 5.2

Effect on lung collagen hydroxyproline, hexosamine, and uronic acid levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Collagen hydroxyproline (μg/mg Protein)</th>
<th>Hexosamine (mg/100 mg tissue)</th>
<th>Uronic acid (μg/100 mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.86 ± 0.07</td>
<td>0.42 ± 0.01</td>
<td>25.74 ± 1.83</td>
</tr>
<tr>
<td>Tumour control</td>
<td>21.25 ± 0.94</td>
<td>3.39 ± 0.18</td>
<td>316.72 ± 17.29</td>
</tr>
<tr>
<td><em>A. lanata (Prophylactically)</em></td>
<td>10.37 ± 0.59*</td>
<td>1.44 ± 0.09*</td>
<td>174.81 ± 7.51*</td>
</tr>
<tr>
<td><em>A. lanata (Simultaneously)</em></td>
<td>8.41 ± 0.52*</td>
<td>1.28 ± 0.07*</td>
<td>158.30 ± 9.86*</td>
</tr>
<tr>
<td><em>A. lanata (After tumour development)</em></td>
<td>16.31 ± 0.94*</td>
<td>2.32 ± 0.12*</td>
<td>217.95 ± 12.47*</td>
</tr>
<tr>
<td><em>10-MC (Simultaneously)</em></td>
<td>6.41 ± 0.29*</td>
<td>1.04 ± 0.06*</td>
<td>128.32 ± 6.74*</td>
</tr>
<tr>
<td><em>Thujone (Prophylactically)</em></td>
<td>14.73 ± 0.95*</td>
<td>1.97 ± 0.08*</td>
<td>174.15 ± 9.46*</td>
</tr>
<tr>
<td><em>Thujone (Simultaneously)</em></td>
<td>15.26 ± 1.25*</td>
<td>2.13 ± 0.10*</td>
<td>183.81 ± 12.93*</td>
</tr>
<tr>
<td><em>Thujone (After tumour development)</em></td>
<td>17.54 ± 1.18*</td>
<td>2.76 ± 0.15*</td>
<td>248.57 ± 16.02*</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1 × 10^6) were injected into each animal via the lateral tail vein and treated with respective test compound in different modalities. Animals were sacrificed on the 21\textsuperscript{st} day; lungs were dissected out, and the levels of lung hydroxyproline, hexosamine, and uronic acid were determined. Values are mean ± SD. *P < 0.05, compared with tumour control group.
### Table 5.3

**Effect on serum sialic acid and γ-glutamyl transferase levels**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sialic acid (μg/ml)</th>
<th>γ-GT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>22.64 ± 1.03</td>
<td>3.93 ± 0.24</td>
</tr>
<tr>
<td>Tumour control</td>
<td>134.76 ± 8.39</td>
<td>104.27 ± 7.94</td>
</tr>
<tr>
<td><em>A. lanata</em> (Prophylactically)</td>
<td>68.93 ± 3.17*</td>
<td>63.71 ± 3.29*</td>
</tr>
<tr>
<td><em>A. lanata</em> (Simultaneously)</td>
<td>62.86 ± 4.68*</td>
<td>58.25 ± 3.65*</td>
</tr>
<tr>
<td><em>A. lanata</em> (After tumour development)</td>
<td>96.08 ± 5.92*</td>
<td>82.64 ± 5.72*</td>
</tr>
<tr>
<td>10-MC (Simultaneously)</td>
<td>50.47 ± 3.64*</td>
<td>47.85 ± 2.65*</td>
</tr>
<tr>
<td>Thujone (Prophylactically)</td>
<td>84.31 ± 5.72*</td>
<td>68.36 ± 4.67*</td>
</tr>
<tr>
<td>Thujone (Simultaneously)</td>
<td>87.59 ± 4.93*</td>
<td>71.08 ± 3.76*</td>
</tr>
<tr>
<td>Thujone (After tumour development)</td>
<td>103.26 ± 6.20*</td>
<td>80.75 ± 5.14*</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells ($1 \times 10^6$) were injected into each animal via the lateral tail vein and treated with respective test compound in different modalities. Animals were sacrificed on the 21st day; blood was collected by heart puncture and serum separated, and the levels of serum sialic acid and γ-glutamyl transferase (γGT), were determined. Values are mean ± SD. *P < 0.05, compared with tumour control group.
± 4.68 μg/ml) and prophylactic administration of Thujone (84.31 ± 5.72 μg/ml) significantly reduced the elevated serum sialic acid levels.

Serum γ-glutamyl transpeptidase level was also significantly (p<0.05) enhanced in metastatic control animals (104.27 ± 7.94 U/L) compared to normal animals (3.93 ± 0.24 U/L). After simultaneous administration of 10-MC and A. lanata the elevated γ-GGT level was reduced significantly (p<0.05) to 47.85 ± 2.65 U/L and 58.25 ± 3.65 U/L respectively. In animals treated with Thujone prophylactically, the serum γ-GGT level was reduced to 80.75 ± 5.14 U/L.

c) Effect on serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 level in metastases bearing animals

Serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 levels are shown in the Table 5.4. Serum levels of proinflammatory cytokines; IL-1β, IL-6 and TNF-α was found to be significantly increased to 58.18 ± 1.58 pg/ml, 461.94 ± 18.74 pg/ml and 327.56 ± 18.37 pg/ml respectively in metastatic tumour bearing animals on 21st day. Administration of A. lanata, 10-MC and Thujone simultaneously with tumour induction could significantly lower this elevated level as time progress. Serum GM-CSF level also showed an elevation in control animals from the normal value. Administration of test compounds was found to significantly lower the elevated level of GM-CSF on 7th day and 21st day.

The normal serum VEGF level was only 18.37 ± 0.82 pg/ml. This was significantly increased (p<0.05) to 163.71 ± 10.2 pg/ml in metastatic tumour-bearing mice. This elevated level of serum VEGF was reduced significantly (p<0.05) in A. lanata (59.60±0.96 pg/ml on 7th day and 83.53 ± 5.64 pg/ml on 21st day) and 10-MC (47.52 ± 1.85 pg/ml on 7th day and 79.35 ± 5.31 pg/ml on 21st day) simultaneously treated animals. The elevated serum VEGF level was also reduced in the animals treated with Thujone (62.38 ± 2.77 pg/ml on 7th day and 101.52±6.95 pg/ml on 21st day). The serum levels of TIMP-1 were restored to near normal range in animals treated with A. lanata (603.21±23.79 pg/ml on 21st day), 10-MC (593.61±29.74 pg/ml on 21st day) and Thujone (496.36±20.38 pg/ml on 21st day).
Table 5.4

**Effect on serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 level in metastases bearing animals**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Tumour control</th>
<th>A. lanata (S)</th>
<th>10-MC (S)</th>
<th>Thujone (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 21</td>
<td>Day 7</td>
<td>Day 21</td>
<td>Day 7</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>16.37±0.39</td>
<td>43.50±0.58</td>
<td>58.18±1.58</td>
<td>41.81±1.13</td>
<td>45.11±0.81*</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>36.16±0.97</td>
<td>326.57±4.67</td>
<td>461.94±18.74</td>
<td>273.65±4.44*</td>
<td>338.01±2.00*</td>
</tr>
<tr>
<td><strong>TNFα</strong></td>
<td>20.94±1.21</td>
<td>265.49±2.82</td>
<td>327.56±18.37</td>
<td>170.13±4.53*</td>
<td>203.04±4.48*</td>
</tr>
<tr>
<td><strong>GM-CSF</strong></td>
<td>17.98±0.96</td>
<td>38.69±0.36</td>
<td>40.41±1.85</td>
<td>28.61±0.25*</td>
<td>32.98±0.17*</td>
</tr>
<tr>
<td><strong>VEGF</strong></td>
<td>18.37±0.82</td>
<td>65.44±1.39</td>
<td>163.71±10.2</td>
<td>59.60±0.96*</td>
<td>83.53±5.64*</td>
</tr>
<tr>
<td><strong>TIMP-1</strong></td>
<td>613.48±23.57</td>
<td>349.12±2.41</td>
<td>384.57±15.62</td>
<td>557.76±3.98*</td>
<td>603.21±23.79*</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1×10⁶) were injected into each animal via the lateral tail vein and treated with respective test compound. Blood was collected by tail vein bleeding on 7th day after tumour induction and on 21st day; levels of serum IL-1β, IL-6, TNF-α, GM-CSF, VEGF and TIMP-1 (pg/ml) were determined. Values are mean ± SD. *P < 0.05, compared with respective day on tumour control group.
d) Histopathological analysis of lungs

The hematoxylin and eosin stained sections of lung tissues are shown in Figure 5.1 (100X magnification). Lungs in the control animals showed infiltration of the neoplastic cells around the main bronchioles extended to the pleura. This together with fibrosis reduces alveolar space, which in turn leads to reduced vital capacity. Animals treated with *A. lanata* and 10-MC simultaneous to tumour induction showed significant reduction in tumour mass. Alveoli and pleura were tumour free, alveolar passage lined with healthy ciliated columnar epithelial cells and almost similar to normal lung. Considerable reduction of tumour mass was also observed in animals treated with Thujone prophylactically to tumour induction.

e) Effect on the expression of various pro and anti metastatic genes

mRNA expression analysis of lungs tissue reveals that iNOS, COX-2, MMPs, prolyl hydroxylase, lysyl oxidase, ERK-1, ERK-2 and VEGF genes was down regulated in the groups where *A. lanata* and 10-MC were administered simultaneously to tumour induction and Thujone administered prophylactically (Figure 5.2) when compared to metastatic tumour bearing control animals. The expression of antimetastatic genes; TIMP-1, TIMP-2 was minimal and nm23 was absent in tumour bearing animals. The treatment with *A. lanata*, 10-MC and Thujone resulted in the upregulation of these gene expressions.

5.3.3. Effect of 10-MC on cell mediated immune responses in metastatic tumour bearing animals

a) Effect on NK cell mediated lysis

The effect of 10-MC on NK cell mediated cytotoxicity in tumour-bearing animals is shown in Figure 5.3.A. The percentage cell lysis of target cells mediated by NK cell activity in naïve mice was 7.84% (± 1.25; mean ± SD). Administration of 10-Methoxycanthin-6-one significantly enhanced NK cell activity in metastatic tumour-bearing hosts; this occurred much earlier when compared with tumour-only controls. In the case of 10-MC treated tumour-bearing mice, maximum lysis of target cells (i.e., 47.24%) was seen on day 5 after tumour induction, whereas in tutor-only control animals, the peak lysis (i.e., 13.50%) was observed only on day 9.
Figure 5.2

Effect on the expression of pro and anti metastatic genes

Lysyl oxidase
Prolyl hydroxylase

COX-2
iNOS
Figure 5.2 (continued)

Effect on the expression of pro and anti metastatic genes

Line 1: Molecular weight marker
Line 2: Metastatic tumour control
Line 3: Tumour + A. lanata simultaneously
Line 4: Tumour + 10-MC simultaneously
Line 5: Tumour + Thujone prophylactically
Figure 5.2 (continued)

Effect on the expression of pro and anti metastatic genes

<table>
<thead>
<tr>
<th>Line 1</th>
<th>Molecular weight marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line 2</td>
<td>Metastatic tumour control</td>
</tr>
<tr>
<td>Line 3</td>
<td>Tumour + A. Janata simultaneously</td>
</tr>
<tr>
<td>Line 4</td>
<td>Tumour + 10-MC simultaneously</td>
</tr>
<tr>
<td>Line 5</td>
<td>Tumour + Thujone prophylactically</td>
</tr>
</tbody>
</table>
b) **Effect on antibody-dependent cellular cytotoxicity**

Treatment with 10-MC significantly augmented ADCC in metastatic tumour-bearing animals (Figure 5.3.B). The percentage cell lysis of target cells mediated by ADCC in naïve mice was 1.69% (± 0.47). In 10-MC treated tumour-bearing hosts, maximum lysis of target cells (i.e., 43.41%) was seen on day 6 post-tumour induction, whereas in tumour control mice the maximum activity (i.e., 11.43%) was observed only on day 15.

c) **Effect on antibody-dependent complement-mediated cytotoxicity**

The effect of 10-MC on ACC is given in Figure 5.3.C. 10-MC treatment significantly enhanced ACC in tumour-bearing mice. The percentage cell lysis of target cells mediated by ACC in naïve mice was 0.37% (± 0.06). In tumour-only controls, maximum cell lysis (i.e., 13.50%) was observed only on day 17 post-tumour induction; a maximum cell lysis of 29.60% was noted in the 10-MC treated tumour-bearing mice on day 15.

5.3.4. **Effect of 10-MC on IL-2 and IFN-γ production in metastatic tumour bearing animals**

The effect of 10-MC on serum levels of IL-2 and IFN-γ during metastasis is shown in Table 5.5. Metastasis induction resulted in decrease in the level of IL-2 from the normal (10.87±0.49 pg/ml) and it was 8.35±0.62pg/ml and 6.84±0.45pg/ml respectively on 7th and 21st days of tumour induction. Administration of 10-MC significantly enhanced the production of IL-2 in metastatic tumour bearing mice to 13.92 ± 1.04pg/ml on 7th day and 11.26 ± 0.89pg/ml on 21st day after tumour induction. The levels of IFN-γ in the metastatic tumour bearing control animals (1365.82±67.21pg/ml on 21st day) were augmented in animals treated with 10-MC to 2731.62±127.44pg/ml on 7th day and 2253.17±93.28pg/ml on 21st day.

5.3.5. **Anti metastatic activity of A. lanata, 10-MC and Thujone in the in vitro system**

a) **Adhesion of B16F-10 melanoma cells to collagen matrix**

The effect of A. lanata, 10-MC and Thujone on the adhesion of B16F-10 melanoma cells to collagen matrix is given in Table 5.6. There was a dose
Figure 5.3

A) Effect on NK cell activity

B) Effect on ADCC

C) Effect on ACC

Metastatic tumour bearing mice were treated with 10-MC for 5 days. Animals were sacrificed on different time points. Spleen cells were used to determine NK cell mediated lysis of chromium labelled K-562 cells and ADCC of chromium labelled EL-4 cells. Serum was used to determine ACC of B16F-10 melanoma cells. All results expressed as percentage cell lysis.
Table 5.5

Effect of 10-MC on IL-2 and IFN-γ production in tumour bearing animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 21</td>
</tr>
<tr>
<td>Normal</td>
<td>10.87 ± 0.49</td>
<td>284.64 ± 11.76</td>
</tr>
<tr>
<td>Tumour control</td>
<td>8.35 ± 0.62</td>
<td>6.84 ± 0.45</td>
</tr>
<tr>
<td>Tumour+10-MC</td>
<td>13.92±1.04*</td>
<td>11.26±0.89*</td>
</tr>
</tbody>
</table>

Metastatic tumour bearing C57BL/6 mice with and without treatment with five dose of 10-MC and nomral mice were used. Blood was collected from all animals on 7th day and 21st day by tail vein bleeding. Serum was separated and the cytokine level was estimated by ELISA method. Values are mean ± SD. *P < 0.05 compared with tumour control.
Table 5.6

Effect on tumour cell adhesion to collagen matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4770 ± 30</td>
<td>-</td>
</tr>
<tr>
<td><em>A. lanata</em> (5μg/ml)</td>
<td>4488 ± 22*</td>
<td>5.91</td>
</tr>
<tr>
<td><em>A. lanata</em> (10μg/ml)</td>
<td>3916 ± 30*</td>
<td>17.90</td>
</tr>
<tr>
<td><em>A. lanata</em> (25μg/ml)</td>
<td>3211 ± 26*</td>
<td>32.68</td>
</tr>
<tr>
<td><em>10-MC</em> (0.5μg/ml)</td>
<td>4451 ± 22*</td>
<td>6.69</td>
</tr>
<tr>
<td><em>10-MC</em> (1μg/ml)</td>
<td>3765 ± 22*</td>
<td>21.08</td>
</tr>
<tr>
<td><em>10-MC</em> (2μg/ml)</td>
<td>3047 ± 23*</td>
<td>36.12</td>
</tr>
<tr>
<td>Thujone (2μg/ml)</td>
<td>4548 ± 11*</td>
<td>4.66</td>
</tr>
<tr>
<td>Thujone (5μg/ml)</td>
<td>4032 ± 17*</td>
<td>15.48</td>
</tr>
<tr>
<td>Thujone (10μg/ml)</td>
<td>3367 ± 13*</td>
<td>29.42</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (5000 cells/well) were seeded into Type I collagen coated wells of flat-bottomed titre plates, in the absence and presence of different concentrations of test compounds and incubated at 37°C for 5h. After incubation cells were washed, the adhering cells were fixed, stained and counted under microscope. Values are mean ± SD. *P < 0.05, compared with untreated cells.
Effect on collagen matrix invasion of B16F-10 cells

a) Control  
c) A. lanata (10µg/ml)  
e) 10-MC (0.5µg/ml)  
b) A. lanata (5µg/ml)  
d) A. lanata (25µg/ml)  
f) 10-MC (1µg/ml)
Figure 5.4 (continued)

Effect on collagen matrix invasion of B16F-10 cells

g) 10-MC (2μg/ml)

h) Thujone (2μg/ml)

i) Thujone (5μg/ml)

j) Thujone (10μg/ml)
dependent inhibition of tumour cell adhesion in cells treated with the test compounds with maximum inhibition in 2μg/ml 10-MC treated cells (36.12%) followed by 25μg/ml A. lanata (32.68%). Thujone at concentrations of 5μg/ml and 10μg/ml could inhibit the tumour cell adhesion by 15.35% and 29.31% respectively.

b) Migration of B16F-10 melanoma cells across polycarbonate membrane

Inhibition of tumour cell migration by A. lanata, 10-MC and Thujone is given in Table 5.7. Treatment with A. lanata at 10μg/ml and 25μg/ml produced 21.13% and 44.26% inhibition in tumour cell migration. 10-MC at concentrations 1μg/ml and 2μg/ml produced more inhibition; 22.33% and 44.81% respectively. Thujone significantly inhibited the migration of B16F-10 melanoma cells across the polycarbonate filters by 7.39%, 18.96% and 42.57%, respectively, in a dose dependent manner.

c) Collagen matrix invasion of B16F-10 melanoma cells

Metastatic B16F-10 melanoma cells show highly invasive property through the collagen matrix. Most of cells were found in the lower surface of the polycarbonate membrane in the control experiment, but there was a significant inhibition in the invasion of the collagen matrix by the tumour cells when they were treated with A. lanata, 10-MC and Thujone (Figure 5.4), in a dose dependent manner. At a concentration of 10μg/ml and 25μg/ml A. lanata produced 22.98% and 40.75% inhibition in the invasion of B16F-10 melanoma cells where as 10-MC at 1μg/ml and 2μg/ml concentrations had 26.31% and 45.38% respectively. Thujone at 10μg/ml concentration significantly inhibited the invasion of B16F-10 melanoma cells by 37.57% where as at 5μg/ml and 2μg/ml the percentage of inhibition of invasion was found to be 21.97% and 8.99% respectively.

d) Gelatin zymographic analysis of MMP production

A. lanata, 10-MC and Thujone inhibited the activation of matrix metalloproteinases produced by B16F-10 melanoma cells as shown in Figure 5.5 and Figure 5.6. Conditioned medium after trypsin activation showed digested clear areas at 92 and 72 kD which was identical to MMP-9 and MMP-2 activity (Lane 2). Gels loaded with conditioned medium, without trypsin activation, did
**Table 5.7**

**Effect on tumour cell migration and invasion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition on migration</th>
<th>% Inhibition on invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. lanata</em> (5μg/ml)</td>
<td>7.06</td>
<td>9.61</td>
</tr>
<tr>
<td><em>A. lanata</em> (10μg/ml)</td>
<td>21.13</td>
<td>22.98</td>
</tr>
<tr>
<td><em>A. lanata</em> (25μg/ml)</td>
<td>44.26</td>
<td>40.75</td>
</tr>
<tr>
<td>10-MC (0.5μg/ml)</td>
<td>6.98</td>
<td>10.33</td>
</tr>
<tr>
<td>10-MC (1μg/ml)</td>
<td>22.33</td>
<td>26.31</td>
</tr>
<tr>
<td>10-MC (2μg/ml)</td>
<td>44.81</td>
<td>45.38</td>
</tr>
<tr>
<td>Thujone (2μg/ml)</td>
<td>7.39</td>
<td>8.99</td>
</tr>
<tr>
<td>Thujone (5μg/ml)</td>
<td>18.96</td>
<td>21.97</td>
</tr>
<tr>
<td>Thujone (10μg/ml)</td>
<td>42.57</td>
<td>37.57</td>
</tr>
</tbody>
</table>

The effect of *A. lanata*, 10-MC and Thujone on the migration of the tumour cells was carried out using the Boyden chamber with polycarbonate filters. The lower compartment of the chamber was filled with serum-free DMEM, and B16F-10 cells (10⁵ cells/chamber) suspended in DMEM were then seeded on to the upper chamber in the presence and absence of test compounds and incubated for 10 hours at 37°C in 5% CO₂. The invasion assay was carried out in Boyden chambers in the similar with by using polycarbonate filter coated with 25 μg Type I collagen. The number of migrated cells to the lower compartment was determined and expressed as percentage inhibition in cell migration/invasion.
**Figure 5.5**

A) Effect of *A. lanata* on MMP production by B16F-10 cells

1. CM from untreated B16F-10 cells without trypsin activation.
2. CM from untreated B16F-10 cells after trypsin activation.
3. CM from pretreated B16F-10 cells (5μg/ml *A. lanata*) after trypsin activation.
4. CM from pretreated B16F-10 cells (10μg/ml *A. lanata*) after trypsin activation.
5. CM from pretreated B16F-10 cells (25μg/ml *A. lanata*) after trypsin activation.
6. CM from untreated B16F-10 cells after trypsin activation+EDTA.

B) Effect of 10-MC on MMP production by B16F-10 cells

1. CM from untreated B16F-10 cells without trypsin activation.
2. CM from untreated B16F-10 cells after trypsin activation.
3. CM from untreated B16F-10 cells after trypsin activation+EDTA.
4. CM from pretreated B16F-10 cells (0.5μg/ml 10-MC) after trypsin activation.
5. CM from pretreated B16F-10 cells (1μg/ml 10-MC) after trypsin activation.
6. CM from pretreated B16F-10 cells (2μg/ml 10-MC) after trypsin activation.
Figure 5.6

Effect of Thujone on MMP production by B16F-10 cells

1. CM from untreated B16F-10 cells without trypsin activation.
2. CM from untreated B16F-10 cells after trypsin activation.
3. CM from untreated B16F-10 cells after trypsin activation + EDTA.
4. CM from pretreated B16F-10 cells (2μg/ml) after trypsin activation.
5. CM from pretreated B16F-10 cells (5μg/ml) after trypsin activation.
6. CM from pretreated B16F-10 cells (10μg/ml) after trypsin activation.
not show any clear areas, indicating the inactive form of the enzyme collagenase (Lane 1). Trypsin activated condition medium loaded gels, after incubation with 10mM EDTA did not show clear areas, which indicate that the enzyme responsible for degradation is metalloproteinase. In conditioned medium from cells treated with A. lanata, no clear bands were observed (Lane 4 and 5) indicating that A. lanata inhibited the enzyme production at concentrations of 10μg/ml and 25μg/ml. Conditioned medium from cell treated with A. lanata at a concentration of 5μg/ml showed two faded bands (Lane 3), indicating a partial inhibition in MMP activation. Similarly 10-MC (1μg/ml and 2μg/ml) and Thujone at concentrations of 10 and 5μg/ml inhibited the production of MMP.

5.4. DISCUSSION

In the present study, intraperitonal administration of A. lanata, 10-MC and Thujone in simultaneous modalities showed maximum reduction in lung colonization by B16F-10 cells, which is evident by reduction in tumour nodule formation. The survival rate of animals treated with A. lanata and 10-MC was significantly higher than tumour bearing control animals.

The level of lung hydroxyproline, the major building block of collagen, corresponds to the fibrous accumulation in lungs of melanoma-induced animals. A. lanata, 10-MC and Thujone treated animals had much lower lung collagen hydroxyproline content comparing to untreated metastatic tumour bearing animals. Histopathological analysis of lungs of metastatic control and that of the animals treated with A. lanata and 10-MC simultaneously correlates with these findings, while Thujone administration showed much less effect. Administration of A. lanata, 10-MC and Thujone significantly reduced the levels of the structural monosaccharides, uronic acid and hexosamine, showing a reduction in the metastatic potential of B16F-10 melanoma cells. γ-GT, a marker of cellular proliferation was increased in the serum of tumour bearing animals compared to normal. Serum γ-GT levels were reduced significantly (p<0.05) by the treatment of A. lanata, 10-MC and Thujone. Metastatic cancer cells often express a high density of sialic acid-rich glycoproteins and sialic acid have been strongly implicated in tumour cell migration, invasion, and metastatic potential (Chang et al., 2006). The level of sialic acid in serum was significantly reduced by the administration of A. lanata, 10-MC and Thujone. Here also maximum reduction
was achieved in simultaneous modalities of *A. lanata* and 10-MC treatment. All these results demonstrate the ability of test compounds to inhibit lung metastasis of melanoma cells.

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. Treatment with *A. lanata*, 10-MC and Thujone significantly downregulated the production of these proinflammatory cytokines.

MMPs are endopeptidases that are capable of degrading the constituents of ECM. Treatment with *A. lanata*, 10-MC and Thujone suppressed the activity of MMP-2 and 9, which is evident from the zymographic analysis.

NM23, the first identified metastasis-suppressor gene (De La Rosa *et al.*, 1995), has been shown to be down regulated in a variety of tumours. Survival, proliferation and invasive responses of tumour cell lines have been shown to be mediated by VEGF through Erk1/Erk2 pathways and activation of ERK-1 and ERK-2 is usually linked to stimulation of cell proliferation (Graells *et al.*, 2004). This pathway is often upregulated in tumours and represents an attractive target for anticancer therapy. Simultaneous administration of *A. lanata*, 10-MC and Thujone down regulated the expression of MMP-2, MMP-9, ERK-1, ERK-2 and VEGF when compared to untreated metastatic control. The expression of TIMP-1, TIMP-2 and nm23 were minimal in metastatic control animals. These gene expression level was increased when the animals were treated with *A. lanata*, 10-MC and Thujone.

The elevated NK cell activity by 10-MC administration to metastatic tumour bearing animals resulted in a significant enhancement of tumour cell killing. A similar enhancement of ADCC and ACC by the administration 10-MC indicates the activation of cell mediated immune system in tumour bearing animals.

Cell migration plays an important role in several pathological processes, such as tumour formation and cancer metastasis. *A. lanata*, 10-MC and Thujone treatment was found to reduce the tumour cell adhesion to Type I collagen-coated wells of flat-bottomed titre plates by B16F-10 melanoma cells in a dose dependent manner. Tumour cell migration and collagen matrix invasion was also significantly inhibited by *A. lanata*, 10-MC and Thujone in a dose dependent manner. The results from these *in vivo* studies confirm the effectiveness of test compounds to inhibits metastasis of tumour cells.