CHAPTER 4

EFFECT OF *AERVA LANATA*, 10-METHOXYCANTHIN-6-ONE AND THUJONE ON IMMUNE SYSTEM
TABLE OF CONTENTS

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS
4.2.1. Animals
4.2.2. Cell lines
4.2.3. Chemicals
4.2.4. Administration of *A. lanata*, 10-MC and Thujone
4.2.5. Determination of the effect of *A. lanata*, 10-MC and Thujone on immune system
   a) Haematological parameters
   b) Relative weight and cellularity of lymphoid organs
   c) Bone marrow cellularity and α esterase positive cells
   d) Proliferation of lymphoid cells; spleenocyte, thymocyte and bone marrow cells
   e) Circulating antibody titre
   f) Plaque forming cells (PFC) in spleen
   g) Cell-mediated immune response in normal and tumour bearing animals
   h) IL-2 and IFN-γ production in normal and tumour bearing animals
   i) Cytotoxic T lymphocyte generation
      1. *In vivo* generation of CTL
      2. *In vitro* CTL generation

4.3. RESULTS
4.3.1. Effect on haematological parameters and body weight
4.3.2. Effect on relative weight and cellularity of lymphoid organs
4.3.3. Effect on bone marrow cellularity and α esterase positive cells
4.3.4. Effect on proliferation of lymphoid cells; spleenocyte, thymocyte and bone marrow cells
a) Spleen blastogenesis
b) Thymocyte blastogenesis
c) Bone marrow blastogenesis

4.3.5. Effect on circulating antibody titre

4.3.6. Effect on plaque forming cells in spleen

4.3.7. Effect on cell-mediated immune response in normal and tumour bearing animals
   a) Natural killer cell mediated target cell lysis
   b) Antibody-dependent cell-mediated cytotoxicity (ADCC)
   c) Antibody-dependent complement-mediated cytotoxicity (ACC)

4.3.8. Effect on IL-2 and IFN-γ production in normal and tumour bearing animals

4.3.9. Effect on cytotoxic T lymphocyte generation
   a) Effect on in vivo generation of CTL
   b) Effect on in vitro CTL generation

4.4. DISCUSSION
4.1. INTRODUCTION

The major drawbacks of current cancer therapeutic practices such as chemotherapy and radiation therapy includes mucosal ulceration, alopecia, pulmonary fibrosis, cardiac and hepatic toxicity and bone marrow suppression resulting in cytopenia (Devasagayam and Sainis, 2002). Chemically synthesized medicines used during chemotherapy are costly and have serious side effects leading to reduction in immune-competence of the host, resulting in further tumour progression. Due to these side effects, use of natural medicines in the treatment of cancer is becoming more and more important. Modulation of immune system by cytotoxic agents is emerging as a major area in pharmacology, especially in cases where undesired immunosuppression is the result of therapy. The use of immunomodulating agents provides distinct advantages over conventional therapies such as radiation and chemotherapy. Most important, the enhancement of the host immune system’s innate ability to combat infections obviates the side effects associated with chemotherapy.

In recent years there is significant interest among academia as well as pharmaceutical companies in plant derived products, considering it as a potential source for new medicines. According to Cragg et al., (1997) 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives. In this study we are focussing on analysis of the immunomodulatory activities on natural products.

4.2. MATERIALS AND METHODS

4.2.1. Animals

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

4.2.2. Cell lines

EL-4, K-562, EAC cells and SRBC were used for the study.

4.2.3. Chemicals

RPIM-1640 medium, LPS, PWM, Con-A, PHA, ³H thymidine, Radioactive sodium chromate, pararosaniline hydrochloride and α-naphthyl acetate were used in this study. All other chemicals and reagents were of analytical grade.
4.2.4. Administration of *A. lanata*, 10-MC and Thujone

*In vivo* dosage of *A. lanata*, 10-Methoxycanthin-6-one (10-MC) and Thujone were selected based on 14 day short term toxicity studies (discussed in Chapter 3). *A. lanata* ethanolic 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. 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.

4.2.5. Determination of the effect of *A. lanata*, 10-MC and Thujone on immune system

a) Haematological parameters

Four groups of BALB/c mice (n=8/group) were used. Group I animals were kept as untreated control. Group II, group III and group IV animals were treated with *A. lanata*, 10-MC and Thujone for 5 consecutive days. Blood was collected from the tail vein and parameters such as total WBC count, differential count and haemoglobin content (as described in Chapter 2) were recorded prior to the administration of test compounds and continued every 3rd day for one month.

b) Relative weight and cellularity of lymphoid organs

BALB/c mice were divided into four groups as described above. Body weights of all groups of animals were recorded and mice were sacrificed 24h after the last dose of test compound administration and weight of the lymphoid organs; spleen and thymus were recorded. Thymus was very carefully dissected to remove adherent perithymic lymph nodes and blotted on filter paper to remove any traces of blood. Red blood cells were removed by centrifuging the pellet in RBC lysing buffer. Spleen and thymus were processed to single cell suspension and the cell number was determined using haemocytometer.
c) Bone marrow cellularity and α-esterase positive cells

Bone marrow cellularity was determined by the method of Sredni et al., (1992). BALB/c mice were divided into four groups as described above. Animals were sacrificed 24h after the last dose of test compound and bone marrow cells from femur was collected into RPMI-1640 medium and cell number determined using haemocytometer. The number of α-esterase positive cells was determined by azodye coupling method as explained in Chapter 2.

d) Proliferation of lymphoid cells; spleenocyte, thymocyte and bone marrow cells

BALB/c mice were divided into four groups as described above. Animals were sacrificed 24h after last dose of treatment; spleen, thymus and bone marrow were collected aseptically. 5×10^4 cells were cultured in the presence and absence of various mitogens; Con-A (10µg/ml) and LPS (10µg/ml), PHA (2.5µg/ml) and PWM (10µg/ml). Rate of proliferation was determined by ³H-Thymidine incorporation assay as explained in Chapter 2.

e) Circulating antibody titre

BALB/c mice were divided into four groups. Group II, group III and group IV animals were treated with A. lanata, 10-MC and Thujone respectively for 5 consecutive days. Along with the 5th dose the animals in all the groups were immunized with SRBC. Group I animal were immunized with SRBC alone and kept as control. Blood was collected from tail vein every 3rd day after antigen administration and continued for one month. Serum was separated and heat inactivated at 56°C for 30 minutes. Antibody titre was determined by haemagglutination assay using SRBC as antigen, as explained in Chapter 2.

f) Plaque forming cells (PFC) in spleen

BALB/c mice were divided into four groups (n=21/group) as described early. The animals in group II, group III and group IV were treated with the test compounds for 5 consecutive days and along with the last dose immunized with SRBC. Group I animals were immunized with SRBC alone and kept as control. Animals were sacrificed on different days starting from 3rd day after immunization up to the 9th day; spleen was processed to single cell suspension
and used for the determination of the antibody producing cells by Jerne's plaque assay (Jerne and Nordin, 1963) as explained in Chapter 2.

**g) Cell-mediated immune response in normal and tumour bearing animals**

BALB/c mice were divided into seven groups (n=36/group). Group I animals received EAC cells (1×10^6 cells/animal, i.p) and kept as tumour control. Group II, group III and group IV animals were treated with A. lanata, 10-MC and Thujone for 5 consecutive days. Group V, group VI and group VII animals received 5 doses of A. lanata, 10-MC and Thujone respectively, and EAC cells (1×10^6 cells/animal, i.p) after the last dose of drug treatment. Animals in the above mentioned groups were sacrificed at different time intervals (n=3/time point/group), beginning at 24h post- tumour induction. Spleen and blood were collected; spleen 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.

**h) IL-2 and IFN-γ production in normal and tumour bearing animals**

BALB/c mice were divided into seven groups as explained above (n=6/group). All groups of animals were sacrificed 24h after the last dose of test compound administration; blood collected and serum used for the estimation of IL-2 and IFN-γ as per ELISA kit manufacturer's protocol.

**i) Cytotoxic T lymphocyte generation**

Cytotoxic T lymphocytes (CTL) were generated using *in vivo* and *in vitro* systems. (a) *in vivo* CTL generation by alloimmunization (System-A); carried out by injecting spleen cells (2×10^7 cells) from C57BL/6 mice subcutaneously to BALB/c mice. The alloimmunized mice were treated with test compounds. Control animals were kept without further treatment. The effector cells were taken from these animals 7 days after alloimmunization. (b) *in vitro* CTL generation by Mixed Lymphocyte Culture (System-B); Effector cells were produced by a 5-day mixed lymphocyte culture of spleen cells (responder cells)
from test compound treated and untreated BALB/c mice with mitomycin C (50μg/ml)-treated spleen cells (stimulator cells) of C57BL/6 mice.

CTL activity was determined by Winn’s neutralization assay according to the method of Kobayashi et al., (1990). 1×10^7 effector cells (CTL) were mixed with 5×10^5 EL-4 cells (target cells) in RPMI medium at an effector target ratio of 20:1 and incubated for 1h at 37° in 5% CO_2 atmosphere. After incubation 5×10^4 cells were injected intraperitoneally to BALB/c mice. The animals were observed daily after tumour inoculation to determine survival rate. Increase in mean survival days of the treated groups, compared with that of tumour controls, was considered as an indicative of CTL activity.

1. **In vivo generation of CTL**

Eight groups of BALB/c mice (n=8/group) were used for this study. Group I animals received EL-4 cells alone (5 × 10^4 cells). Groups II, group III and group IV animals received EL-4 (5×10^4 cells) and 5 doses of *A. lanata*, 10-MC and Thujone respectively. Group V animals received EL-4 cells after Winn’s neutralization with spleen cells from normal alloimmunized animals. Groups VI animals received EL-4 cells after Winn’s neutralization with spleen cells from *A. lanata* treated alloimmunized mice while animals in group VII received EL-4 cells after Winn’s neutralization with spleen cells from 10-MC treated alloimmunized mice. Group VIII animals received EL-4 cells after Winn’s neutralization with spleen cells from Thujone treated alloimmunized mice. All the animals were observed for the survival time.

2. **In vitro CTL generation**

Five groups of animals were used (n=8/group) for this study. Group I animals received EL-4 cells alone (5×10^4 cells). Group II received EL-4 cells after Winn’s neutralization with effector cells generated using mixed lymphocyte culture of normal spleen cells in system-B. Groups III animals received EL-4 cells after Winn’s neutralization with effector cells generated using mixed lymphocyte culture of spleen cells from *A. lanata* treated spleen cells. Groups IV animals received EL-4 cells after Winn’s neutralization with effector cells generated using mixed lymphocyte culture of spleen cells from 10-MC treated spleen cells. Groups V animals received EL-4 cells after Winn’s neutralization with effector
cells generated using mixed lymphocyte culture of spleen cells from Thujone treated spleen cells. All the animals were observed for their survival.

4.3. RESULTS

4.3.1. Effect on haematological parameters and body weight

Administration of the ethanolic extract of A. lanata, 10-MC and Thuone resulted in increased total WBC count in normal BALB/ c mice (Fig. 4.1). The maximum WBC count obtained in the extract treated animals was 14238 ± 608.72 cells/mm³ on the 12th day, while 10-MC treated animals had a peak value of 13976 ± 386.48 cells/mm³ on the 12th day. Administration of 5 doses of Thujone increased the total WBC count to 11631.3 ± 386.32 cells/mm³. There was no significant difference in the ratio of lymphocytes to neutrophil (data not shown) as well as haemoglobin level (data not shown) after treatment with test compounds.

4.3.2. Effect on relative weight and cellularity of lymphoid organs

The effect of test compound treatment on the weight and cellularity of lymphoid organs is given in Table 4.1.A. Control animals had an average thymus weight of 0.105g. Treatment with A. lanata, 10-MC and Thujone for 5 consecutive days resulted in 32.38%, 41.90% and 15.23% increase in the weight of thymus when compared to control animals. The cellularity of thymus was also significantly increases by test compound treatment. The size and weight of spleen was also enhanced significantly (p<0.05) by the administration of A. lanata, 10-MC and Thujone. Control animals had 0.397 ± 0.024g while, treated animals had spleen weight of 0.468 ± 0.017g, 0.462 ± 0.028g and 0.441± 0.033g respectively.

4.3.3. Effect on bone marrow cellularity and α-esterase positive cells

The effect of treatment of A. lanata, 10-MC and Thujone on bone marrow cellularity and α-esterase positive cells is shown in Table 4.1.B. Control animals had 15.16 ± 0.98 × 10⁶ cells/femur and administration of A. lanata and 10-MC for 5 consecutive days significantly increased the bone marrow cellularity by 47.29% and 52.24% respectively. Thujone treated animals had 18.83 ± 1.36 × 10⁶ cells/femur. Moreover, the number of α-esterase positive cells was found to
Figure 4.1

Effect on total WBC count

Mice were treated with test compounds for 5 days. Blood was collected from tail vein before treatment and on every 3rd day for a period of one month.
Table 4.1

A) Effect on relative weight and cellularity of lymphoid organs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight (g)</td>
<td>Cellularity (x10^6)</td>
</tr>
<tr>
<td>Control</td>
<td>0.397 ± 0.024</td>
<td>16.2 ± 0.83</td>
</tr>
<tr>
<td>A. lanata</td>
<td>0.468±0.017*</td>
<td>20.86 ± 1.85*</td>
</tr>
<tr>
<td>10-MC</td>
<td>0.462±0.028*</td>
<td>20.16 ± 1.34*</td>
</tr>
<tr>
<td>Thujone</td>
<td>0.441±0.033*</td>
<td>19.47 ± 1.19*</td>
</tr>
</tbody>
</table>

B) Effect on bone marrow cellularity and α esterase positive cell number

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bone marrow cellularity (cells/femur x 10^6)</th>
<th>No of α esterase positive cells per 4000 bone marrow cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.16 ± 0.98</td>
<td>851.3 ± 12.24</td>
</tr>
<tr>
<td>A. lanata</td>
<td>22.33 ± 1.03*</td>
<td>1276 ± 9.97*</td>
</tr>
<tr>
<td>10-MC</td>
<td>23.08 ± 0.86*</td>
<td>1283.16 ± 21.10*</td>
</tr>
<tr>
<td>Thujone</td>
<td>18.83 ± 1.36*</td>
<td>1084.6 ± 21.81*</td>
</tr>
</tbody>
</table>

Legend for Table A and B

BALB/c mice were treated with five dose of test compounds and were sacrificed 24h after the last dose. Spleen and thymus were processed to obtain single cell suspension and cellularity determined. Organ weight expressed as g/100g body weight. Bone marrow cells were collected from femur and made into single cell suspension. The bone marrow cellularity and α-esterase positive cells were determined. Values are mean ± SD. *P < 0.05.
be increased significantly to 1276 ± 9.97, 1283.16 ± 21.10 and 1084.6 ± 21.81 cells/4000 cells in A. lanata, 10-MC and Thujone treated animals.

4.3.4. Effect on proliferation of lymphoid cells; spleenocyte, thymocyte and bone marrow cells

a) Spleen blastogenesis

The effect of ethanolic extract of A. lanata, 10-MC and Thujone on blastogenesis of spleen cells is given in Table 4.2.A. Significant enhancement in the proliferation of spleen cells from Aerva extract (4155 ± 41 counts per minute - cpm) and 10-MC (4293 ± 193 cpm) treated animals was observed when compared with the normal spleen cells (1752 ± 46 cpm). Spleen cells from control animals incubated along with LPS had rate of proliferation of 4150 ± 33 cpm, which was increased by 37.08%, 35.05% and 14.16% respectively in spleen cells from A. lanata, 10-MC and Thujone treated animals. Administration of A. lanata, 10-MC and Thujone also enhanced the mitogenic potential of PWM (28.29%, 31.19% and 8.46% respectively), PHA (35%, 33.14% and 21.11% respectively), and Con-A (29.27%, 27.91% and 13.94% respectively) on spleen cell proliferation.

b) Thymocyte blastogenesis

The effect of A. lanata, 10-MC and Thujone on thymocyte proliferation is presented in Table 4.2.B. Thymocyte blastogenesis observed after stimulation by the mitogens such as PHA (4043 ± 41 cpm), Con A (4508 ± 33 cpm) and PWM (4255 ± 50 cpm) was significantly increased to 4511 ± 36 cpm, 4744 ± 60 cpm and 4675 ± 24 cpm, respectively in A. lanata treated animals. Treatment of animals with 10-MC and Thujone for 5 days augmented the mitogenic potential of PWM (14.99% and 5.89% respectively), PHA (12.98% and 5.34% respectively), and Con-A (9.78% and 7.64% respectively) on thymocyte proliferation.

c) Bone marrow blastogenesis

Treatment with A. lanata, 10-MC and Thujone significantly enhanced proliferation of normal bone marrow (2776 ± 24 cpm, 2637 ± 102 cpm and 1932 ± 81 cpm respectively) when compared with the untreated bone marrow cells (1268 ± 29 cpm). There was further enhancement in the rate of proliferation of spleen cells from test compound treated treated animals when they were
Table 4.2

A) Effect on proliferation of spleenocytes

<table>
<thead>
<tr>
<th></th>
<th>Without mitogens</th>
<th>Con-A treated</th>
<th>PHA treated</th>
<th>PWM treated</th>
<th>LPS treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1752 ± 46</td>
<td>4381 ± 24</td>
<td>3859 ± 24</td>
<td>4594 ± 24</td>
<td>4150 ± 33</td>
</tr>
<tr>
<td>A. lanata</td>
<td>4155 ± 41*</td>
<td>5659 ± 19*</td>
<td>5210 ± 21*</td>
<td>5894 ± 29*</td>
<td>5689 ± 22*</td>
</tr>
<tr>
<td>10-MC</td>
<td>4293 ± 193*</td>
<td>5604 ± 117*</td>
<td>5138 ± 152*</td>
<td>6027 ± 97*</td>
<td>5604±268*</td>
</tr>
<tr>
<td>Thujone</td>
<td>2074 ± 79*</td>
<td>4992 ± 58*</td>
<td>4674 ± 83</td>
<td>4983 ± 107</td>
<td>4783 ± 81*</td>
</tr>
</tbody>
</table>

B) Effect on proliferation of thymocytes

<table>
<thead>
<tr>
<th></th>
<th>Without mitogens</th>
<th>Con-A treated</th>
<th>PHA treated</th>
<th>PWM treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1511 ± 20</td>
<td>4508 ± 33</td>
<td>4043 ± 41</td>
<td>4255 ± 50</td>
</tr>
<tr>
<td>A. lanata</td>
<td>1946 ± 31*</td>
<td>4744 ± 60*</td>
<td>4511 ± 36*</td>
<td>4675 ± 24*</td>
</tr>
<tr>
<td>10-MC</td>
<td>1982 ± 115*</td>
<td>4949 ± 129*</td>
<td>4568 ± 132*</td>
<td>4893 ± 79*</td>
</tr>
<tr>
<td>Thujone</td>
<td>1697 ± 48*</td>
<td>4852 ± 72*</td>
<td>4259 ± 63</td>
<td>4506 ± 37</td>
</tr>
</tbody>
</table>

C) Effect on proliferation of bone marrow cells

<table>
<thead>
<tr>
<th></th>
<th>Without mitogens</th>
<th>Con-A treated</th>
<th>PHA treated</th>
<th>PWM treated</th>
<th>LPS treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1268 ± 29</td>
<td>1969 ± 32</td>
<td>3530 ± 38</td>
<td>2216 ± 46</td>
<td>3718 ± 36</td>
</tr>
<tr>
<td>A. lanata</td>
<td>2776 ± 24*</td>
<td>2832 ± 41*</td>
<td>4404 ± 32*</td>
<td>3788 ± 36*</td>
<td>4215 ± 46*</td>
</tr>
<tr>
<td>10-MC</td>
<td>2637 ± 102*</td>
<td>2782 ± 193*</td>
<td>4295 ± 140*</td>
<td>3842 ± 89</td>
<td>4169 ±124*</td>
</tr>
<tr>
<td>Thujone</td>
<td>1932 ± 81</td>
<td>2395 ± 126</td>
<td>4027 ± 64</td>
<td>2785 ± 93</td>
<td>4052 ± 147</td>
</tr>
</tbody>
</table>

BALB/c mice were treated with five dose of test compounds and were sacrificed 24h after the last dose. Spleen, thymus and bone marrow were processed to obtain single cell suspension. Cells were cultured in the presence and absence of various mitogens for 48h in RPMI-1640 medium. Rate of proliferation determined by $^3$H-thymidine incorporation assay.
incubated with mitogens. Mitogenic activity of PHA (3530 ± 38 cpm), PWM (2216 ± 46 cpm), Con A (1969 ± 32 cpm) and LPS (3718 ± 36 cpm) on proliferation of bone marrow cells from control animals were significantly increased in animals treated A. lanata, 10-MC and Thujone (Table 4.2.C).

4.3.5. Effect on circulating antibody titre

Enhancement in the total antibody production by the administration of A. lanata, 10-MC and Thujone is shown in Figure 4.2.A. The level of antibody reached a titre value of 1024 on 15th day and maintained till day 18 in A. lanata treated animals. 10-MC treated animals had a peak value of 1024 on 12th day and maintained up to 15th day, while the control animals showed a maximum antibody titre value of 128 on 15th day after antigen administration. A maximum antibody titre value of 512 was observed on 15th day in Thujone treated animals and this level was maintained up to 18th day.

4.3.6. Effect on plaque forming cells in spleen

The effect of ethanolic extract of A. lanata, 10-MC and Thujone on the number of plaque forming cells (PFC) is shown in Figure 4.2.B. Aerva extract treated animals had a maximum of 243.33 PFC/10^6 spleen cells on 6th day after antigen administration while 10-MC treated animals had 250.67 PFC/10^6 spleen cells at the same time point. The maximum number of plaque forming cells in the Thujone treated group (205.33 ± 4.37 PFC/10^6 spleen cells) was observed on the 6th day while in control animals a maximum of 160.83 ± 4.22 PFC/10^6 spleen cells was observed on the same day.

4.3.7. Effect on cell-mediated immune response in normal and tumour bearing animals

a) Natural killer cell mediated target cell lysis

The effect of A. lanata, 10-MC and Thujone on NK cell mediated target cell cytotoxicity in normal and tumour-bearing animals is shown in Figure 4.3.A. Administration of A. lanata and 10-MC significantly enhanced the NK cell activity in tumour-bearing (2.17 fold on 5th day and 1.42 fold on 7th day respectively) as well as normal animals (2.34 fold on 5th day and 1.52 fold on 7th day respectively) and this occurred much earlier when compared with tumour
**Figure 4.2**

**A) Effect on circulating antibody titre**

Mice were treated with test compounds for five consecutive days followed by immunization with SRBC along with the last dose. Blood was collected from tail vein before treatment and on every 3rd day for a period of one month. Antibody titre determined using SRBC as antigen.

**B) Effect on plaque forming cells in spleen**

Mice were treated with test compounds for five consecutive days followed by immunization with SRBC along with the last dose. Animals were sacrificed on different days starting from 3rd day after immunization up to 9th day. Spleen cells were used to determine antibody producing cells by Jerne's plaque assay.
control animals. In Thujone treated tumour-bearing animals, maximum lysis of target cells (33.24%) was seen on 5th day after tumour induction, whereas in tumour control animals, the peak lysis (18.82%) was observed only on day 9. Normal animals treated with Thujone also showed an earlier enhancement of NK cell mediated lysis and the peak activity (34.30%) was observed on day 5.

b) Antibody-dependent cell-mediated cytotoxicity (ADCC)

Treatment with *A. lanata*, 10-MC and Thujone significantly augmented ADCC in tumour-bearing as well as normal animals (Figure 4.3.B). In Aerva extract and 10-MC treated tumour-bearing animals, maximum lysis of target cells was 43.28% on 9th day and 30.76% on 19th day respectively, whereas in tumour control animals maximum activity (14.77%) was observed only on 15th day. Peak lysis of target cells in *A. lanata* (39.93%) and 10-MC (38.59%) treated normal animals was observed on 9th day and 11th day respectively. Thujone treated tumour-bearing animals had 2.01 fold increase in ADCC while Thujone treated normal animals had 1.82 fold increase on 9th day when compared with control.

c) Antibody-dependent complement-mediated cytotoxicity (ACC)

The effect of *A. lanata*, 10-MC and Thujone on ACC in normal and tumour bearing animals is given in Figure 4.3.C. Thujone treatment significantly enhanced ACC in tumour-bearing as well as normal animals. In tumour control animals, maximum cell lysis (13.2%) was observed only on day 17, while *A. lanata*, 10-MC and Thujone treated tumour bearing animals animals had the peak value at an earlier time point itself (27.9% on 15th day, 23.8 on 11th day and 17.9% on 15th day respectively).

4.3.8. Effect on IL-2 and IFN-γ production in normal and tumour bearing animals

The effect of *A. lanata*, 10-MC and Thujone on serum IL-2 and IFN-γ levels is presented in Table 4.3. Animals treated with *A. lanata* had a significantly increased IL-2 and IFN-γ levels (18.29 ± 0.48 pg/ml and 3183.90 ± 14.03 pg/ml) when compared to normal animals (10.44 ± 0.58 pg/ml and 262.71 ± 12.08 pg/ml). In tumour control group, IL-2 level were markedly reduced on 6th day (7.77 ± 0.32 pg/ml) after tumour inoculation, whereas treatment with *A. lanata*,
Figure 4.3

A) Effect on NK cell activity

B) Effect on ADCC

C) Effect on ACC

Tumour bearing and normal mice were treated with test compounds 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 EAC cells. All results expressed as percentage cell lysis.
Table 4.3

Effect on IL-2 and IFN-γ production in normal and 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>Control</td>
<td>10.44 ± 0.58</td>
<td>262.71 ± 12.08</td>
</tr>
<tr>
<td>Tumour control</td>
<td>7.77 ± 0.32*</td>
<td>1993.22 ± 117.36*</td>
</tr>
<tr>
<td>A. lanata</td>
<td>18.29 ± 0.48*</td>
<td>3183.90 ± 14.03*</td>
</tr>
<tr>
<td>Tumour+A. lanata</td>
<td>12.80 ± 0.30*</td>
<td>2888.14 ± 26.79*</td>
</tr>
<tr>
<td>10-MC</td>
<td>17.36 ± 0.72*</td>
<td>3092.37 ± 132.08*</td>
</tr>
<tr>
<td>Tumour+10-MC</td>
<td>13.41 ± 0.56*</td>
<td>2791.24 ± 94.26*</td>
</tr>
<tr>
<td>Thujone</td>
<td>13.77 ± 0.87*</td>
<td>3019.49 ± 49.71*</td>
</tr>
<tr>
<td>Tumour+Thujone</td>
<td>11.05 ± 0.37*</td>
<td>2710.17 ± 52.54*</td>
</tr>
</tbody>
</table>

Normal and tumour bearing BALB/c mice were treated with five dose of test compounds and were sacrificed on 6th day after tumour induction. Serum was separated and the cytokine level was estimated by ELISA method. Values are mean ± SD. *P < 0.05 compared with control.
10-MC and Thujone restored IL-2 production in tumour bearing mice (12.80 ± 0.30 pg/ml, 13.41 ± 0.56 pg/ml and 11.05 ± 0.37 pg/ml respectively). The serum IFN-γ levels were also enhanced by the treatment with test compounds. In tumour bearing animals the level of IFN-γ was found to be elevated after administration of A. lanata (2888.14 ± 26.79 pg/ml), 10-MC (2791.24 ± 94.26 pg/ml) and Thujone (2710.17 ± 52.54 pg/ml) when compared with tumour bearing control animals (1993.22 ± 117.36 pg/ml).

4.3.9. Effect on cytotoxic T lymphocyte generation

a) Effect on in vivo generation of CTL

Effect of Thujone on CTL generation in vivo is given in Table 4.3.A. Administration of A. lanata, 10-MC and Thujone significantly enhanced the life span of tumour bearing animals. The life span of untreated EL-4 tumour bearing animals was 31.4 days. Administration of A. lanata significantly enhanced the life span to 43.4 days, with an increase of 38.22% in their life spans, whereas EL-4 bearing animals treated with 10-MC and Thujone had 33.15% and 15.57% increase in life span. When animals received EL-4 cells after Winn’s neutralization with normal alloimmunized effector cells, they survived for 37.8 days only (%ILS 20.38). Animals injected with EL-4 cells incubated with alloimmunized spleen cells from A. lanata, 10-MC and Thujone treated mice also showed enhancement in survival (44.27%, 51.49% and 36.46%).

b) Effect on in vitro CTL generation

The effect of A. lanata, 10-MC and Thujone on the in vitro CTL generation by mixed lymphocyte culture is presented in Table 4.3.B. The animals induced with EL-4 cells alone survived for 31.4 days. The life span of animals were increased when they received EL-4 cells after Winn’s neutralization with effector cells from cocultured normal spleen cells (%ILS 26.43%). Survival was increased when the animals received EL4 cells incubated with effector cells from A. lanata, 10-MC and Thujone treated spleen cells (50.32%, 59.64% and 57.17%).
Table 4.4

A) Effect on \textit{in vivo} generation of CTL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days survived</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL-4 alone</td>
<td>31.4 ± 2.6</td>
<td>-</td>
</tr>
<tr>
<td>EL-4 + Normal alloimmunized effector cell</td>
<td>37.8 ± 1.2</td>
<td>20.38</td>
</tr>
<tr>
<td>EL-4 + \textit{A. lanata}</td>
<td>43.4 ± 3.6*</td>
<td>38.22</td>
</tr>
<tr>
<td>EL-4 + \textit{A. lanata} -treated alloimmunized spleen cells</td>
<td>45.3 ± 2.7#</td>
<td>44.27</td>
</tr>
<tr>
<td>EL-4 + 10-MC</td>
<td>41.81 ± 1.82*</td>
<td>33.15</td>
</tr>
<tr>
<td>EL-4 + 10-MC -treated alloimmunized spleen cells</td>
<td>47.57 ± 2.64#</td>
<td>51.49</td>
</tr>
<tr>
<td>EL-4 + Thujone</td>
<td>36.29 ± 3.26 *</td>
<td>15.57</td>
</tr>
<tr>
<td>EL-4 + Thujone -treated alloimmunized spleen cells</td>
<td>42.85 ± 4.96#</td>
<td>36.46</td>
</tr>
</tbody>
</table>

B) Effect on \textit{in vitro} generation of CTL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days survived</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL-4 alone</td>
<td>31.4 ± 2.6</td>
<td>-</td>
</tr>
<tr>
<td>EL-4 + Normal cocultured spleen cells</td>
<td>39.7 ± 1.6</td>
<td>26.43</td>
</tr>
<tr>
<td>EL-4 + \textit{A. lanata} -treated cocultured spleen cells</td>
<td>47.2 ± 2.4*</td>
<td>50.32</td>
</tr>
<tr>
<td>EL-4 + 10-MC -treated cocultured spleen cells</td>
<td>50.13 ± 3.28*</td>
<td>59.64</td>
</tr>
<tr>
<td>EL-4 + Thujone -treated cocultured spleen cells</td>
<td>44.62 ± 4.07*</td>
<td>57.17</td>
</tr>
</tbody>
</table>

All groups of animals received EL-4 cells (5×10$^4$ cells) with and without treatment with effector cells produced \textit{in vivo} by different types of treatments (Table A)/ \textit{in vitro} by mixed lymphocyte culture (Table B). CTL activity was determined by Winn’s neutralization assay and animals were observed for survival. Values are mean ± SD. *P < 0.05 compared with EL-4 alone group. #P < 0.05 compared with EL-4 alone group.
4.4. DISCUSSION

In the past 50 years, cancer therapy has adopted a direct strategy in the form of cytotoxic drugs for therapeutic purposes. Drawbacks of this method, such as drug-resistant clone selection and the destruction of normal cells, denote a window of opportunity for immunostimulatory natural products.

The humoral and cell mediated immune responses are involved in fighting against the altered self-cells and pathogens. Parameters like total WBC count, weight and cellularity of lymphoid organs, bone marrow cellularity and number of α- esterase positive bone marrow cells were found to be significantly increased with treatment of ethanolic extract of *A. lanata* (25mg/Kg body weight), 10-MC (0.5mg/Kg body weight) and Thujone (1mg/Kg body weight, which indicates the immunostimulatory activity in BALB/c mice. The radioactive $^3$H-thymidine incorporation assay shows that increase in cell count is mediated by enhancement in the rate of proliferation of splenocytes, thymocytes and bone marrow cells. This effect on proliferation of splenocytes may be due to reduction in the threshold levels for the mitogens needed to induce cell proliferation or by unmasking of the mitogen receptors on cell surface (Blitstein-Willinger *et al.*, 1976). The humoral immune response is depended on antibody production by cells of B-lymphocyte lineage. Animals treated with *A. lanata*, 10-MC and Thujone showed a significant enhancement in the circulating antibody titre on the 15th and 18th day after antigen administration. The elevated levels of titre value remained for several days indicating that immunological activity is sustained even after the treatment. A similar set of *in vivo* study with BALB/c mice using sheep RBC as antigen showed a significant increase in the antibody-producing cell in the spleen of antigen stimulated- *A. lanata*, 10-MC and Thujone treated animals, which indicates stimulated humoral response.

Cell mediated immune reactions are mediated by cells of T-lymphocyte lineage, mainly composed of NK cells and CTLs along with macrophages, and forms the first line of innate defence against cancer cells and virus-infected cells (Liu *et al.*, 2006). NK cells are large granular lymphoid cells that mediate non-specific target cell killing without prior sensitization (Cooper *et al.*, 2001). NK cells have Fc receptors on their surface that allow them to kill antibody-coated target cells by ADCC, thus providing another form of immune response.
Experimental data shows that treatment with *A. lanata*, 10-MC and Thujone significantly increased the NK cell activity in normal as well as tumour bearing animals. The lymphokine IL-2, alone or in combination with interferons are capable of promoting the lytic activity of NK cells (Kuyljenstierna *et al.*, 2011). Administration of *A. lanata*, 10-MC and Thujone significantly enhanced the production of IFN-γ and IL-2 in normal as well as tumour-bearing mice, and this in turn contributes to NK cell mediated tumour cell destruction. Augmentation of ADCC by test compounds administration contributed to significant enhancement cellular immune responses in both normal and tumour bearing animals. A similar enhancement of ACC by the administration *A. lanata*, 10-MC and Thujone indicates the activation of cell mediated immune system.

Cytotoxic T lymphocytes, the major effector cells with lytic capability, do not require co-stimulation to kill infected targets though perforin/granzyme B pathway. CTLs are capable of lysing a variety of tumour cells *in vitro* (Kobayashi *et al.*, 1990). EL4 is dimethylbenzanthracine induced lymphoma of C57BL/6 (Gorer, 1950). In this study, CTL were generated *in vivo* as well as *in vitro*, both in presence and absence of test compounds, and target cell lytic activity was determined by Winn's neutralization assay. The increased life span of treated animals compared with control suggests that *A. lanata*, 10-MC and Thujone could increase the CTL production and activity, thereby killing the tumour cells.

In the present study, the test compounds revealed their ability to stimulate the humoral arm of immune system under normal conditions and also to boost the cell mediated immune responses resulting in inhibition of tumour progression.