Chapter 3

Emilia sonchifolia (L.) DC: Effect on the immune system
3.1. Introduction

Immune system acts as a vital defence system against tumours, cancerous growth, and infectious diseases. Many of the plant based products are capable of positively altering the host immune response by triggering the defence cells of the immune system, and thereby act as potent immunomodulatory agents. In some disease treatments immunostimulatory agents are required to overcome the immunosuppression induced by drugs or environmental factors, and in others when there is undesired immunopotentiation, immunosuppressants are required. Immunomodulatory agents that can improve the immune system are necessary to quash the immunosuppressive effects or in situations where there is an impaired immune responsiveness. Immune response modulatory effects of medicinal plants can provide additional support to conventional therapeutic approaches like chemotherapy (Lin et al., 2015). Study on the immune response modulatory effect of *E. sonchifolia* (George and Kuttan, 2015) was done in order to provide a scientific basis for the conventional use of this plant in the traditional Indian Ayurvedic medicine possibly through modulation of the host immune defence. Previous investigations revealed the antitumor and anti-inflammatory potential of *E. sonchifolia* (Shylesh et al., 2005; Shylesh and Padikkala, 2000; Muko and Ohiri, 2000; Nworu et al., 2012). Thus, the plant will be a better target to be subjected to more studies, which can reveal its multifunctional roles in the physiological system. In the present study, we have focused on analyzing the immunomodulatory effects of the plant on humoral and cell-mediated immune responses in experimental animal models, which could contribute additional evidences to validate the pharmacological uses of the plant. The modification of immune response favourably by enhancement of immunological and nonspecific host defences is an exciting development in the field of immunopharmacology (George and Kuttan, 2015).
3.2. Materials and Methods

Plant material: - *E. sonchifolia* whole plant methanolic extract 25mg/kg b.wt., resuspended in 1% gum acacia.

Dosage: - Plant material was intraperitoneally (i.p) administered for five consecutive days.

Animals: - BALB/c and C57BL/6 mice (4-6 weeks old)

Cell lines: - B16F10 melanoma cells, EL4 thymoma cells and K562 leukemic cells.

3.2.1. Toxicity study

*E. sonchifolia* in different concentrations (200, 100, 50 and 25mg/kg b.wt.) were administrated intraperitoneally in BALB/c mice for 14 days. Animals were observed for mortality, behavioural changes, and change in body weight. On 15th day, all the animals were sacrificed and selected organs such as liver, spleen, thymus, kidney and lungs were dissected out and weights were recorded. Blood was collected by heart puncture; serum separated and used for the analysis of hepatic and renal functions. Liver function markers, such as alkaline phosphatase (ALP) (King, 1965), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980), and kidney function markers such as creatinine (Toro, 1975) and blood urea (Murray, 1984) were determined.

3.2.2. Hematological parameters

Blood was collected from the caudal vein of all the animals (BALB/c mice, n= 6/group) prior to the administration of *E. sonchifolia*, which was continued every third day for one month. The various parameters such as total white blood cell (WBC) count (hemocytometer), differential count (DC) (Leishman’s stain), and hemoglobin content by cyanmethemoglobin method were assessed. In addition, change in body weight was also recorded.
3.2.3. Relative organ weights, bone marrow cellularity, and α-esterase activity

Animals (BALB/c mice, n= 6/group) were weighed 24 h after the last dose of *E. sonchifolia* administration and were sacrificed. Weights of vital organs such as liver, kidney, lungs, spleen, and thymus were recorded and expressed as relative organ weights. Bone marrow cells from the femur were collected and the cell number was determined and expressed as total live cells/femur (Sredni et al., 1992). The numbers of α-esterase-positive cells were determined by the azodye coupling method (Bancroft and Cook, 1984). The numbers of α-esterase-positive cells were expressed out of 4000 cells.

3.2.4. Hemagglutinating antibody titer

The BALB/c mice (n=6/group) were immunized with SRBCs (2.5 × 10^8 cells/animal, i.p.) along with the last dose of *E. sonchifolia* administration. Blood was collected from the caudal vein every third day for a period of 30 days. Serum was separated and heat inactivated at 56^oC for 30 min. Antibody titer was determined by hemagglutination assay using SRBC as antigen (Singh et al., 1984).

3.2.5. Plaque forming cell assay

BALB/c mice (n=21/group) immunized with SRBC (2.5×10^8 cells/animal, i.p.) along with the last dose of *E. sonchifolia* administration. The animals were sacrificed on different days starting from the third day up to the ninth day. Spleens were collected and processed into single cell suspension and the numbers of plaque forming cells (PFCs) were determined by the Jerne’s Plaque assay (Jerne and Nordin, 1963).
3.2.6. Blastogenesis assay

Mitogens can stimulate resting lymphocytes to undergo a series of changes and are converted to blast-like cells. This process leads to cell division and can be quantified by $^{3}$H thymidine incorporation assay. The animals (BALB/c mice, n=6/group) were sacrificed 24h after the last dose of *E. sonchifolia* administration. Lymphoid organs such as spleen, thymus, and bone marrow were collected and processed aseptically into single cell suspension. Then, $5\times10^4$ cells were cultured in a 96-well round-bottomed titer plate in the presence and absence of various mitogens such as phytohemagglutin in (PHA) 2.5μg/ml, concanavalin A (Con A) 10μg/ml, pokeweed mitogen (PWM) 10μg/ml, and ipopolysaccharide (LPS) 10μg/ml in a humidified 5% CO$_2$ atmosphere at 37°C and incubated for 48h. All the cells were labelled with 1μCi of $^{3}$H-thymidine and further incubated for 18h. After incubation, DNA was precipitated using 10% ice-cold perchloric acid, centrifuged and the pellets were dissolved in 0.5ml of 6 N NaOH and transferred to 5ml scintillation fluid. Radioactivity was measured using Rack Beta fluid scintillation counter.

3.2.7. Determination of the effect of *E. sonchifolia* on the cell-mediated immune response by cytotoxic T lymphocytes (CTL) production

**Winn’s neutralization test:** CTL activity was assessed using Winn’s neutralization test according to the method of (Kobayashi et al., 1992). Briefly, 1ml (1×10$^7$) of effector cells (spleen cells) was mixed with the same volume containing 5×10$^5$ EL4 cells (target cells). The cells were incubated for 1h at 37°C in 5% CO$_2$ atmosphere, and 0.2ml of this mixture was injected intraperitoneally to BALB/c mice. The animals were observed for survival time period. The survival times of treated animals were compared with those animals that received tumour cells alone. Increase in survival days was directly related to the CTL activity.

Alloimmunization was carried out by injecting spleen cells (2×10$^7$) from C57BL/6 mice, subcutaneously to BALB/c mice. Two systems were used to generate effector cells from the spleen cells of BALB/c mice.
System A

Spleen cells (effector) were obtained 7 days after alloimmunization of BALB/c mice with spleen cells from C57BL/6 mice as described above. Four groups of BALB/c mice (n = 10/group) were used. Group I received EL4 cells alone (5×10^5/0.1 ml), group II animals received *E. sonchifolia* along with EL4 cells. Group III animals received EL4 cells incubated with normal spleen cells (0.2ml) from the BALB/c mice and group IV animals received EL4 cells incubated with spleen cells from *E. sonchifolia*-treated alloimmunized mice. All the animals were observed for survival. Blood was collected from the caudal vein of each animal 2 days after the last dose of *E. sonchifolia* administration, and levels of IL-2 and IFN-γ were determined using ELISA kits according to the manufacturer’s protocol.

System B

Effector cells were produced by a 5 day mixed lymphocytes culture (MLC) of spleen cells (responder cells) from *E. sonchifolia* treated and untreated BALB/c mice and mitomycin treated (50μg/ml) spleen cells (stimulator cells) from C57BL/6 mice. Three groups of animals (n= 10/group) were used. Group I animals received EL4 cells alone, group II animals received EL4 cells cocultured with effector cells generated using normal spleen cells and group III animals received EL4 cocultured with effector cells generated using spleen cells from *E. sonchifolia* treatment.

3.2.8. Determination of the effect of *E. sonchifolia* on cell mediated immune response (CMI) in B16F10 melanoma bearing animals

Mice (C57BL/6) were divided into 3 groups (n=36/group). Group I animals received B16F10 cells (1x10^6 cells/animal) intravenously through lateral tail vein and kept as untreated control, group II animals were treated with *E. sonchifolia* for 5 consecutive days and group III animals received B16F10 cells (1x10^6 cells/animal) intravenously after the last dose of *E. sonchifolia*
administration. Starting from 24 hour after tumour induction three animals from each group were sacrificed; spleen and blood were collected on different time intervals. Spleen cells were processed to single cell suspension and used as effector cells for Natural Killer (NK) cell activity and antibody dependent cellular cytotoxicity (ADCC) by $^{51}$Cr release assay (Kim et al., 1980). $^{51}$Cr binds to cytoplasmic proteins after diffusing through the cell membrane and is released only when the cell membrane is sufficiently damaged. Serum was used for the estimation of antibody dependent complement mediated cytotoxicity (ACC) by trypan blue exclusion method (Talwar, 1983).

Labelling of target cells

Target cells K562 ($10^6$) and SRBC ($10^7$) was done by adding 100µCi of Na$_2^{51}$CrO$_4$ and incubation for 1 hr at 37°C on a shaker. Cells were washed and further incubated at 4°C for 1 hr.

Percentage of cell lysis = \[
\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total releases} - \text{Spontaneous release}} \times 100
\]

Spontaneous release (SR) - wells contained only target cells and medium.
Total release (TR) - wells contained target cells, medium and 100 µl of 1N HCl.

A. Determination of NK cell mediated cytotoxicity

100µl of labelled target cells K562 ($1\times10^4$ cells/ml) were mixed with effector spleen cells in 96-well round bottom titre plates to yield an effector: target ratios of 100:1. Final volume was adjusted to 200µl and incubated at 37°C for 4 h and $^{51}$Cr release assay was performed. For this titre plates were centrifuged for 15 minutes, 100µl of supernatant was collected and radioactivity measured using gamma counter. Control tubes were kept for each experiment.
B. Determination ADCC

By mixing 100µl of labelled SRBC (1x10^4 cells/ml) that act as target cells and spleen cells (effector cells) in an effector-target ratio of 100:1. 100µl of anti-sera against SRBC was added to this and incubated at 37°C for 4h, and ^51^Cr release assay was done as explained above.

C. Determination ACC

ACC was done using the heat inactivated (56°C for 30 min) serum. Fresh rabbit serum was used as a source of complement for the reaction. Serum samples were mixed with 100µl of B16F10 (1x10^4 cells) and 50µl of complement. Final volume was made up to 2ml and incubated at 37°C for 3h. Cytotoxicity was assessed by trypan blue exclusion method

3.3. Results

3.3.1. Toxicological evaluation of *E. sonchifolia*

The no-observed-adverse-effect level (NOAEL) of *E. sonchifolia* was 50mg/kg b.wt. *E. sonchifolia*, when administered at doses of 25 and 50 did not produce any mortality and change in behaviour, body weight, relative organ weight, hepatic and renal functions when compared with control group. Meanwhile, administration of 100mg/kg b.wt. produced a nonsignificant increase in the serum GPT and blood urea when compared to control and 50mg/kg b.wt. groups. *E. sonchifolia* at 200mg/kg b.wt. showed observable toxicity showing behaviour changes, hypoactivity, weight loss, and decrease in the relative organ weight with altered hepatic and renal functions (table 3.1). Hence, the immunomodulatory study was conducted using three different concentrations of *E. sonchifolia* (10, 25, and 50mg/kg b.wt.), which are observed to be nontoxic. Since *E. sonchifolia* 25 and 50mg/kg showed similar effects on the immune parameters and also the concentration of 10mg/kg did not show any significant
Table 3.1. Toxicity profile of *E. sonchifolia*

<table>
<thead>
<tr>
<th>Concentrations of <em>E. sonchifolia</em> (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

Mortality (D/T)  
None  
None  
None  
None  
None

Behavioural change  
None  
None  
None  
None  
Hypo activity

Change in body weight (g)  
+2.01±0.2  
+2.08±0.8  
+2.05±1.1  
+1.76±0.6  
-0.98±0.5

Relative organ weights (g/100 g body weight)

1. Liver  
5.39±0.23  
5.38±0.17  
5.34±0.19  
5.07±0.18  
4.77±0.25

2. Spleen  
0.39±0.02  
0.41±0.02  
0.40±0.02  
0.36±0.02  
0.31±0.03

3. Thymus  
0.12±0.01  
0.11±0.01  
0.11±0.01  
0.10±0.01  
0.09±0.02

4. Kidney  
1.34±0.15  
1.34±0.13  
1.30±0.12  
1.27±0.12  
1.23±0.07

5. Lungs  
0.61±0.02  
0.62±0.02  
0.60±0.04  
0.59±0.03  
0.57±0.03

Serum ALP (U/ml)  
13.37±0.42  
13.31±0.5  
13.96±0.78  
14.42±0.78  
24.57±2.01

Serum GPT (U/ml)  
58.1±3.62  
54.9±2.6  
58.72±3.22  
67.96±7.10  
129.28±2.7

Blood urea (mg/dl)  
42.88±1.17  
43.06±1.1  
43.93±1.12  
50.17±2.38  
53.22±1.07

Serum creatinine (mg/dl)  
0.92±0.01  
0.91±0.02  
0.92±0.03  
0.95±0.03  
1.37±0.15

Values are the mean ± standard deviation.

Abbreviations: D/T, dead/treated mice; ALP, alkaline phosphatase; GPT, glutamate pyruvate transaminase.

“None” means that no toxic symptoms were seen during the observation period.
effects, the data of minimum dose, which is nontoxic but immunologically effective (25mg/kg b.wt.), were shown.

3.3.2. Effect on hematological parameters

The total WBC count of *E. sonchifolia* treated normal BALB/c mice were increased significantly (p<0.001) to 9995±535 cells/mm$^3$ on the sixth day compared to control animals (figure 3.1). There was no significant difference in the DC, hemoglobin content, and body weight (data not shown) of the animals after treatment with *E. sonchifolia* compared to control groups.

3.3.3. Effect on relative organ weights, bone marrow cellularity, and α-esterase activity

There was a significant (p<0.001) increase in the weight of thymus and spleen after administration of *E. sonchifolia* when compared with the control groups. There was no significant change in the weight of other vital organs such as liver, kidney, and lungs (table 3.2). This shows the stimulatory effect of *E. sonchifolia* on the lymphoid organs such as spleen and thymus. The effect of *E. sonchifolia* on the bone marrow cellularity and the increase in the number of α-esterase positive cells were also significant (p< 0.001) compared to control groups (table 3.3).

3.3.4. Effect on hemagglutinating antibody titer and PFC assay

*Emilia sonchifolia* administration produced a titer value of 1024, up to which the agglutination of the antibody formed against SRBC antigen was recorded. This value was obtained in the serum collected on the 12th day. The control animals showed a maximum antibody titer value of 128 on the same day (figure 3.2). There was a significant (p<0.001) increase in the number of PFCs, which showed a clear area of lysis of SRBC in the *E. sonchifolia* treated group (257±23 PFC/10$^6$ spleen cells) on the sixth day compared to the control groups of animals (149.3±9.4 PFC/10$^6$ spleen cells) (figure 3.3).
Figure 3.1. Total WBC count of animals treated with *E. sonchifolia*

![Graph showing Total WBC count over time for different treatments.](image)

***p < 0.001

Figure 3.2. Hemagglutinating antibody titer of animals treated with *E. sonchifolia*

![Graph showing Hemagglutinating antibody titer over time for different treatments.](image)
Figure 3.3. Plaque forming cell assay of animals treated with *E. sonchifolia*

***p < 0.001, **p < 0.01, *p < 0.05
Table 3.2. Effect of *E. sonchifolia* on the relative organ weights (g/100g body weight)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.43±0.01</td>
<td>0.13±0.01</td>
<td>5.76±0.12</td>
<td>1.56±0.07</td>
<td>0.63±0.03</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.44±0.02</td>
<td>0.12±0.02</td>
<td>5.73±0.11</td>
<td>1.56±0.06</td>
<td>0.61±0.04</td>
</tr>
<tr>
<td><em>E. sonchifolia</em></td>
<td>0.52±0.01 ***</td>
<td>0.18±0.01 ***</td>
<td>5.75±0.10</td>
<td>1.54±0.04</td>
<td>0.64±0.02</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *** p< 0.001 compared with control groups.

Table 3.3. Effect of *E. sonchifolia* on the Bone marrow cellularity and the number of α-esterase positive cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bone marrow cellularity (1x10^6 Cells /femur)</th>
<th>α-esterase positive cells (No of +ve cells/4000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16.08±0.74</td>
<td>817.00±25.13</td>
</tr>
<tr>
<td>Vehicle</td>
<td>17.08±0.38</td>
<td>825.33±29.87</td>
</tr>
<tr>
<td><em>E. sonchifolia</em></td>
<td>22.92±1.69 ***</td>
<td>1186.00±118.24 ***</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *** p< 0.001 compared with control groups.
3.3.5. Effect on blastogenesis assay of splenocytes, thymocytes, and bone marrow cells

Significant (p<0.05) enhancement in the rate of proliferation of spleen, thymus, and bone marrow cells was observed in the *E. sonchifolia* treated group of animals compared with control groups. Administration of *E. sonchifolia* also enhanced significantly (p<0.01) the mitogenic potential of various mitogens such as LPS, PWM, PHA, and Con A in spleen and bone marrow cell proliferation. In *E. sonchifolia*-treated group of animals, the rate of thymocytes proliferation was increased significantly (p<0.001) in the presence of mitogens excluding LPS (table 3.4).

3.3.6. Effect on CTL production

**System A**

Effect of *E. sonchifolia* on *in vivo* CTL generation is given in table 3.5A. There was a 44% increase in the lifespan of animals treated with five doses of *E. sonchifolia* when compared with the untreated tumour bearing group (EL4 alone). However, in animals injected with EL4 cells incubated with normal alloimmunized effector cells, the percentage increase in lifespan was only 23%. But when the animals received EL4 cells incubated with *E. sonchifolia* treated alloimmunized spleen cells, the number of survival days was significantly (p<0.001) increased to 69%, showing the *in vivo* generation of CTL that can destroy sensitive thymoma cells (EL4) and decrease mortality due to tumour burden. The effect of *E. sonchifolia* on the production of IL-2 and IFN-γ by EL4-bearing animals incubated with *in vivo* generated effector cells is presented in table 3.5B. Administration of *E. sonchifolia* significantly increased the level of cytokines, which are involved in immunomodulation, when compared to administration of tumour alone and tumour plus normal alloimmunized spleen cells.
### Table 3.4. Effect of *E. sonchifolia* on spleen, thymus and bone marrow blastogenesis

<table>
<thead>
<tr>
<th></th>
<th>Without mitogens</th>
<th>Con A</th>
<th>PHA</th>
<th>PWM</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Splenocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1736±113</td>
<td>4582±127</td>
<td>3487±123</td>
<td>3745±176</td>
<td>4062±208</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1709±104</td>
<td>4668±101</td>
<td>3545±130</td>
<td>3697±145</td>
<td>4048±227</td>
</tr>
<tr>
<td><em>E. sonchifolia</em></td>
<td>1940±98*</td>
<td>4857±128</td>
<td>3850±163</td>
<td>4128±176</td>
<td>4469±128**</td>
</tr>
<tr>
<td><strong>Thymocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1686±96</td>
<td>3648±105</td>
<td>3026±108</td>
<td>3524±102</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1720±121</td>
<td>3765±110</td>
<td>3181±103</td>
<td>3652±108</td>
<td>-</td>
</tr>
<tr>
<td><em>E. sonchifolia</em></td>
<td>1896±103*</td>
<td>4680±125***</td>
<td>4201±213***</td>
<td>4881±101***</td>
<td>-</td>
</tr>
<tr>
<td><strong>Bone marrow cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1325±101</td>
<td>1472±104</td>
<td>2835±126</td>
<td>2165±128</td>
<td>3396±161</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1346±109</td>
<td>1502±105</td>
<td>2869±159</td>
<td>2121±158</td>
<td>3438±129</td>
</tr>
<tr>
<td><em>E. sonchifolia</em></td>
<td>1524±139*</td>
<td>1765±134**</td>
<td>3156±103**</td>
<td>2492±190**</td>
<td>3712±108**</td>
</tr>
</tbody>
</table>

Values are mean of the rate of proliferation in Counts per minute (CPM) ± SD.

***p < 0.001, **p < 0.01, *p < 0.05 compared with control groups
### Table 3.5A. Effect of *E. sonchifolia* on the CTL generation and survival

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of days survived</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>System A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EL4 alone</td>
<td>31 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>EL4 + <em>E. sonchifolia</em></td>
<td>48.6 ± 2.1(^a)</td>
<td>44</td>
</tr>
<tr>
<td>EL4 + normal alloimmunized effector cell</td>
<td>39.3 ± 3</td>
<td>23</td>
</tr>
<tr>
<td>EL4 + <em>E. sonchifolia</em> treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alloimmunized spleen cells</td>
<td>53.1 ± 3.8(^b)</td>
<td>69</td>
</tr>
<tr>
<td>System B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EL4 alone</td>
<td>32.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>EL4 + normal cocultured spleen cells</td>
<td>39.6 ± 2.4(^a)</td>
<td>20</td>
</tr>
<tr>
<td>EL4 + <em>E. sonchifolia</em> treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cocultured spleen cells</td>
<td>53.2 ± 2.6(^b)</td>
<td>62</td>
</tr>
</tbody>
</table>

System A: All data are expressed as means ± SD. \(^a\) p<0.001 compared with EL4 alone \(^b\) p<0.01 compared with EL4 + normal alloimmunized effector cell. System B: All data are expressed as means ± S.D. \(^a\) p<0.001, compared with EL4 alone, \(^b\) p<0.001 compared with EL4 + normal co-cultured spleen cells.

### Table 3.5B. Effect of *E. sonchifolia* on the cytokine production System A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (pg/mL)</th>
<th>IFN-(\gamma) (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11.9 ± 1.8</td>
<td>2957.4 ± 222.86</td>
</tr>
<tr>
<td>EL4 alone</td>
<td>7.04 ± 0.23</td>
<td>1362.6 ± 101.4</td>
</tr>
<tr>
<td>EL4 + <em>E. sonchifolia</em></td>
<td>19.9 ± 2.38(^a)</td>
<td>3452.43 ± 138.12(^a)</td>
</tr>
<tr>
<td>EL4 + normal alloimmunized effector cell</td>
<td>8.4 ± 1.31</td>
<td>1356.84 ± 92.81</td>
</tr>
<tr>
<td>EL4 + <em>E. sonchifolia</em> treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alloimmunized spleen cells</td>
<td>28.96 ± 2.68(^b)</td>
<td>3690.49 ±104.63(^b)</td>
</tr>
</tbody>
</table>

Results were expressed as means ± S.D. \(^a\) p<0.001 compared with EL4 alone. \(^b\) p<0.001 compared with EL4 + normal alloimmunized effector cell.
System B

The effect of *E. sonchifolia* on the generation of CTL by system B is given in table 3.5A. There was a 20% increase in the lifespan of animals that received EL4 cells incubated with effector cells from cocultured normal spleen cells. When EL4 cells were incubated with *E. sonchifolia* treated alloimmunized effector cells, the lifespan of animals was increased by 62%.

3.3.7. Effect on NK cell activity, ADCC and ACC

*E. sonchifolia* administration enhanced NK cell activity (p < 0.001) in normal as well as in tumour bearing animals as shown in figure 3.4A. On 5th day after tumour induction maximum lysis of target cells 43.2% and 44% was seen in *E. sonchifolia* treated, and in *E. sonchifolia* treated tumour bearing mice respectively. At the same time in untreated tumour bearing control animals, the maximum cell lysis of 18.8% was observed only on day 9. Effect of *E. sonchifolia* on ADCC is represented in figure 3.4B. *E. sonchifolia* treatment augmented ADCC (p <0.001) in tumour-bearing as well as in normal animals. On day 9 in *E. sonchifolia* treated normal as well as in tumour-bearing animals 37% maximum lysis of target cells was seen compared to untreated tumour-bearing control animals that showed a peak lysis of only 9.6%. *E. sonchifolia* administration significantly (p <0.001) enhanced ACC in tumour-bearing as well as in normal animals as shown in figure 3.4C. Maximum activity was observed on day 15 with 21.7% and 22.1% of cell death in normal and tumour bearing *E. sonchifolia* treated mice. Whereas in untreated tumour-bearing control animals, the peak activity of 13.6% cell death was observed on day 17.

3.4. Discussion

Maintenance of health and prevention of and recovery from diseases are important functions attributed to a healthy immune system whose activities are well regulated by cross-linked pathways of cytokines. During pathological conditions such as cancer, the immune tolerance induced by tumour microenvironment helps tumour cell proliferation, survival, and migration
Figure 3.4A. Effect of *E. sonchifolia* on NK cell activity

![Graph showing the effect of *E. sonchifolia* on NK cell activity over days after tumour challenge, with significant p-value (**p < 0.001**).](image)

Figure 3.4B. Effect of *E. sonchifolia* on ADCC

![Graph showing the effect of *E. sonchifolia* on ADCC over days after tumour challenge, with significant p-value (**p < 0.001**).](image)
Figure 3.4C. Effect of *E. sonchifolia* on ACC

**Tumour control**

**E. sonchifolia**

**E. sonchifolia+Tumour**

***p < 0.001***
without being attacked by the immune system (Zou, 2005). Hence, the immune cells should be boosted to get activated to maintain an antitumor defence state, thus enabling the physiological system to overcome the immune suppressive tumour microenvironment (Kwon et al., 2009). The investigations on novel antitumor substances with immunomodulatory effects should be encouraged, and this type of immune response modulation can be considered as an alternative against the currently known toxic side effects of cancer treatment (Xu et al., 2009).

The hematopoietic stem cells present in the bone marrow can give rise to myeloid and lymphoid progenitor cells. These cells then differentiate into various blood cells of corresponding lineages. The preliminary observation itself regarding an increased production of total WBC by *E. sonchifolia* treatment thus signifies the stimulation of the hematopoietic system. Analysis of the treatment effects on the production of bone marrow cells further confirmed the above results, where the bone marrow cells were found actively increased along with the increased rate of their subsequent differentiation in the bone marrow. In order to check whether or how treatment with *E. sonchifolia* could influence the lymphoid organs, lymphoid organ weights as well as their mitogen-induced proliferation were studied. The results were in fact promising, which strongly suggest that *E. sonchifolia* stimulated the proliferation of the cells of lymphoid organs, which in turn caused an increase in organ weights. Since the lymphoid cells (B and T cells) are the major effectors of the immune system, these results directly correlate to the immune stimulatory effects of *E. sonchifolia*. These regulatory effects on the immune cells can be utilized in therapy, especially when the immune system must be alert, as in the case of infections or immune suppressed conditions.

Antibody functions as the effector of humoral immune response by binding to the antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of *E. sonchifolia* on humoral immune response, sheep erythrocyte antigen specific hemagglutinating antibody (HA) production was assayed in SRBC treated mice. In mice treated with *E. sonchifolia*, enhancement in HA titer was
obtained, with a peak value on the 12th day, reflecting an overall elevation in humoral immune response. The PFC assay is considered to be one of the most highly predictive single assays used to assess potential modulation of the humoral immune response, which quantifies the number of B cells producing SRBC specific IgM (Wilson et al., 1999). Treatment with E. sonchifolia markedly increased the number of PFCs, indicating its enhancing effect on humoral immunity. The radioactive 3H-thymidine incorporation assay clearly showed a significantly enhanced proliferation of both B and T lymphocytes in the presence and absence of specific mitogens. The increased proliferation of these lymphoid cells specially points out the influence of E. sonchifolia in the modulation of cell mediated immune response. This effect of E. sonchifolia on proliferation may be due to the reduction in the threshold levels for the mitogens needed to induce cell proliferation or by unmasking of the mitogen receptors on cell surface. From these results, it is confirmed that E. sonchifolia can stimulate the humoral arm of the immune system via B lymphocytes and cell-mediated arm via T lymphocytes.

Cell-mediated immune responses are the major immune effectors during pathogenesis of tumour, which might be mediated mainly by T cells, including NK cells. These cells will produce many factors like macrophage mobile factor, lymphotoxin, and interferon, which will lead to the proliferation and differentiation of immune cells and to macrophage phagocytosis and increase the capacity of killing target cells, and all these will have active participation in preventing the tumour (Kim et al., 2001). Alloimmunization of mice will produce CTLs, resulting in the augmentation of the cytotoxic activity of NK cells (Suzuki et al., 1985). In the present study, Winn’s neutralization assay was used for the generation and activity determination of CTLs. The increased lifespan of treated animals compared with control groups suggests that E. sonchifolia could increase CTL production and thereby cause cytotoxicity to the tumour cells. Mixed lymphocyte culture (MLC) is an in vitro test of lymphocyte recognition and proliferation. The experimental analysis using MLC reaction also showed that E. sonchifolia augmented T-cell-mediated immune response by modulating CTL activity. It was reported that the activated T cells in MLC will produce cytokines, especially IL-2, which was responsible for the differentiation
of precursor into mature effector CTL (Kern et al., 1981). Since cytokines are the key intermediators in the regulation of immune response, we also assessed the effect of *E. sonchifolia* on the production of specific cytokines, such as IL-2 and INF-γ. The proliferating CD4+ will secrete IL-2, which stimulates the proliferation of CTL and helper T lymphocytes, and potently augment the cytolytic activity of NK cells, lymphokine-activated killer cells, and macrophages, all of which can participate in immunological antitumor mechanisms (Mizuno et al., 2000). NK cells, CD8+T cells, and the Th1 subclass of CD4+T cells produce IFN-γ, which is responsible for antitumor activity either by promoting the apoptotic death of target cells through the Fas/Fas ligand pathway or through secretion of perforin and granzymes (Tanaka et al., 2004; Wang et al., 2004). Analysis of serum from tumour bearing mice after treatment with *E. sonchifolia* revealed an increased secretion of IL-2 and IFN-γ. These observations indicated that these cytokines have a key role in regulating the increased CTL response, which in turn results in cytotoxicity to the tumour cells (Kelly et al., 2002; George and Kuttan, 2015). NK cells mediate nonspecific target cell killing, without prior sensitization and they do not need to recognize antigen/MHC on the target cell (Cooper et al., 2001). Fc receptors on NK cell surface allow them to kill antibody-coated target cells by ADCC, thus providing another form of immune defence (Qu and Li, 2010). Treatment with *E. sonchifolia* significantly enhanced the NK cell activity in normal as well as tumour bearing animals along with an enhancement in ADCC and ACC altogether proved the activation of cell mediated immune system.