CHAPTER 6

TO INVESTIGATE THE ANTI-CANCER ACTIVITY OF *Acacia ferruginea* EXTRACT AGAINST DALTON’S LYMPHOMA ASCITES (DLA) INDUCED TUMOR MODELS

6.1 INTRODUCTION

Cancer is the leading cause of death worldwide and the World Health Organization (WHO) reported there were 7.6 million deaths (≈ 13% of all deaths) in 2008 and they estimated this will reach 13.1 million deaths by 2030. Chronic inflammation could lead to cellular proliferation – a process that in and of itself increased the risk for aberrant cell formation and, ultimately, development of cancer (Mantovani et al, 2008; Grivennikov et al, 2010). During tumorigenesis, tumor-infiltrating inflammatory cells will produce variety of cytokines. It has been reported that pro-inflammatory cytokines including TNF-α, Interleukins (IL-1β, IL-6) and GM-CSF contribute to carcinogenesis by persuading the survival, growth, proliferation, differentiation and metastasis of tumor cells (Lazar et al, 2000; Lawrence, 2007).

Conventional cancer therapies include surgery and radiation if the tumor is diagnosed at initial stage and chemotherapy is the treatment of choice for advanced tumors. Although these treatments are effective, it associated with severe adverse events include drug resistance and dose-limiting toxicities such as immunosuppression. Thus, there is need to develop new therapeutic options with low toxicity and minimal side effects. In fact, single antitumor drug may be ineffective because of its unique molecular target with in the tumor cell.
Therefore, presence of multiple compounds in well characterized plant extract with synergistic activities may tackle this difficulty.

Developments of drugs from natural sources that prevent or inhibit tumor growth by down-regulating select inflammatory factors has become of keen interest in the field of drug discovery and anti-cancer therapies. Throughout history, plants have been the most consistently successful source of traditional medicines and continue to provide new remedies and to promote human health and well-being. Several traditionally-used medicinal plants and plant products have become potential sources of anti-cancer agents.

Extensive research on Acacia has been carried out over the past few decades because of their reputed pharmacological effects and low toxicity. Our previous investigation of A. ferruginea extract revealed potent immunomodulatory effect against CTX-induced toxicity/immunosuppression. Hence in the present study, we made an attempt to evaluate the protective effect of A. ferruginea extract against DLA induced solid as well ascites tumor models.

6.2 MATERIALS AND METHODS

6.2.1 Collection of plant material

The fresh aerial parts of the plant were collected from Coimbatore, India. The plant was identified and authenticated at Botanical Survey of India, Coimbatore (No: BSI/SRC/5/23/2011-12/Tech-687). A voucher specimen was retained in the Department of Biotechnology, Karunya University, Coimbatore. The harvested plant samples were washed thoroughly with water and shade-dried at room temperature.
6.2.2 Preparation of extract

The shade-dried aerial parts of the plant were subjected to mechanical size reduction. The powdered material (≈ 25 g) was then extracted with methanol in a Soxhlet apparatus. Traces of solvent were then removed by evaporation and the final extract concentrated in a vacuum rotary system; the percentage yield of extract was 12% [w/w]. Based on toxicity studies, a dose of 10 mg/kg B.wt. was found to be non-toxic and selected for use in the current experiments. For each exposure, the extract was re-suspended in 1% gum acacia for subsequent administration to the mice.

6.2.3 Animals

Male Balb/c mice (4-6-wk-old, 22-25 g) were obtained from the College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University (Mannuthy, Kerala, India). All mice were maintained in a controlled sterile environment maintained at a constant temperature (24±2°C), 50% relative humidity, and a 12-hr light/dark cycle. All mice had ad libitum access to standard diet pellets (Sai Durga Feeds, Bangalore, India) and filtered water. After 2 weeks of acclimatization, the mice were randomly allocated into respective groups. All animal experiments were performed after obtaining approval from Institutional Animal Ethics Committee, Karunya University (Approval No: IAEC/KU/BT/2013/12).

6.2.4 DLA cell line

DLA cells were obtained from the Amala Cancer Research Institute (Thrissur, India) and propagated in the peritoneal cavity of naive BALB/c mice. For instillation into mice in the various treatment groups, cells freshly-aspirated
from these mice were washed with phosphate-buffered saline (PBS, pH 7.4) to remove cell debris and dead cells under sterile conditions. The viability of the cells were checked by Trypan Blue assay and the viable cells ($10^6$ cells) were inoculated via intraperitoneal (IP) injection.

6.2.5 DLA-induced ascites tumor studies

Ascites tumors were induced by IP injection of DLA cells ($1.5 \times 10^6$ cells/mouse). In these studies, mice were in one of four groups ($n = 6$/group): Group I mice served as Normal (Untreated); Group II mice served as solid tumor control and received PBS vehicle only (i.e., no drug, no extract); Group III was treated with methotrexate (3.5 mg/kg B.wt.); Group IV mice were treated with extract (10 mg/kg B.wt.). All treatments were given IP (as 100 µl injections) starting 24 hr after DLA injection and were done daily for 14 consecutive days thereafter.

6.2.6 Determination of the effect of A. ferruginea extract on Mean Survival Time (MST), % Increase of Life Span (ILS) and average body weight changes in mice with DLA-induced ascites tumor

For these studies, dedicated sets of mice were treated with tumor cells and the various drugs/extract regimens as outlined above; the animals were then monitored daily for 50 days. Anti-tumor effects of the extract were determined by monitoring mortality due to tumor (MST) and any percentage increase in lifespan (% ILS) relative to the survival of mice that received tumor cells but no other treatment. The latter was calculated as: $\text{ILS (\%) = } 100 \times \frac{\text{mean survival of treated group} - \text{mean survival of control}}{\text{mean survival of control group}}$. Body weights (B.wt.) of all animals were measured from Day 1 to Day 15 and the average increase in body weight on Day 15 was determined.
6.2.7 Determination of the effect of *A. ferruginea* extract on key liver marker and oxidative stress marker enzymes in mice with DLA-induced ascites tumor

Blood was collected from each animal (via tail-vein) on Days 10 and 15. Total WBC count and hemoglobin content (Hb) were estimated and the remaining blood was centrifuged and serum prepared for estimation of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transferase (GGT) activities by using a kit from Span Diagnostics (Surat, India). Nitric oxide (NO) in the serum was measured by the method of (Green et al, 1982). Ascites fluid was also aspirated from the peritoneal cavity on Days 10 and 15. The aspirated cells were washed to remove dead cells, and then the remaining cells were suspended in RPMI 1640 medium and placed in sterile glass dishes to allow resident macrophages to adhere. After 2 hr at 37°C, the culture medium containing the ‘purified’ DLA cells was gently removed, and then centrifuged to pellet the cells. The cells were then re-suspended at 10^7 cells/ml in RPMI 1640; 100 µl aliquots of the sample were then removed and sonicated for 30 sec to rupture the cells present. This material was then assessed for NO and reduced glutathione (GSH) content using the protocol of (Szasz et al, 1976).

6.2.8 Histopathological analysis

At Day 15 of the study, mice with DLA-induced ascites were euthanized via cervical dislocation. At necropsy, a small portion of liver was recovered from each mouse and fixed in 5% formaldehyde solution. After several steps to induce dehydration in alcohol, sections of 4-μm thickness were prepared and stained with haematoxylin and eosin (H&E). Thereafter, histopathological analysis was carried out using a EVOS-xl CORE light microscope (AMG,
Bothell, WA). All samples were analyzed in a blinded manner. A certified histopathologist performed all analyses/interpreted the observed outcomes.

6.2.9 DLA-induced solid tumor studies

Solid tumors were induced by intramuscular injection of the DLA cells (1.5 × 10^6 cells per mouse) into the right hind limb of the mice. In these studies, mice were in one of four groups (n = 6/group): Group I mice served as Normal (Untreated); Group II mice served as solid tumor control and received PBS vehicle only (i.e., no drug, no extract); Group III was treated with methotrexate (3.5 mg/kg B.wt.); Group IV mice were treated with extract (10 mg/kg B.wt). All treatments were given IP (as 100 µl injections) starting 24 hr after DLA injection and were done daily for 14 consecutive days thereafter.

6.2.10 Determination of the effect of A. ferruginea extract on Solid tumor volume and Body weight

Limb initial diameter was measured using a vernier caliper. From Day 3 post-DLA injection onwards, tumor diameter was measured every 3 day, up to Day 30. Tumor volume was calculated as: \( V = \frac{4}{3} \pi r_1^2 r_2 \), where \( r_1 \) and \( r_2 \) are tumor radii measured in two planes. Body weight (B.wt.) of all mice were measured at 3 day intervals from Day 0 to Day 30.
6.2.11 Determination of the effect of *A. ferruginea* extract on cytokines TNF-α, iNOS, IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and VEGF using Enzyme-Linked Immunosorbent Assay (ELISA) kit in mice with DLA-induced solid tumor

To study the effect of *A. ferruginea* extract on cytokines level, blood samples obtained from the above experiment at two time intervals, (i.e., day 15 and 30) was centrifuged and serum separated for estimation of TNF-alpha, iNOS (USCN Life science, USA), IL-1β, IL-2, IL-6, IFN-γ, GM-CSF and VEGF (Koma Biotech, Korea) using standard sandwich ELISA kit specific for murine cytokines according to the manufacturer’s instruction.

6.2.12 Statistical analysis

All values are expressed as mean ± SD. Statistical analysis were performed using a one-way analysis of variance (ANOVA) followed by Dunnett’s test, using Graphpad InStat version 3.0, (GraphPad Software, San Diego, CA). Results from DLA tumor + *A. ferruginea* treated group were considered statistically significant compared to those from DLA tumor control hosts (no-drug treated) at *p*-values **< 0.05 (or in some cases, *< 0.01).

6.3 RESULTS

6.3.1. Effect of *A. ferruginea* on mean survival time (MST), % increase of lifespan (ILS) and % increase in body weight in mice with DLA-induced ascites

Administration of extract for 14 consecutive days significantly prolonged lifespans/survival time (MST) of treated mice (27.16±1.47) days compared to
that of their untreated tumor-injected counterparts (16.66±1.03) days; this represented a 63% increase in lifespan. The percentage increase in lifespan of methotrexate-treated mice was 79%. The percentage increases in body weights in the ascites-bearing mice were also analyzed. The results indicate that there was a significant reduction in the net changes between the extract vs. non-extract-treated DLA-injected hosts control (17.1 vs. 38.8%) over the study period, whereas mice treated with methotrexate shown 13.6%. (Table.6.1).

Table.6.1 Effect of A. ferruginea on Body weight, Mean Survival Time, Increase in life span in DLA bearing ascites tumor models

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment design</th>
<th>MST (in days)</th>
<th>Increase in Lifespan (%)</th>
<th>Percentage increase in Bodyweight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>&gt;50</td>
<td>-</td>
<td>10.29</td>
</tr>
<tr>
<td>2.</td>
<td>DLA tumor Control</td>
<td>16.66 ±1.03</td>
<td>-</td>
<td>38.77</td>
</tr>
<tr>
<td>3.</td>
<td>Tumor + Methotrexate (3.5 mg/kg B.wt.)</td>
<td>29.83 ±1.16**</td>
<td>79.05</td>
<td>13.58</td>
</tr>
<tr>
<td>4.</td>
<td>Tumor + A. ferruginea (10mg/kg B.wt.)</td>
<td>27.16 ±1.47**</td>
<td>63.02</td>
<td>17.10</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD (n=6/group). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).

6.3.2 Effect of A. ferruginea on hematological parameters in mice with DLA-induced ascites tumor

Inoculation with DLA cells resulted in a significant increase in the of total WBC levels (14.9±0.7 and 15.5±0.4 x 10^3 cells/ml) on Days 10 and 15 day, respectively, as compared to values seen in naive mice. Administration of extract mitigated these changes; values only reached 12.2±0.6 and 12.8±0.4 x 10^3
cells/ml on Days 10 and 15, respectively. These outcomes were comparable with results produced by methotrexate. (11.6±0.5 and 12.5±0.8 x 10^3 cells/ml) on Days 10 and 15, respectively. Hemoglobin content was also significantly reduced in DLA-injected mice (10.0± 0.5 and 10.2±0.3 gm% on Days 10 and 15, respectively (Figure.6.1), when compared with values in naive animals. Both extract and standard drug significantly prevented the anaemic condition (Hb values of, respectively, 14.6±0.4 and 14.6±0.4 gm% on Day 15).

6.3.3 Effect of A. ferruginea on serum AST, ALT, ALP, GGT and NO levels in mice with DLA-induced ascites tumor

The effect of A. ferruginea on serum AST, ALT, ALP, GGT, and NO levels in DLA-injected mice are presented in Table.6.2. Serum levels of AST, ALT, and ALP on Day 10 and 15 post-injected were significantly increased in DLA-tumor control mice (i.e., 75.25±2.93, 57.29±1.91, 171.87±2.84, respectively, on Day 15) as compared to values in naive hosts (Group I), (45.9±1.3, 32.9±1.8, 94.1±2.1, respectively, on Day 15). In extract-treated tumor-injected hosts, the levels of AST, ALT and ALP were significantly reduced (i.e., 59.80±1.98, 45.05±2.12, 110.46±3.31, respectively, on Day 15) as compared to those in the tumor-injected controls. Methotrexate also produced a similar result (i.e., 57.1±1.9, 43.8±1.9, 104.8±2.3, respectively, on Day 15).

On Day 15 after tumor injection, elevated levels of GGT and NO (27.4±2.12 U/L and 36.22±1.22µM, respectively) were found in the serum of DLA-injected controls as compared to in naive hosts 15.9±1.4 U/L and 24.4±0.9 µM, respectively) (Table 6.3). These levels were significantly reduced to 17.43±2.21 U/L and 29.6±2.37 µM, respectively due to administration of the A. ferruginea extract. This reduction was comparable to that achieved with methotrexate (i.e., 18.53±2.86 U/L and 28.68±1.69 µM, respectively).
6.3.4 Effect of *A. ferruginea* extract on GSH and NO levels in ascites fluid cells

The GSH content in aspirated DLA tumor cells on Day 15 after injection was found to be 17.2±2.8 nmol/mg protein. In extract- and methotrexate-treated animals, the cellular GSH level was significantly reduced to 8.7±0.8 and 9.3±1.5 nmol/mg protein, respectively (Figure 6.2). The nitric oxide level in the aspirated cells on Day 15 was 13.1±0.5 μM. In extract and drug-treated mice, the NO levels were seen to be lowered to 9.4±0.8 and 10.2±0.7 μM, respectively.

![Graph showing effects on haematological parameters](image)

**Figure 6.1.** Effect of methanolic extract of *A. ferruginea* on haematological parameters.

Treated animals received 14 doses of extract (at 10 mg/kg B.wt.). Blood samples were collected from tail vein on Day 10 and 15 after start of extract administration. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).
Table 6.2 Effect of *A. ferruginea* on serum AST, ALT and ALP levels in DLA-bearing and extract-treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 10</td>
<td>Day 15</td>
<td>Day 10</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>45.9 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>DLA tumor Control</td>
<td>71.54 ± 2.09</td>
<td>75.25 ± 2.93</td>
<td>52.34 ± 1.74</td>
</tr>
<tr>
<td>Tumor + Methotrexate (3.5 mg/kg B.wt.)</td>
<td>53.43 ± 1.93**</td>
<td>57.14 ± 1.92**</td>
<td>37.95 ± 1.92**</td>
</tr>
<tr>
<td>Tumor + <em>A. ferruginea</em> (10mg/kg B.wt.)</td>
<td>52.12 ± 2.49**</td>
<td>59.80 ± 1.98**</td>
<td>40.13 ± 1.59**</td>
</tr>
</tbody>
</table>

Blood samples were collected from tail vein on Days 10 and 15. AST, ALT, ALP levels were assessed. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).
Table 6.3. Effect of *A. ferruginea* on serum GGT and NO levels in DLA-bearing and extract–treated mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GGT (U/L)</th>
<th>NO (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 10</td>
<td>Day 15</td>
</tr>
<tr>
<td>Normal</td>
<td>-</td>
<td>15.9±1.4</td>
</tr>
<tr>
<td>DLA tumor Control</td>
<td>22.47 ± 2.35</td>
<td>27.4 ± 2.12</td>
</tr>
<tr>
<td>Tumor + Methotrexate (3.5 mg/kg B.wt.)</td>
<td>16.21 ± 2.24**</td>
<td>18.53 ± 2.86**</td>
</tr>
<tr>
<td>Tumor + <em>A. ferruginea</em> (10mg/kg B.wt.)</td>
<td>17.43 ± 2.21**</td>
<td>19.49 ± 2.75**</td>
</tr>
</tbody>
</table>

Blood samples were collected from tail vein on Days 10 and 15. GGT and NO levels were assessed. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05,**p < 0.01).
Figure 6.2 Effect of *A. ferruginea* on cellular GSH and NO levels in DLA bearing tumor mice.

Ascites fluid was collected on day 10 and 15 after intraperitoneal injection of DLA cells. The isolated solution was sonicated and lysates then assayed for GSH and NO. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).
6.3.5 Effect of *A. ferruginea* on liver histopathology in ascites-bearing hosts

Representative liver sections from normal (PBS), DLA tumor control, methotrexate-, and extract-treated mice that were collected at the end of the experimental periods (i.e., Day 15 post-DLA injection) are presented in Figure 6.3. PBS mice yielded tissues with normal lobular architecture and with an intact central vein and sinusoids, normal portal tracts, and intact hepatocytes. DLA-injected mice (control) samples evidenced necrosis, fibrosis, nuclear debris, and a perivenular inflammation containing several polymorphonucleated cells. Vacuole formation and local inflammation was significant in the tissues from DLA-injected hosts. In comparison, mice treated with *A. ferruginea* extract or methotrexate had livers with a reduced vacuole presence and inflammation and an almost normal hepatocellular architecture.
**Figure 6.3** Histopathological changes in liver of experimental mice.

Pictures presented are representative liver sections collected at end of experimental periods (i.e., Day 15 after DLA injection). (A) Normal (no tumor, no extract); (B) tumor only; (C) tumor + *A. ferruginea* extract; (D) tumor + methotrexate.
6.3.6 Effect of *A. ferruginea* extract on solid tumor development and host body weight

Treatment of DLA-injected mice with *A. ferruginea* extract on 14 consecutive days led to a significant reduction in tumor volume (0.81±0.07 mm$^3$) compared to that seen in tumor-injected mice that did not receive any extract at all (2.52±0.06 mm$^3$) by Day 30 of study; Figure 6.4 A). Mice treated with the methotrexate also displayed effective reduction (0.88±0.07 mm$^3$) in tumor development. Body weight was measured at 3 day intervals throughout the period of experiment. By Day 30, there was a significant increase (vs. Day 0) in weights (up to 29.17± 0.33g) of the tumor-bearing controls (Figure 6.4 B). In contrast, in extract-treated tumor-injected mice, significantly lower comparative weights (25.55±0.19 g) were noted. Mice treated with methotrexate were also of a similar ‘lower’ weight by this time (24.92± 0.29g). It is likely that the greater weights in the non-extract-treated mice were attributable to increases in the tumor mass itself.

6.3.7 Determination of the effect of *A. ferruginea* extract on cytokines profile and inflammatory markers in mice with DLA-induced solid tumor

The effect of *A. ferruginea* extract on TNF-α, iNOS, IL-1β and IL-6 production on day 15 and 30 in mice with DLA-induced solid tumor is depicted in Figure 6.5. Serum TNF-α, iNOS, IL-1β, IL-6, GM-CSF and VEGF level was found to be elevated where as IFN-γ and IL-2 level were reduced in DLA-injected hosts on day 15 and 30 respectively. Administration of *A. ferruginea* extract in DLA-injected hosts significantly reduced the Serum TNF-α, iNOS, IL-1β, IL-6, GM-CSF and VEGF level on day 15 and 30. Similarly, reduced level of IFN-γ and IL-2 were significantly enhanced in the DLA-injected mice treated with *A. ferruginea* extract on day 15 and 30. The effect of *A. ferruginea* extract on GM-CSF, IL-2, IFN-γ, and VEGF level on day 15 and 30 in mice with DLA-induced...
solid tumor is depicted in Figure 6.6. Mice treated with the methotrexate also displayed effective result in cytokine profiling.
Figure 6.4 Effect of *A. ferruginea* extract on tumor volume and body weight on indicated days after DLA intramuscular injection.

Treated animals received 14 doses of extract (10 mg/kg B.wt.). Tumor radii in extract- and non-extract-treated controls were measured at 3-d intervals using a vernier caliper. Body weights of treated and non-extract-treated controls were also measured at 3-d intervals. Values shown are means (± SD; in mm³) from 6 mice/treatment group. Values significantly different from tumor (non-extract-treated) control (*p<0.05, **p<0.01).
**Figure 6.5** Effect of *A. ferruginea* extract on TNF-α, iNOS, IL-1β and IL-6 production in mice with DLA-induced solid tumor.

Blood samples were collected from tail vein on Days 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).
Figure 6.6 Effect of *A. ferruginea* extract on GM-CSF, IL-2, IFN-γ, and VEGF level in mice with DLA-induced solid tumor.

Blood samples were collected from tail vein on Days 15 and 30, the level of cytokines were assessed using standard ELISA method. Values shown are means (± SD). Values significantly different from tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).
6.4. DISCUSSION

Cancer is a complex disease, and has become a major public health problem around the world. As a treatment for cancer, chemotherapy is successful but still faces a variety of challenges due to poor selectivity and/or toxicities that affect all rapidly proliferating and dividing cells, including lymphatic, red blood, epithelia, and bone marrow cells (Mahato et al, 2011). Several natural product drugs of plant origin have been proposed for use against cancer; galantamine, nitisinone, and tiotropium have been examined in late-phase clinical trials (Balunas and Kinghorn, 2005). Our interest in recent years has been in examining the potential anti-cancer effects of natural products based on their abilities to act against inflammatory mediators. There are several reports that plants belonging to genus Acacia have been widely used in the management of pain, inflammation, and treatment of cancer in folk medicine (Bukhari et al, 2010). In the current study, we for the first time provide evidence that A. ferruginea extract has potent anti-tumor activity in vivo.

Our preliminary phytochemical analysis of the A. ferruginea methanolic extract revealed a presence of numerous flavonoids, phenolics, steroids, terpenoids, alkaloids, saponins, and tannins. Polyphenols, which include mainly flavonoids and phenolic acids, have been reported to impart a protective role against a wide range of cancers, including those of the lung, breast, colon, stomach, and mouth (Araujo et al, 2011). These bioactive natural products act either by blocking initiation or suppressing/arresting promotion and progression of cancers (Ziech et al, 2012). Polyphenols and alkaloids exhibit considerable activity against a wide range of cancers. Of these, mahanine (a plant carbazole alkaloid) has been shown to inhibit cancer cells by inducing apoptosis of both androgen-sensitive (LNCaP) and androgen-independent (PC-3) cancer cells by
reducing phosphorylation of PIP₃ dependent kinase-1 (PDK-1), deactivating Akt, and down-regulating expression of pro-apoptotic Bcl-XI (Yue and Wang, 2011). The wide range of biological and pharmacological activities of flavonoids in tumor cells is also well known (Hodek et al, 2002). Overall, emerging evidence has shown that the diverse classes of plant metabolites like flavonoids, phenolics, alkaloids, etc. can interfere with the promotion and progression phases of carcinogenesis, thereby inhibiting the premalignant\malignant transformation of initiated cells (Zhao et al, 2010).

In DLA tumor-bearing mice, a regular and rapid increase in tumor volume (i.e., accumulation of ascites fluid) and reduced host lifespan is usually noted. Administration of A. ferruginea extract prolonged host life span; a concomitant reduction in body weight (reflecting a reduced tumor burden) in extract-treated DLA-injected mice animals also suggests the induction of a significant anti-tumor activity by mechanisms as-yet to be defined. Many reports have provided evidences that a presence of tumors in experimental animals affects functions of vital organs, particularly the liver and kidney, even when the tumor does not have direct contact with these organs. Often, the hepatocellular necrosis observed in cancer-bearing host’s results in significant elevations in serum AST and ALT (released from liver). Increased serum levels of ALP (hyper alkaline phosphatasemia) has also been observed with intrahepatic cholestasis, bile duct obstruction, or infiltrative diseases of the liver (Gaze, 2007). In the current study, elevated level of serum AST, ALT, and ALP were noted in DLA-injected control mice. The significant reversal of these changes towards normal values once again indicated the anti-cancer effect of the A. ferruginea extract as well as a hepatoprotective action against potential damage induced by the DLA tumor cells. Lastly, in hosts with cancer (i.e., ascites), myelosuppression and reductions in hemoglobin levels (anemia) due to iron deficiency are also frequently observed. The prevention of a drop in hemoglobin content and a
reversal of the tumor-induced changes in total WBC counts towards a normal range after administration of the extract again indicate that the extract imparts a significant anti-cancer activity. These results also support the notion that the *A. ferruginea* extract might also be providing a protective role for the hematopoietic system as well as some measure of immunostimulatory activity.

Cancer cells have higher total glutathione (GSH) levels than normal cells; this is a characteristic of a higher cell proliferation rate (and often a resistance to chemotherapy). Studies have shown that combining GSH depletion using 1,3-bis(2-chloroethyl)-1-nitrosourea along with superoxide dismutase gene therapy could be successful in the treatment of breast cancer (Weydert et al, 2008). The theory behind this is that when intracellular GSH levels are low, the cancer cells are more susceptible to the effects of reactive oxygen species (ROS). This seems somewhat counter-intuitive in that while ROS might activate different intracellular oncogenic pathways that lead to activation of tumorigenic processes, the excessive levels of ROS can also be toxic to the cells that have already been transformed. In this study, administration of *A. ferruginea* extract resulted in significant reductions in the intracellular GSH levels in DLA cells recovered from the treated mice. It is known that plant extracts containing antioxidants have been shown to cytotoxicity among cancer cells by inducing apoptosis (Trachootham et al, 2009). Whether their reduction in GSH content might have caused the DLA cells in our mice to be more susceptible to ROS generated by peritoneal/local macrophages (and thus more susceptible to apoptotic events) remains to be resolved. Similarly, effects of the extract on local macrophage formation of ROS need to be examined. While it is known that methotrexate has an inhibitory effect on ROS formation by some cell types (i.e.,synoviocytes) (Sung et al, 2000) other studies showed that it stimulates ROS formation/release by immune cells (lymphocytes, monocytes) (Herman et al., 2005).
At this point, it is uncertain what impact the extract has on ROS formation by macrophages. Once this information is in hand, it will be easier to establish if the significant reductions in ascites (as well as solid tumor growth/size) seen with the extract treatments was a product of a two-pronged ‘attack’, i.e., increased susceptibility of tumor cells to the ROS that are now being produced at even greater levels by local macrophages, etc.

γ-Glutamyl transferase (GGT), directly involved in GSH metabolism (catalyzing transfer of γ-glutamyl moieties between glutamate and cysteine), is often significantly increased under tumor-bearing conditions and its role in tumor progression and invasion has been reported. The studies here clearly showed that increases in levels of GGT triggered by the injection of the DLA cells were mitigated by the extract treatment. As indicated in the expansive review by (Zhang et al., 2009), the regulation of GGT genes under various conditions such as oxidative stress, though established in rodent models, is still vaguely understood. Knowledge about which GGT genes are regulated, what signaling pathways are involved, and what is the expression profile of different GGT transcripts in responsive to oxidative stress would help in understanding how GGT is involved in normal physiology as well as in diseases like cancer. Thus, whether the outcomes here (i.e., reductions in GGT) are simply a useful marker of extract anti-tumor activity or if the changes in GGT activity themselves play any critical run in the anti-tumor activity itself are important points that remain to be clarified.

Lastly, nitric oxide (NO), released during various pathophysiological processes (including inflammation and carcinogenesis) and an important mediator of tumor growth was reduced in in tumor cells recovered from A. ferruginea extract-treated hosts. As was noted above with regard to the GSH and ROS parameters, whether there is a concomitant change in the formation of NO
by local macrophages (used to kill tumor cells) remains a critical piece of data to obtain in order to understand how the extract might impart an anti-tumor activity (Ruttimann, 2007).

To ascertain whether this effect of *A. ferruginea* extract on DLA cells was local (cytotoxic effect) or systemic, a second experimental system, i.e., a DLA-induced solid tumor model, was employed. The results showing tumor growth inhibition and a normalization of host body weight (relative to that in non-extract/non-methotrexate-treated hosts) confirmed that the anti-cancer effect was systemic. Immune cells execute many of their functions through production of numerous cytokines. Cytokines (large family of soluble proteins) serve as mediators of immune response and have been linked with tumorigenesis process. Extensive studies have indicated that tumor cells exhibit an elevation in constitutive production of several proinflammatory cytokines such as TNF-α, GM-CSF, IL-1β and IL-6 (Dinarello, 1996). Studies on murine models suggests that TNF-α is a key mediator of cancer cachexia, in addition with IL-1β and IL-6. Release of TNF-α cause’s polymorphonuclear neutrophil influx and release of various inflammatory mediators from multiple cell types (Song et al, 2003; Chen et al, 1999).

The pleiotropic cytokine IL-1β induces immunosuppression in different experimental conditions *in vivo*, also it potentiates tumor invasiveness and metastasis by elevating the level of various growth factors and angiogenesis-promoting factors (VEGF). Increased expression of proinflammatory cytokines IL-1β and IL-6 have been shown in patients with head and squamous carcinoma (Suzuki et al, 1992). GM-CSF, a hematopoietic growth factor belongs to glycoproteins family and plays a pivotal role in regulation of bone marrow progenitor cells proliferation. It is mainly produced by T lymphocytes or non-hematopoietic cells and highly expressed in solid tumors and enhances tumor cell
proliferation as well as angiogenesis (Gasson, 1991). Moreover, the results obtained in this study indicates that *A. ferruginea* extract could inhibit the production of IL-1β and IL-6 in tumor bearing mice and also exerts its regulatory effect on TNF-α, GM-CSF and iNOS, moreover inhibition of VEGF level also shows prevention of tumor-directed new blood vessel formation by downregulating these molecules. The lymphokine, IL-2 stimulates Natural Killer (NK) cell and T cell proliferation, further activated NK cells secretes IFN-γ exerts direct antitumor activity by interfering with killing of tumor cells by upregulating class I major histocompatibility complex molecules and inhibiting angiogenesis (Theze et al, 1996). Significant increase in level of both IL-2 and IFN-γ in serum after treatment with *A. ferruginea* extract in tumor bearing animals indicate its stimulatory effect on immune system.

Compounds (quinone, quinoline, imidazolidine, pyrrolidine, pyrazole, thiazole, cyclopentenone, catechin and coumarin derivatives) identified by the GC/MS and LC/MS analysis *A. ferruginea* methanolic extract have been reported to possess various biological and pharmacological activities, that could result in other forms of immune stimulation and antitumor activity against DLA tumor cells. For example, it was reported that anthraquinone derivatives (group of polyphenolic constituents) has been shown to exhibit antitumorigenic activity by activation of ERK pathway and increased expression of enhancer binding protein β (C/EBPβ), followed by Nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1) expression and subsequently induces apoptosis in human colorectal cancer (Nualsanit et al, 2012).

Recently a report shown imidazolidine derivatives are involved in modulation of immune system and exhibits anti-inflammatory and antinociceptive activities (Guerra et al, 2011). It is well known; medicinally important coumarins and catechins comprise a large class of compounds found
throughout the plant kingdom. Coumarin derivatives are able to arrest cell cycle at G2/M stage and in addition induce apoptosis of human cancer cells (Kim et al, 2009). For example, Eryciboside, a coumarin derivative was isolated from the plant *Laguncularia racemose* showed significant antioxidative activity and potent inhibition of human tumor related protein kinases FLT3 and SAK (Shi et al, 2010). Interestingly, a recent report evidenced that catechin rich fractions from *Acacia catechu* inhibited the 7,12-Dimethylbenz[a] anthracene-Induced Mammary Carcinoma in murine models (Monga et al, 2012). It is possible that the coumarin and catechin derivatives present in the *A. ferruginea* extract may have afforded protection to the treated mice towards DLA tumor cells.

The present investigation clearly indicated that treatment with *A. ferruginea* extract was effective in inhibiting inflammation and tumor progression *in vivo*. This is most likely due to high content and synergistic activity of specific constituents such as flavonoids, phenolics, steroids, terpenoids, alkaloids, saponins, quinones and tannins. Nevertheless, the precise molecular mechanism by which *A. ferruginea* extract mediates anti-tumor activity remains to be determined. Further investigations are in progress in our laboratory to isolate the specific bioactive agents in the extract with potential for use in anti-cancer therapy, and to elucidate their associated mechanisms of therapeutic action.