CHAPTER-7

TO INVESTIGATE THE EFFECT OF *Acacia ferruginea* EXTRACT ON INHIBITION OF PULMONARY METASTASIS INDUCED BY B16F-10 MELANOMA CELLS

7.1 INTRODUCTION

Cancer cells are characterized by failure of cell cycle control which leads to over proliferation of these cells (i.e. Primary tumor). The second important process that leads to cancer is that, the over proliferation of cancerous cells acquire the ability to leave the primary site and form colonies at secondary sites termed as metastasis. Metastasis is an extremely complex process that remains to be a major problem in the management of cancer (Young et al., 2012). Metastatic cancer cells are generally identical as cells of the primary cancer i.e. breast cancer that spreads to the lungs and forms a metastatic tumour is known as metastatic breast cancer, not lung cancer (Talmadge and Fidler, 2010). The completion of complex metastasis cascade include (1) the epithelial cells in primary tumor invade locally through surrounding extracellular matrix (ECM), (2) intravasate into the lumina of blood vessels, (3) transport through vasculature, (4) arrest at distant organ sites, (5) extravasation in to distant tissues, (6) micrometastases (survival in foreign tissue microenvironments and (7) re-initiation of their proliferation rate at metastatic sites, thereby creating macroscopic, clinically detectable neoplastic growths, often called as metastatic colonization process (Valastyan and Weinberg, 2011).
Despite advancement in early cancer diagnosis and treatment included surgery, chemotherapy, radiotherapy and adjuvant therapies. Around 90% of cancer deaths are caused by metastasis that are resistant to conventional therapies (Gupta and Massague, 2006). Although there are several drugs that are used for cancer therapy, however there are no drugs available at present that blockade any single step in the metastatic process. Many experimental studies and clinical trials showed that many natural plants played an important role in blocking of lung metastasis from primary tumors (Leyon and Kuttan, 2004; Leyon et al., 2005; Thejass and Kuttan, 2006).

Our previous investigation of A. ferruginea extract revealed potent anti-tumor effect against Dalton’s Lymphoma Ascites induced solid as well ascites tumor models. Interestingly it was found that A. ferruginea extract possess anti-proliferative action against B16F-10 melanoma cells in vitro. Hence in the present study, we made an attempt to evaluate the inhibition of pulmonary metastasis induced by B16F-10 melanoma cells in vivo.

7.2 MATERIALS AND METHODS

7.2.1 Plant material collection and preparation of extract

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 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.

7.2.2 Animals

Male C57BL/6 mice (4-6-wk-old, 22-25 g) were obtained from Sri Venkateshwara Enterprises (Bangalore, 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/13/07).

7.2.3 Cell line

B16F-10 melanoma cells were procured from National Center for Cell Science, Pune, India. The cells were maintained in DMEM media supplemented with 10% fetal calf serum (FCS) and antibiotics (1% penicillin and streptomycin). The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

7.2.4 Chemicals and Kits

Hydroxyproline and glucuronic acid lactone were procured from Sigma Chemicals (St. Louis, USA). N-acetyl neuraminic acid and papain were
purchased from Sisco Research Laboratory (Mumbai, India). Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from HiMedia (Mumbai, India). All other reagents were of analytical reagent grade. Gamma Glutamyl transpeptidase kit was purchased from Merck (Mumbai, India). Highly specific quantitative sandwich ELISA kits for mouse IL-1β, IL-6, TNF-α and IL-2 were procured from Koma Biotech (Seoul, Korea). iNOS and COX-2 kits were purchased from USCN Life science (Houston, USA) and Bluegene Biotech (Shangai, China) respectively. Transcription factor profiling kit for NF-kB (p50 and p65 subunits) was procured from Cayman (Michigan, USA).

7.2.5 In vitro cytotoxicity assay

The anti-proliferative activity of *A. ferruginea* methanolic extract against the B16F-10 melanoma cells were determined by using conventional MTT reduction assay. This assay relies primarily on the mitochondrial metabolic capacity of viable cells (i.e. mitochondrial succinate-terazolium reductase system to convert MTT to a blue colored formazan) and hence it reflects the intracellular redox state. Cells were seeded in to 96-well plate at 5x10⁴ cells/well density and treated with escalating concentrations of *A. ferruginea* extract (5-200 µg/ml) for 48 hours. After incubation of 48 hr, cells were treated with MTT solution (10µL) for 4 hr at 37°C. The dark blue formazan crystals formed in intact cells were dissolved by adding solubilization solution DMSO (100µL) in to each well and incubated overnight. The absorbance was measured at 570 nm in a microplate ELISA reader. All experiments were performed in triplicate. IC₅₀ value, the concentration of extract required to inhibit 50% cell growth was determined by nonlinear regression analysis of the corresponding dose response curve.
7.2.6 Determination of the effect of *A. ferruginea* on *in vivo* inhibition of experimental lung tumor metastasis

The log-phase cell culture of the B16F-10 cells were harvested, washed with serum-free DMEM and resuspended to give the appropriate concentrations in PBS. An amount of 0.2 ml of the resultant B16F-10 cell suspension (10^6 cells) was injected via the tail vein of the C57BL/6 mice (Day 0). Drug administration: The animals were divided into five groups. Group 1 (n=6) kept as normal (untreated), Group 2 to 5 (n=14 in each group) were injected with B16F-10 cells. Group 1 (n=6) kept as normal (untreated), Group 2 to 5 (n=14 in each group) were injected with B16F-10 cells. Group I received vehicle only (PBS), Group 2 received no treatment, whereas Groups 3, 4 and 5 received 10 mg/kg B.wt of *A. ferruginea* extract intraperitoneally in three different modalities as follows:

1. **Prophylactic administration:** Animals were treated with 10 consecutive doses of the drug prior to tumor inoculation (i.e. induction of metastasis)

2. **Simultaneous administration:** The drug was given to the animals simultaneously with metastatic tumor cells and continued for 10 consecutive days.

3. **Developed administration:** Ten days after tumor inoculation drug was administered on 10 consecutive days.

Eight mice from Group 2 to 5 were sacrificed 21 days after being inoculated with B16F-10 tumor cells. The animals were euthanized and lungs were excised. The number of B16F-10 colonies present on the surface of each set of lungs were determined by visual inspection using a stereoscopic dissecting microscope. The blood samples were collected and serum separated used for biochemical experiments.
7.2.7 Determination of the effect of A. ferruginea extract on Mean Survival Time (MST) and Percentage increase of life Span (ILS) in mice with B16F-10 tumor cells

For these studies, dedicated sets of mice (n=6 in Group 2 to 5) were treated with tumor cells and the extract regimen as outlined above; the animals were then monitored daily for 90 days. Anti-tumor effect of the extract was 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: ILS (%) =100 x (mean survival of treated group - mean survival of control)/mean survival of control group.

7.2.8 Determination of the effect of A. ferruginea on the lung collagen hydroxyproline, Uronic acid and Hexosamine content during metastasis

Hydroxyproline content in lungs were measured by quantifying lung collagen content and was measured colorimetrically using the method described by Bergman and Loxley (1970). After sacrificing, the lungs were excised, pulmonary airways and blood vessels were removed and discarded. The lung tissue was homogenized in 4 ml of PBS, after that equal volume of 6N Hcl was added and the sample were hydrolyzed in sealed tubes (specially designed hydrolyzing tube) at 110°C and kept for 24 hours. The hydrolyzate solution was neutralized by adding five times equal volume of H2O. Then 0.5 ml of each sample was mixed with 2.5 ml of isopropanol and 1 ml of oxidant solution (oxidant solution buffer and chloramine- T by the ratio 4:1) which was kept at room temp for 4 min, Ehrlich’s solution was added and sample were incubated at 60°C for 2 min. Absorbance was measured at 560 nm. Standard curves were
generated by using reagent hydroxyproline as a standard with different concentration (10 µl-100 µl).

The uronic acid content present in lung tissue were estimated by the method of Bitter and Muir (1962). 1 gram of tissue were digested with papain and acetate buffer and which was hydrolyzed at 100°C after hydrolysis, the hydrolysate was allowed to react sulfuric acid reagent (Sulfuric acid 5ml + Sodium tetraborate 1g) and carbozole reagent (0.125% of carbazole in alcohol) the uronic acid level was estimated at 530 nm. Standard graph was plotted using glucouronic acid lactone.

The Hexosamine content in lung tissue were estimated by method of Elson and Morgan (1933). Tissue samples were hydrolyzed with 2N HCL and boiled with 100°C. The hydrolysate was evaporated to dryness. The residue was dissolved with water and which was treated with freshly prepared 2% acetyl acetone in 5M Na₂CO₃ in capped tubes and kept in boiling water both for 15 min after cooling 95% ethanol and Ehrlich’s reagent were added. The purple red colour development was measured at 530 nm. The standard graph was plotted with various concentration of glucosamine standard.

7.2.9 Determination of the effect of *A. ferruginea* on the inhibition of serum sialic acid and gamma glutamyl transpeptidase level during metastasis

The sialic acid level was determined by thiobarbituric acid assay method (Bhavanandan et al., 1981) serum samples where hydrolyzed with 2N sulfuric acid. The hydrolysate were oxidized with periodic acid and incubated at 37°C. After incubation oxidation was terminated using sodium arsenate. The sialic acid level was estimated after adding 6% thiobarbituric acid at 549 nm with reference to 632 nm after adding DMSO. Sialic acid content was determined from the
standard graph plotted using n-acetyl neuraminic acid. Serum \( \gamma \)-glutamyl transpeptidase (GGT) activity was measured using kit from Span Diagnostics (Surat, India). The GGT content was determined from the graph platted using p-nitroaniline as the standard.

7.2.10 Determination of the effect of *A. ferruginea* on the serum cytokines (TNF-\( \alpha \), IL-1\( \beta \), IL-6 and IL-2) during metastasis

Blood was collected from each animal by cardiac puncture immediately after the sacrifice on day 21. Serum was separated and used for the estimation of TNF-\( \alpha \), IL-1\( \beta \), IL-6 and IL-2 levels using respective ELISA kits according to the manufacturers’ instructions (Koma Biotech, Seoul, Korea).

7.2.11 Preparation of Nuclear Extracts

Lung tissues were homogenized in cold PBS, and then were centrifuged at 500 \( \times \) g for 5 min at 4\(^\circ\)C. The resulting supernatants were discarded. The cell pellet was resuspended in ice cold cell lysis buffer (200\( \mu \)L; pH:7.9) containing HEPES (10mM), MgCl\(_2\) (1.5 mM), KCl (10mM), phenylmethyl sulfonyl fluoride (1mM), dithiothreitol (DTT) (1mM), Nonidet P40 (0.5%) and EGTA (1mM) followed by centrifugation at 5000 rpm for 15 min. The cell pellet was resuspended in double the volume of lysis buffer and the cells were disrupted by repeated single rapid stroke using a sterile syringe. The nuclear pellet was resuspended in extraction buffer (200\( \mu \)L) containing HEPES (20mM), glycerol (25%), MgCl\(_2\) (1.5mM), NaCl (420mM), PMSF (0.1mM) and DTT (1mM) and incubated in ice for 30 min. The nuclear suspension was centrifuged at 10,000 rpm for 15 min at 4\(^\circ\)C and the supernatant (nuclear extract) was frozen in aliquots at -80\(^\circ\)C for the transcription factor profiling (Pratheeshkumar et al., 2010).
7.2.12 Transcription factor profiling NF-κB (p65/p50)

Transcription factor profiling was done using the Cayman TM transcription factor kit (Cayman™, Michigan, USA). The kit provided rapid, high-throughput detection of specific transcription factors, namely subunits of NF-κB such as p65 and p50. Using an ELISA based format, the transcription kit detected the DNA bound transcription factors. Bound transcription factors in the DNA were detected by specific primary antibodies towards NF-κB p65 and NF-κB p50 sub units. A horseradish peroxidase-conjugated secondary antibody was then used to detect the primary antibody. The enzymatic product was then measured using an ELISA reader and the percentage inhibition was calculated using the formula: 100 – ([OD of treated/ OD of control] x 100), where OD is optical density (Pratheeshkumar et al., 2010).

7.2.13 Histopathological analysis of lungs

At the end of the experiment, (i.e., 21 days after being inoculated with B16F-10 tumor cells) the lung tissues were collected, then fixed in 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin, dehydrated in alcohol, and then sections of 4-μm thickness were prepared and stained with haematoxylin and eosin (H&E). Thereafter, analyses were carried out using an EVOS-xl CORE light microscope (AMG, Bothell, WA). A certified histopathologist performed all analyses/interpreted the observed outcomes.

7.2.14 Immunohistochemical localization of p53 and Bcl-2

Lung tissue were collected from each group, fixed in 4% neutral formalin, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5-μm thick) were mounted on slides, cleaned,
hydrated. The sections were treated with buffered blocking solution (3% bovine serum albumin in PBS) for 15 min, then co-incubated with primary antibody against p53 or Bcl-2 (polyclonal murine anti-p53 or Bcl-2 antibody; dilution = 1:400 in PBS [v/v]) (Abcam, Cambridge, MA, USA) at room temperature for 1 hr. The samples were then gently washed with PBS and then co-incubated with secondary antibody (SS Polymer HRP/DAP; dilution = 1:500 in PBS [v/v]) (BioGenex, Fremont, CA), at room temperature for 1 hr. Thereafter, the sections were washed as before and with Tris-HCl (0.05 M, pH 7.6), and then co-incubated with 3,3’-diamino-benzidine solution in the dark at room temperature for 10 min. The sections were then washed with Tris-HCl, stained with hematoxylin, mounted with glycerin, and examined under a light microscope at 40X magnification.

7.2.15 Statistical Analysis

All values are expressed as mean ± SD. For each endpoint, group means were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett’s test using Instat Version 3.0 software (Graphpad, San Diego, CA). p-Values: p < 0.05 were considered to be statistically significant compared with metastasis alone control group (no drug treatment).

7.3 RESULTS

7.3.1 Effect of A. ferruginea extract on B16F-10 cells inhibition

The cytotoxicity activity of A. ferruginea methanolic extract against B16F-10 melanoma cells were shown in Figure.7.1. A. ferruginea extract was found to have a dose dependent toxicity towards B16F-10 melanoma cells. IC₅₀ value of A. ferruginea extract was found to be 52.94 µg/ml.
Figure 7.1 MTT assay with different concentrations (5-200 µg/ml) of *A. ferruginea* methanolic extract was measured at 570 nm. Values are expressed as mean ± SD of triplicate experiments.

7.3.2 Effect of *A. ferruginea* extract on lung tumor colony formation, Mean Survival Time (MST) and Percentage increase of life Span (ILS) in mice with B16F-10 tumor cells

Effect of *A. ferruginea* on the lung tumor colony formation and survival rate of animals is shown in Table 7.1. Injection of B16F-10 melanoma cells via tail vein resulted in the formation of lung tumor nodules. Treatment with *A. ferruginea* significantly (*p*<0.01) reduced the number of colonies to (32.66±3.21) in prophylactic modality, (34.65±3.78) in simultaneous modality and
to 43±6.24 in animals with developed tumor when compared with metastatic control group (76.33±6.65).

The survival rate of *A. ferruginea* treated animals was also increased when compared to metastatic tumor bearing control animals. The survival rate was increased to 78.33±5.52 days by *A. ferruginea* treatment compared to metastatic control animals which survived up to only 49.33±6.5 days. Percentage increase in life span was calculated as T-C/ Cx100, where T and C are the number of days survived by the treated and control group of animals respectively. Maximum survival rate was observed when *A. ferruginea* was administered prophylactically (58.78%) followed by simultaneous (74±5.56%) and then after tumor development (40.54%) as shown in Table 7.1.
Table 7.1. Effect of *A. ferruginea* extract on lung tumor colony formation and survival of B16F-10 melanoma bearing animals.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Number of lung tumor nodules</th>
<th>Percentage inhibition of nodule formation</th>
<th>Number of days animal survived</th>
<th>Percentage increase in life span (% ILS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>Nil</td>
<td>Nil</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>Metastasis control</td>
<td>76.33±6.65</td>
<td>-</td>
<td>49.33±6.5</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Metastasis + <em>A. ferruginea</em> (Prophylactic with tumor induction)</td>
<td>32.66±3.21**</td>
<td>57.21</td>
<td>78.33±5.52**</td>
<td>58.78</td>
</tr>
<tr>
<td>4.</td>
<td>Metastasis + <em>A. ferruginea</em> (Simultaneous with tumor induction)</td>
<td>34.65±3.78**</td>
<td>54.6</td>
<td>74±5.56**</td>
<td>50</td>
</tr>
<tr>
<td>5.</td>
<td>Metastasis + <em>A. ferruginea</em> (After tumor development)</td>
<td>43±6.24**</td>
<td>43.66</td>
<td>69.33±6.42**</td>
<td>40.54</td>
</tr>
</tbody>
</table>

The lungs were dissected out and observed for metastases on 21st day after induction of B16F-10 melanoma (10⁶ cells). Tumor cell injection was done through lateral tail vein. Extract treatment was done in three different modalities (i.e. 10 doses at 24 hr. interval, intraperitonal). Values are expressed as Mean ± SD (n=6/group). Values significantly different from metastasis (non-extract-treated) control (**p < 0.01).
7.3.3 Effect of *A. ferruginea* on the lung collagen hydroxyproline, uronic acid and hexosamine content during B16F-10 induced tumor metastasis

The effect of *A. ferruginea* treatment on lung collagen hydroxyproline, uronic acid and hexosamine content during B16F-10 induced tumor metastasis were represented in Table 7.2. In metastases bearing control group of animals, hydroxyproline concentration was increased significantly up to 24.4 ± 1.4 mg/mg protein compared to the normal animals (0.98 ± 0.08 mg/mg protein). Treatment with *A. ferruginea* in metastasis bearing animals, the concentrations of hydroxyproline were reduced significantly (p < 0.01) to 11.6 ± 1.7 mg/mg protein in prophylactic, 15.8 ± 2.1 mg/mg protein in simultaneous and 14.6 ± 1.6 mg/mg protein in developed modalities compared to the metastasis bearing control group.

Tumor-bearing control animals had a high level of lung hexosamine content (5.2 ± 0.4 mg/100 mg tissue dry weight) compared to normal animals 0.5 ± 0.04 mg/100 mg tissue dry weight). Treatment with *A. ferruginea* extract could reduce the hexosamine content significantly in all the modalities (1.3 ± 0.3 mg/100 mg tissue dry weight in prophylactic, 1.82 ± 0.5 mg/100 mg tissue dry weight in simultaneous and 1.91 ± 0.4 mg/100 mg tissue dry weight in animals with developed tumor).

The uronic acid level in lungs of metastasis bearing control group of animals also were very high (318 ± 14.8 µg/100 mg tissue wet wt) compared to the normal group (35 ± 2.6 µg/100 mg tissue wet wt). Treatment with *A. ferruginea* could significantly (p < 0.01) reduce the elevated level to 106 ± 12.2 µg/100 mg tissue wet weight in prophylactic and 167 ± 17.4 µg /100 mg tissue wet weight in simultaneous modality and 158 ± 20.3 µg /100 mg tissue wet weight in developed treatment.
Table 7.2 Effect of *A. ferruginea* extract on lung biochemical parameters of B16F-10 melanoma bearing animals.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Hydroxyproline (µg/mg protein)</th>
<th>Uronic acid (µg/100 mg tissue wet wt)</th>
<th>Hexosamine (mg/100 mg tissue dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Normal</td>
<td>0.98 ± 0.08</td>
<td>35 ± 2.6</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>2.</td>
<td>Metastasis control</td>
<td>24.4 ± 1.4</td>
<td>318 ± 14.8</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>3.</td>
<td>Metastasis + <em>A. ferruginea</em> (Prophylactic with tumor induction)</td>
<td>11.6 ± 1.7**</td>
<td>106 ± 12.2**</td>
<td>1.3 ± 0.3**</td>
</tr>
<tr>
<td>4.</td>
<td>Metastasis + <em>A. ferruginea</em> (Simultaneous with tumor induction)</td>
<td>15.8 ± 2.1**</td>
<td>167 ± 17.4**</td>
<td>1.82 ± 0.5**</td>
</tr>
<tr>
<td>5.</td>
<td>Metastasis + <em>A. ferruginea</em> (After tumor development)</td>
<td>14.6 ± 1.6**</td>
<td>158 ± 20.3**</td>
<td>1.91 ± 0.4**</td>
</tr>
</tbody>
</table>

The animals were sacrificed on the 21st day and their lungs were exercised. Using lung tissue homogenate, hydroxyproline, uronic acid and hexosamine were estimated. Values are expressed as Mean ± SD (n=6/group). Values significantly different from metastasis (non-extract-treated) control (**p < 0.01).
7.3.4 Effect of *A. ferruginea* on the inhibition of serum sialic acid and gamma glutamyl transpeptidase level during B16F-10 induced tumor metastasis

The effect of *A. ferruginea* on serum biochemical parameters is presented in Table 7.3. The serum sialic acid level of control metastatic tumor-bearing animals was highly increased (116.2 ± 5.6 µg/mL serum) as compared with normal values (26.2 ± 2.4 µg/mL serum). Here also the prophylactic administration of *A. ferruginea* significantly *(p < 0.01)* reduced the elevated serum sialic acid level to 62.±3.4 µg/mL serum, followed by the simultaneous modality (79.6±4.2 µg/mL serum); in the developed-modality group, it was reduced up to 81.4±6.8 µg/mL serum.

Serum γ-GGT level was also considerably enhanced in metastatic control animals (110.4 ± 8.2 nmol p-nitroaniline/ml serum) compared with normal animals (25.6 ± 2.8 nmol p-nitroaniline/ml serum). After the administration of *A. ferruginea* treatment, the elevated γ-GGT level was reduced significantly *(p<0.01)* to 49.7±2.9 nmol p-nitroaniline/ml serum in the prophylactic group and 54.8±5.3 nmol p-nitroaniline/ml serum in the simultaneous-modality group. In animals with developed tumor, the serum γ-GGT level was reduced to 56.6±3.6 nmol p-nitroaniline/ml serum by *A. ferruginea* treatment.
Table 7.3. Effect of *A. ferruginea* extract on serum biochemical parameters of B16F-10 melanoma bearing animals.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment</th>
<th>Sialic acid (µg/ml serum)</th>
<th>GGT (nmol p-nitroaniline/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>26.2 ± 2.4</td>
<td>25.6 ± 2.8</td>
</tr>
<tr>
<td>2</td>
<td>Metastasis control</td>
<td>116.2 ± 5.6</td>
<td>110.4 ± 8.2</td>
</tr>
<tr>
<td>3</td>
<td>Metastasis + <em>A. ferruginea</em> (Prophylactic with tumor induction)</td>
<td>62.±3.4**</td>
<td>49.7±2.9**</td>
</tr>
<tr>
<td>4</td>
<td>Metastasis + <em>A. ferruginea</em> (Simultaneous with tumor induction)</td>
<td>79.6±4.2**</td>
<td>54.8±5.3**</td>
</tr>
<tr>
<td>5</td>
<td>Metastasis + <em>A. ferruginea</em> (After tumor development)</td>
<td>81.4±6.8**</td>
<td>56.6±3.6**</td>
</tr>
</tbody>
</table>

The animals were sacrificed on the 21st day. Blood was collected, and the serum was separated and used for the estimation of sialic acid and GGT activity. Values are expressed as Mean ± SD (n=6/group). Values significantly different from metastasis (non-extract-treated) control (**p < 0.01).
7.3.5 Effect of *A. ferruginea* on the serum cytokines TNF-α, IL-6, IL-1β and IL-2 during B16F-10 induced tumor metastasis

The serum TNF-α, IL-6, IL-1β and IL-2 level during B16F-10 induced tumor metastasis and after treatment with *A. ferruginea* extract were represented in Figure 7.2 and 7.3. Serum levels of TNF-α, IL-6 and IL-1β were found to be significantly increased to (325 ± 28.2, 480 ± 40.6 and 58.2 ± 5.6 pg/ml) respectively in tumor metastasis control group. These levels were significantly reduced (p<0.01) in animals treated with *A. ferruginea* extract prophylactically (194.6 ± 24.2, 212.8 ± 35.4 and 28.4 ± 4.8 pg/ml), simultaneously (211.6 ± 28.4, 252.2 ± 29.5 and 29.4 ± 5.2 pg/ml) and in animals with developed tumor (269.6 ± 18.9, 342.8 ± 38.4 and 32.6 ± 4.8 pg/ml) respectively. But the level of IL-2 was found to be significantly decreased in tumor metastasis control group (17.8 ± 4.2 pg/ml) when compared with treatment prophylactically (26.3 ± 2.9 pg/ml), simultaneously (23.4 ± 3.4 pg/ml) and in animals with developed tumor (22.9 ± 3.6 pg/ml) respectively.
Figure 7.2 Effect of *A. ferruginea* on the serum cytokines TNF-α and IL-6 during B16F-10 induced tumor metastasis.

B16F-10 melanoma cells were induced through lateral tail vein (1 × 10⁶ cells). Tumor control animals were kept as such without any treatment. *A. ferruginea* treatment was done in different modalities (10 doses at 24 h interval, intraperitoneally). On 21st day of tumor induction, blood was collected, serum separated to determine the levels of TNF-α, IL-1β, IL-6 and IL-2 levels using respective ELISA kits. Values are mean + SD.
Figure 7.3 Effect of *A. ferruginea* on the serum cytokines IL-1β and IL-2 during B16F-10 induced tumor metastasis.

B16F-10 melanoma cells were induced through lateral tail vein (1 × 10^6 cells). Tumor control animals were kept as such without any treatment. *A. ferruginea* treatment was done in different modalities (10 doses at 24 h interval, intraperitoneally). On 21st day of tumor induction, blood was collected, serum separated to determine the levels of TNF-α, IL-1β, IL-6 and IL-2 levels using respective ELISA kits. Values are mean + SD.
7.3.6 Effect of *A. ferruginea* on Transcription factor profiling NF-κB (p65/p50) during B16F-10 induced tumor metastasis

DNA-bound transcription factor NF-κB (p65/p50) was detected by the primary antibody. A horseradish peroxidase-conjugated secondary antibody was then used to detect the primary antibody. The enzymatic product was measured spectrophotometrically. *A. ferruginea* treatment significantly inhibited the activation and nuclear translocation of transcription factor NF-κB subunit p65 (70.53 %) in prophylactic treatment, (67.63 %) in simultaneous modality and (65.97 %) in treatment with developed tumor respectively. Similarly in NF-κB subunit p50 (64.13 %) in prophylactic treatment, (56.92 %) in simultaneous modality and (53.51 %) in treatment with developed tumor respectively as represented in Table 7.4.

7.3.7 Effect of *A. ferruginea* on Histopathological analysis of lungs

The H&E-stained sections of lung tissues are shown in Figure 7.4. Lungs in the control animals showed infiltration of the neoplastic cells around the main bronchioles extended to the pleura. Together with fibrosis, reduces alveolar space, which in turn leads to reduced vital capacity. Animals treated with *A. ferruginea* extract prophylactically, simultaneously with tumor induction, after tumor development showed a significant reduction in the tumor mass, mild infiltration of the neoplastic cells, Alveoli and pleura were tumor free; the alveolar passage was lined with healthy ciliated columnar epithelial cells and was almost similar to the normal lung.
Table 7.4 Effect of *A. ferruginea* extract on transcription factors profiling NF-κB (p65/p50) subunits.

<table>
<thead>
<tr>
<th>Transcription Factors</th>
<th>% inhibition by <em>A. ferruginea</em> treatment (Prophylactic with tumor induction)</th>
<th>% inhibition by <em>A. ferruginea</em> treatment (Simultaneous with tumor induction)</th>
<th>% inhibition by <em>A. ferruginea</em> treatment (After tumor development)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB p65</td>
<td>70.53 %</td>
<td>67.63 %</td>
<td>65.97 %</td>
</tr>
<tr>
<td>NF-κB p50</td>
<td>64.13 %</td>
<td>56.92 %</td>
<td>53.51 %</td>
</tr>
</tbody>
</table>

NF-κB = nuclear factor Kappa B, OD is optical density. Nuclear extracts from lung tissue were prepared separately and subjected for transcription factor assay as per manufacturer’s instruction using Cayman™ transcription factor kit (Cayman™, Michigan, USA). Values shown are Mean ± SD. Values significantly different from tumor (non-extract-treated) control (**p < 0.01).
Figure 7.4 Histopathology of the lung of metastatic tumor-bearing animals.

Lungs of the metastasis-induced animals were fixed in neutral buffered formalin, and 4 mm sections were taken and stained with hematoxylin and eosin: (A) normal lung, (B) metastasis-bearing control, (C) *A. ferruginea* treated prophylactically, (D) *A. ferruginea* treated simultaneously, (E) *A. ferruginea* treated 10 days after tumor induction.
7.3.8 Effect of *A. ferruginea* on Immunohistochemical localization of p53 and Bcl-2 in lungs

One aim of the present study was to compare levels of immunohistochemical (IHC) expression of p53 tumor suppressor gene and Bcl-2 oncoprotein (anti-apoptotic protein) between metastasis alone bearing animals and metastasis bearing animals treated with *A. ferruginea* in three different modalities, along with normal lung tissue to determine its biological significance during metastasis. The results showed that, there was a great number of p53 immunoreactive cells (p53⁺; brown staining) in the lung tissues of mice in three different modalities treated with *A. ferruginea*. Metastasis alone bearing animals (no-drug treated) displayed a noticeable reduction of p53 immunoreactive cells. In contrast, there was a great number of Bcl-2 immunoreactive cells (Bcl-2⁺; brown staining) in the lung tissues of metastasis control group (no-drug treated). Mice treated with *A. ferruginea* in three different modalities reduced the overexpression of Bcl-2 immunoreactive cells (Figure 7.5 and 7.6).
Figure 7.5 Immunohistochemical localization of p53 tumor suppressor gene.

(A) normal lung, (B) metastasis-bearing control, (C) *A. ferruginea* treated prophylactically, (D) *A. ferruginea* treated simultaneously, (E) *A. ferruginea* treated 10 days after tumor induction.
Figure 7.6 Immunohistochemical localization of Bcl-2 protein.

(A) normal lung, (B) metastasis-bearing control, (C) *A. ferruginea* treated prophylactically, (D) *A. ferruginea* treated simultaneously, (E) *A. ferruginea* treated 10 days after tumor induction.
7.4 DISCUSSION

Metastatic cascade, the series of multistep-cell biological process involves dissemination of tumor cells to anatomically distant organ sites and subsequent adaptation to foreign microenvironments (Valastyan and Weinberg, 2011). Among the metastatic cancers, lung become the first organ to be encountered by the tumor cells and making it as major site for tumor metastasis. Earlier reports shown that, the survival rate for patients with metastatic melanoma is less than 10% (Hyoudou et al., 2004 and Bhatia et al., 2009). The tumor metastasis is treated by surgery, chemo- and radiation therapy which is effectively control many cancers only at the primary site and the development of metastatic tumors signals a poor diagnosis. In the present study, the cytotoxicity results shown that *A. ferruginea* extract have effective anti-proliferative activity against B16F-10 melanoma cells *in vitro*. The intraperitoneal administration of *A. ferruginea* extract in prophylactically, simultaneously with tumor inoculation and after development of tumor showed maximum reduction in lung colonization which results in significant reduction in tumor nodule formation. The survival rate of *A. ferruginea* treated animals in three different modalities was found to be increased by ≈ 40 to 58% than the control animals.

The major building block of collagen is hydroxyproline which plays a significant role in maintenance of lung structure and function. In the lung, collagen is found associated with bronchi, with blood vessels and with the alveolar interstitium (Nelson and Cox, 2005). During lung metastasis, the elevated levels of hydroxyproline indicate the fibrosis due to high collagen levels in the alveoli of lungs which results in reduced pulmonary function (i.e. inability of lung to perform normal gaseous exchange). Hence, the estimation of hydroxyproline has been used as an indicator to determine collagen content and lung fibrosis (Pradeep and Kuttan, 2002). *A. ferruginea* treated group of animals
in different modalities has much lower lung collagen hydroxyproline content compared with metastatic control animals (no-drug treated) which indicates prevention of fibrosis and normal alveolar function. This was validated in histopathological analysis of lung tissue from metastatic control and treated animals. The reduction in number of lung tumor colony formation (metastatic colonies of melanoma cells) was also correlates with these findings.

The acidic and basic modifications of primary alcohol group of aldoses in the tumor cells yield uronic acids (glucoronic acid) and amino sugar (hexosamine). Prolyl hydroxylase enzyme converts the prohydroxyproline to hydroxyproline in the presence of glucuronic acid lactone (uronic acid) and further activates the fiber formation during lung fibrosis. The elevated level of hexosamine and uronic acid was observed in several types of tumors and it is a well-known promoter of metastasis by opening up the spaces for tumor cells to migrate through ECM (Extra cellular matrix). The excess hexosamine and uronic acid level is directly correlated with the active growth and proliferation of malignant cells (Lipponen et al., 2001). The treatment with A. ferruginea extract in different modalities significantly inhibited the excess synthesis of hexosamine by blocking the ECM thus preventing malignant cells metastasis and lung fibrosis.

Hexosamine plays a major role in synthesis of N-acetyl neuraminic acid (i.e. sialic acid) which is a component of glycolipids present on the surface of the metastatic cells. The elevated level of these compounds in the metastasis bearing control animals indicates rapid tumor growth. The amount of sialic acid present on the tumor cell surface directly correlates with their metastatic ability (Vedralova and Borovansky, 1994; Sprenger and Duncan, 2012). After treatment with A. ferruginea, the amount of hydroxyproline, hexosamine, uronic acid and sialic acid reduced significantly which indicates reduced tumor metastasis.
Highly elevated level of GGT, the marker for cellular proliferation was observed in patients with either primary or secondary neoplasms. This metastatic tumor marker GGT level was increased in serum of tumor-bearing animals compared to normal group (Pradeep and Kuttan, 2002). The increased level of GGT was reduced significantly in all three modalities of *A. ferruginea* extract treatment, whereas prophylactic and simultaneous modalities showed maximum reduction.

Several studies have indicated that cancer cells exhibit a drastic elevation in the constitutive production of proinflammatory cytokines which include TNF-α, IL-1ß and IL-6 (Choo et al. 2005). The pleiotropic cytokine TNF-α has wide range of biological activities include regulation of tumor promotion and progression. The growth factor IL-6 was found to be stimulate vascular endothelial growth factor which promotes angiogenesis and metastasis (Siveen and Kuttan, 2011). Similarly IL-1ß is found to be constitutively overexpressed by tumor cells which is involved in regulation of host defense and immune responses and also regulates various genes such as Interferon regulatory factor 1 (IRF-1), plasminogen-activator inhibitor-1 (PAI1), IL-6, and inflammatory mediator cyclooxygenase-2 (COX-2) (Dudas et al. 2011). Our results indicate that administration of *A. ferruginea* extract could inhibit the expression of these cytokines in metastasis bearing animals. Moreover, there was a drastic reduction in the level of these cytokines in serum of tumor bearing animals after treatment with extract specifically in prophylactic and simultaneous treatment modalities which indicates its potent inhibition of pulmonary metastasis. IL-2 is involved in regulation of natural immunity and stimulates cytotoxic T-lymphocyte production and natural killer cells (Boyman et al., 2012). In contrast to other cytokines we measured in our present investigation, IL-2 level was found to increase after treatment with *A. ferruginea* extract which indicates stimulation of immune system against tumor progression during pulmonary metastasis.
The transcription factor NF-κB proteins plays a significant role in cellular transformation by providing rapid cell growth stimulus which is mediated by several cytokines (Mantovani, 2010). Overexpression of heterodimer NF-Kb p50 and p65 subunits is required for tumor invasion and progression in melanomas has been reported (Madonna et al., 2012). Thus, NF-Kb proteins become one of the main target for anti-cancer agents. In resting stage, NF-κB normally localizes to the cytoplasm, where it is bound by IκB proteins. During tumor progression, IκB is phosphorylated by IκB kinase, subsequently degraded by proteasome, then get released and translocates in to nucleus, where it triggers the transcription of multiple genes involved in metastatic cascade (Sathish and Guruvayoorappan, 2014). In the present study, we observed that treatment with A. ferruginea extract could inhibit the activation and nuclear translocation of NF-kb p50 and p65 subunits.

The tumor suppressor gene p53 is also termed as “Guardian of the Genome” because of its vital role involved in inducing apoptotic effects on cancer cells ad regulating genomic stability by controlling the cell cycle. It is clear that, the activation of p53 deregulates many oncogene expression which leads to suppression of tumor formation (Yang et al., 2014). Immunohistochemical analysis of p53 protein revealed increased expression in tumor control as well as treated one bearing metastatic tumor suggests that administration of extract activates p53 tumor suppressor gene. In contrast, administration of A. ferruginea extract suppressed the expression of Bcl-2 (B-cell lymphoma) the anti-apoptotic protein in tumor bearing metastasis hosts and also we observed overexpression of Bcl-2 protein in tumor control. Preclinical evidence suggests that consistent aberrant expression of Bcl-2 has been associated with variety of tumor types (Mukherjee et al., 2015).
In conclusion, the present study suggests that administration of extract could inhibit pulmonary metastasis induced by B16F-10 melanoma cells by regulating proinflammatory cytokines, could inhibit the activation and nuclear translocation of NF-kB p50 and p65 subunits. Therefore, *A. ferruginea* extract may be used as therapeutic target to inhibit pulmonary metastasis. Further investigations on understanding the molecular mechanisms involved in the anti-metastatic effect of *A. ferruginea* extract and its phytoconstituents will guide further to combat melanoma with effective treatment strategy.