CHAPTER – 7


EFFECT OF *Decalepis hamiltonii* AND *Solanum muricatum* ON EXPERIMENTAL METASTASIS

7.1 Introduction

Cancer is a term used for disease in which abnormal cells proliferate without control and invade other tissues. The major clinical challenge for cancer therapy remains the eradication of metastasis. Metastasis is an extremely complex process that remains to be a major problem in the management of cancer (Young et al., 2012). Tumour cells break away from the primary tumour site and degrade proteins that make up the surrounding extracellular matrix (ECM) that separates tumour from neighboring tissues. Cancer cells degrade the protein, breach the ECM and metastasize to form secondary tumour at distant organs (Nguyen and Massague, 2007). 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 competence of metastatic cells depends on the host’s immune cells at the niche, blood circulation and capillary beds, but most of the cancer cells are been trapped by these barriers, but only few cancer cells prevail over these barriers and metastasize. Sometime metastasized cancer cells can be dormant at distance niche for many years and could redevelop in later stage (Luzzi et al., 1998; Argon-Ching and Zujewski, 2007). Moreover there are many factors involved in metastasis such
as adhesion molecules, proteases, cell mobility, ECM, growth factors, oncogenes, signal transductions and transcription factors.

Despite advancement in early cancer diagnosis and treatment includes 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, there are no drugs available at present in the market that can block the steps involved in the metastatic process. Natural products contribute more than 50% of all the drugs in clinical use of the world. (Conte and Guarneri, 2012). Many experimental studies and clinical trials showed that many natural products played an important role in blocking of lung metastasis from primary tumours (Romagnolo and Selmin, 2012; Schumacher et al, 2011; Guruvayoorappan and Kuttan, 2008).

In the present study we had provided crucial evidence for the inhibition of metastasis by *D.hamiltonii* and *S.muricatum* and studied its regulatory effect on the inflammatory mediators and nuclear factor-kappa B subunits.
7.2 Materials and methods

7.2.1 Preparation and administration of plant extract

*D. hamiltonii* root extract and *S. muricatum* fruit extract were prepared according to the procedure described in chapter 3. For *in vivo* studies *D. hamiltonii* extract and *S. muricatum* extract were resuspended separately in 1% gum acacia and administered at a concentration of 20 mg/kg B.wt., (i.p.) and 10mg/kg B.wt., (i.p.) respectively.

7.2.2 Animals

In this study, male C57BL/6 mice (20-25g) were purchased from the National Institute of Nutrition (Hyderabad, India). The animals were kept on an equal light and dark cycle, diet with normal mouse chow (Sai Durga Feeds, Bangalore, India) and given water *ad libitum*. All animal were handled according to the guidelines obtained from Institutional Animal Ethics Committee, Karunya University.

7.2.3 Cell lines

The B16F-10 melanoma cells were procured from National Centre for Cell Sciences, Pune, India.
7.2.4. Induction of lung metastasis

B16F-10 melanoma cells ($10^6$ cells/animal) were injecting through tail vein for induction of lung metastasis.

7.2.5 Groups

C57BL/6 Mice (20 to 25 grams) were divided into four groups (14 mice/group). Group I was kept as normal control, Group II, III and IV were injected with B16F-10 cells as explained above. Group II served as metastatic control, group III was treated with *D.hamiltonii* and group IV was treated with *S.muricatum* as explained above.

7.2.6 Chemicals

N-acetyl neuraminic acid and papain were purchased from Sisco Research Laboratory, Mumbai, India. Hydroxyproline and glucuronic acid lactone were obtained from Sigma Chemicals, St. Louis, USA. DMEM was obtained from HiMedia (Mumbai, India). All other reagents were of analytical reagent grade.

7.2.7 ELISA kits

ELISA kits for mouse IL-1β, IL-2, IL-6, TNF-α, and GM-CSF were procured from Koma Biotech™, Gangseo-gu Seoul, Korea. Gamma glutamyl
transpeptidase kit was purchased from Merck™, Mumbai, India. iNOS and COX-2 kits were procured from USCN Life science Inc™, Houston, USA and Bluegene Biotech™, Shangai, China respectively. Transcription factor profiling kit for p50 and p65 was procured from Cayman™, Michigan, USA.

7.2.8 Assessment of metastasis

7.2.8.1 Lung nodule formation

All groups of animals (8 animals/each group) were sacrificed after 21 days of tumour induction, their blood was collected and lungs also were excised, washed with saline and were used for lung nodule count, biochemical analysis and histopathological examination.

7.2.8.2 Survival rate

All groups of animals (6 animals/group) were observed for their survival rate, and the percentage increase in life span (ILS) was calculated using the formula: %ILS=T-C/C x 100, where T: number of survival days of the treated animals and C: number of survival days of the control animals.
7.2.8.3 Biochemical studies

The lung tissue and serum was used for the assessment of lung hydroxyproline (Bergman et al., 1970), lung uronic acid (Bitter and Mair, 1962), lung hexosamine (Elson and Morgan, 1933), serum sialic acid (Bhavanandhan et al., 1981) serum GGT [ELISA] (Merck™, Mumbai, India) lung iNOS and COX-2 (USCN Life Science Inc, Houston, USA), serum TNF-α, IL-1β, IL-6, GM-CSF and IL-2 [ELISA] (Koma Biotech™, Gangseo-gu Seoul, Korea) and serum nitrite (Green et al., 1982). Nuclear extract for prepared and NF-kB p65 and NF-kB p50 level were quantified according to (Cayman™, Michigan, USA)

7.2.8.4 Phagocytic index

Phagocytic Index was determined by carbon clearance test. The phagocytic index K was calculated using the equation.

\[ K = \frac{(\ln OD_1 - \ln OD_2)}{(t_2 - t_1)} \]

where, OD_1 and OD_2 are the optical densities at time t_1 and t_2 respectively (Gupta et al., 2010)
7.2.8.5 Histopathological studies

The lung tissue from the metastasis group was fixed in 10% formaldehyde, dehydrated and embedded in paraffin, and routine 4µm sections were prepared. Tissues were stained with eosin and hematoxylin. The images were captured at 40X magnification.

7.2.9 Statistical analysis

Results were expressed as mean ± standard deviation. Statistical evaluation was performed via one-way analysis of variance (ANOVA) followed by Dunnett’s test using Graph Pad Software (Instat version 3.0). The minimum level of statistical significant was considered at \( p < 0.05 \).

7.3 Results

7.3.1 Macroscopical results

Treatment with *D.hamiltonii* (42 ± 5.2) and *S.muricatum* (66 ± 6.2) significantly reduce in lung tumour nodule formation when the animals were administered simultaneously with the tumour cells. The metastatic control animals had an increase number of tumour nodules (250 ± 25) on their lungs (Table 7.1).
The survival rate of metastatic control group of mice was found in up to 33 days. The extract treated mice were still alive after the termination of the experiment (90 days) (Table 7.1).

7.3.2 Hydroxyproline, uronic acid and hexosamine concentration

Figure 7.1 shown as significant increase in lung hydroxyproline level (24.4 µg/mg protein) in metastatic control animals. Administration of *D.hamiltonii* (7.6 µg/mg protein) and *S.muricatum* (8.8 µg/mg protein) showed significant reduction in lung collagen hydroxyproline level.

The level of uronic acid in metastatic control animals is 318 µg/100mg tissue. The normal control animals levels of uronic acid in their lung tissue (35µg/100mg tissue), and was significantly reduced in the *D.hamiltonii* (62.2 µg/100mg tissue) and *S.muricatum* (76 µg/100mg tissue) treated animals, indicating decreased lung fibrosis. The metastatic control animals showed an increased level of lung hexosamine (5.2 mg/100mg lyophilized tissue) and the *D.hamiltonii* and *S.muricatum* treatment showed a significantly reduced level (1.2 mg/100mg lyophilized tissue and 1.8 mg/100mg lyophilized tissue), indicating a decreased tumour burden (Figure 7.1, 7.2 and 7.3).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animal Per group</th>
<th>No. of nodules per lung</th>
<th>% inhibition of tumour nodules</th>
<th>No. of animals Survived after 90 days (%ILS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>250 ± 25.0</td>
<td>-</td>
<td>0/6</td>
</tr>
<tr>
<td>D. hamiltonii</td>
<td>14</td>
<td>42 ± 5.2**</td>
<td>83.2</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>S. muricatum</td>
<td>14</td>
<td>66 ± 6.2**</td>
<td>73.6</td>
<td>6/6 (100%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D. **p<0.01 compared with control group.

**Table 7.1: Effect of *D. hamiltonii* and *S. muricatum* on the lung tumour nodule formation and survival rate of metastatic-tumour-bearing animals**
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.1: Effect of *D. hamiltonii* and *S. muricatum* on the lung collagen hydroxyproline, content during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

**Figure 7.2:** Effect of *D.hamiltonii* and *S.muricatum* on the lung uronic acid level during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

**Figure 7.3: Effect of *D.hamiltonii* and *S.muricatum* on the lung hexosamine content during metastasis**
7.3.3 Serum sialic acid and GGT concentration

The methanolic extract of *D. hamiltonii* and *S. muricatum* significantly decreased the serum sialic acid level to 39.4 µg/ml and 43.4 µg/ml respectively, compared to metastatic tumour-bearing animals (116.2 µg/ml) (Figure 7.4.). Similarly the higher level of GGT (110.4 nmol p-Nitroaniline/ml) in control animals was significantly reduced to 42.2 8 nmol p-Nitroaniline/ml and 47.8 nmol p-Nitroaniline/ml after the treatment with *D. hamiltonii* and *S. muricatum* respectively (Figure 7.5).

7.3.4 Serum nitrite concentration

The effect of *D. hamiltonii* and *S. muricatum* on the nitrate level of serum is shown in Figure 7.6. The increased level of nitrite was observed in metastasis control group (40.2 µmol). Administration of *D. hamiltonii* and *S. muricatum* significantly reduced the elevated level of serum nitrite to 28.4 and 24.2 µmol respectively.
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.4: Effect of *D.hamiltonii* and *S.muricatum* on the inhibition of serum sialic acid level during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.5: Effect of *D. hamiltonii* and *S. muricatum* on the inhibition of serum gamma glutamyl transpeptidase level during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.6: Effect of *D.hamiltonii* and *S.muricatum* on the serum nitrite level during metastasis
7.3.5 iNOS and COX-2 quantification

The effect of *D. hamiltonii* and *S. muricatum* on the lung iNOS and COX-2 level is shown in Figure 7.7 and Figure 7.8 respectively. The elevated level of iNOS in control animals (65.6 ng/g tissue) was significantly reduced to 40.2 and 43.6 ng/ml after *D. hamiltonii* and *S. muricatum*. Similarly *D. hamiltonii* and *S. muricatum* treatment could significantly reduce the level of COX-2 from 74.6 ng/g tissue (control) to 39.4 ng/g tissue (*D. hamiltonii*) and 42.2 ng/g tissue (*S. muricatum*) respectively (Figure 7.7 and 7.8).

7.3.6 TNF-α, IL-1β, IL-6, GM-CSF and IL-2 level

In metastatic control animals, the TNF-α level was significantly increased to 325 pg/ml. Administration of *D. hamiltonii* and *S. muricatum* could significantly decrease the TNF-α level to 182.6 pg/ml and 176.4 pg/ml respectively (Figure 7.9). The normal level of IL-1β is 18 pg/ml. In metastatic control animals it was increased to 58.2 pg/ml on 21st day after tumour challenge. Administration of *D. hamiltonii* and *S. muricatum* could significantly reduce to 33.2 pg/ml and 36.4 pg/ml respectively (Figure 7.10). Serum IL-6 level was found to be significantly increased to 480 pg/ml in metastatic control animals which was significantly lower level to 276 pg/ml and 285 pg/ml on 21st day after tumour challenge due to *D. hamiltonii* and *S. muricatum* (Figure 7.11). In metastatic control animals the serum GM-CSF level
was significantly increased (40.6 pg/ml) from the normal control animal value of 20.2 pg/ml. Treatment of *D.hamiltonii* and *S.muricatum* was found to significantly decreased the elevated level of GM-CSF to 31.0 pg/ml and 33.2 pg/ml (Figure 7.12).

We also evaluated the effect of *D.hamiltonii* and *S.muricatum* on the immunopotentiating cytokine, T-cell growth factor (IL-2) during metastasis. The IL-2 levels in the *D.hamiltonii* (29.8 pg/ml) and *S.muricatum* (30.6 pg/ml) treated animals on day 21 was found to be significantly enhanced compared with the metastatic control group (21.4 pg/ml). The IL-2 level in normal control group of animals was found to be 24 pg/ml (Figure 7.13).

### 7.3.7 Phagocytic activity

The effect of *D.hamiltonii* and *S.muricatum* on the phagocytic index is shown in Figure 7.14. The decreased phagocytic index (K) in the control animals was found to be increased to 71.42% and 50 % after the treatment with *D.hamiltonii* and *S.muricatum*. 
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.7: Effect of *D.hamiltonii* and *S.muricatum* on the lung iNOS level during metastasis
Values are expressed as mean ± S.D. **$p<0.01$ compared with control group.

Figure 7.8: Effect of D. hamiltonii and S. muricatum on the lung COX-2 level during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.9: Effect of *D.hamiltonii* and *S.muricatum* on the serum TNF-α during metastasis
Values are expressed as mean ± S.D. ** p<0.01 compared with control group.

Figure 7.10: Effect of *D. hamiltonii* and *S. muricatum* on the serum IL-1β during metastasis
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

Figure 7.11: Effect of *D.hamiltonii* and *S.muricatum* on the serum IL-6 during metastasis
Values are expressed as mean ± S.D. *p<0.05, **p<0.01 compared with control group.

**Figure 7.12:** Effect of *D. hamiltonii* and *S. muricatum* on the serum GM-CSF during metastasis
Values are expressed as mean ± S.D. *p<0.05 compared with control group.

Figure 7.13: Effect of *D.hamiltonii* and *S.muricatum* on the serum IL-2 during metastasis
Values are expressed as mean ± S.D. *p<0.05 compared with control group.

**Figure 7.14: Effect of *D. hamiltonii* and *S. muricatum* on the phagocytic index during metastasis**
7.3.8 Translocation of transcription factors

*D.hamiltonii* and *S.muricatum* treatment significantly inhibited the translocation of NF-kB subunits p65 and p50 compared with metastatic control group (Table 7.2).

7.3.9 Histopathological results

The tissue specimen from metastatic control animal showed prominent tumour nodules around the terminal bronchiole. A clear area of necrosis has been observed. The alveolar passage could not be distinguished because of massive infiltration of neoplastic cells (Figure 7.15A). Tumour mass has been decreased in *D.hamiltonii* and *S.muricatum* treated animals. The alveoli and pleura were tumour free and the alveolar passage was lined with healthy columnar epithelial cells (Figure 7.15B and 7.15C).
Values are expressed as mean ± S.D. **p<0.01 compared with control group.

**Table 7.2: Effect of *D.hamiltonii* and *S.muricatum* on the translocation of transcription factors**

<table>
<thead>
<tr>
<th>Transcription</th>
<th>OD of Control (B16F-10 alone)</th>
<th>OD after <em>D.hamiltonii</em> Treatment</th>
<th>OD after <em>S.muricatum</em> Treatment</th>
<th>% of inhibition by <em>D.hamiltonii</em> Treatment</th>
<th>% of inhibition by <em>S.muricatum</em> Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB p65</td>
<td>0.552 ± 0.026</td>
<td>0.145 ± 0.022 **</td>
<td>0.152 ± 0.036 **</td>
<td>73.73</td>
<td>72.46</td>
</tr>
<tr>
<td>NF-κB p50</td>
<td>0.645 ± 0.042</td>
<td>0.192 ± 0.032 **</td>
<td>0.242 ± 0.082 **</td>
<td>70.23</td>
<td>62.48</td>
</tr>
</tbody>
</table>
Figure 7.15: Effect of *D. hamiltonii* and *S. muricatum* on the lung

Histopathology analysis (40X magnification)

A) Metastasis Control  
B) Metastasis + *S. muricatum*

C) Metastasis + *S. muricatum*
7.4 Discussion

One of the most challenging tasks in cancer research is to acquire more insight into mechanisms involved in the metastatic process, in which detached cells from the primary tumour can establish secondary tumour in distant sites (Lang et al., 2006). The process of cancer metastasis involves a cascade of genetic alterations, and expression of the metastatic phenotypes depends on a balance between regulatory elements that promote or inhibit the process at one or more of the sequential steps (Hanahan and Weinberg, 2000). Although several drugs are available for cancer therapy, there are no drugs which can satisfactorily inhibit the metastatic process. Medicinal plants can play a prominent role in the treatment of cancer. However, most of these plants have not been scientifically evaluated for their therapeutic efficacy and safety. In the present study we had analyzed the effect of *D.hamiltonii* and *S.muricatum* on the inhibition of lung metastasis: when administered simultaneously there was 83.2% and 73.6% inhibition of lung nodules. Compared with metastatic control group.

The state of lung fibrosis was evaluated by estimating the lung collagen hydroxyproline content because collagen is deposited massively in the alveolus of lungs during lung fibrosis (Tormey and Waalkes, 1980). Deposition of collagen during lung fibrosis results in the reduced pulmonary function (Voet and Voet, 1995). One of the major component in collagen is hydroxyproline which constitutes
about fifteen to thirty percentage (Prabhu and Guruvayoorappan, 2011). The treatment with *D. hamiltonii* and *S. muricatum* showed significant reduction in lung hydroxyproline content, indicating less incidence of lung fibrosis.

In the presence of glucuronic acid lactone, prolyl hydroxylase enzyme converts the prohydroxyproline to hydroxyproline. Glucuronic acid lactone also activates the fiber formation during lung fibrosis. Hexosamine plays an important role in the synthesis of sialic acid (N-acetyl neuraminic acid). The increased levels of these compounds in the metastatic control animals indicate the constructive work going on in the tissue, that is, the tumour growth. On treatment with *D. hamiltonii* and *S. muricatum*, the amount of uronic acid, hydroxyproline and hexosamine reduced significantly indicating reduced tumour growth.

The serum sialic acid and GGT levels were found to be higher in the tumour bearing control animals. Sialic acid is an acylated derivative of neuraminic acid, usually occurring as a terminal component of carbohydrate chains of glycoprotein and glycolipids. The amount of sialic acid present on the tumour cell surface correlates directly with their metastatic ability (Vedralova and Borovansky, 1994). GGT, a marker for cellular proliferation, was found to be increased in the serum of metastatic control animals compared with the normal animals. GGT catalyzes the GSH breakdown. Treatment with *D. hamiltonii* and *S. muricatum* could reduce the GGT level significantly thereby indicating its inhibitory effect on metastasis.
iNOS is overly expressed in several tumours and any natural product which can inhibit the expression of iNOS is of practical value (Prabhu and Guruvayoorappan, 2010). The role of Nitric oxide in tumour biology is ambiguous. NO has been reported to induce vasodilation or, as second messenger of other growth factors, gives rise to enhanced permeability in tumour vasculature. In such cases, the blood flow in the tumour will be increased, which would provide a favourable condition for the tumour (Guruvayoorappan, 2008).

More recently, some cytokines have also been implicated as putative mediators of metastasis (Coussens et al., 2013). TNF-α may regulate many critical processes of tumour promotion and progression and is an important cytokine mediator of cancer cachexia, in addition to IL-1β, IL-6 and interferon-γ (Oliff et al., 1987). TNF-α release causes influx of polymorphonuclear neutrophil, expression of vascular endothelial growth factor and release of inflammatory mediators from multiple pulmonary cell types (Tracey et al., 1988). IL-1β is a pleiotropic cytokine (Dinarello, 1996). It easily diffuses into the tumour microenvironment, in which it potentiates tumour invasiveness and metastasis by increased secretion of growth factors, matrix metalloproteases, angiogenesis-promoting factors and adhesion molecule expression (Song et al., 2003). In patients with head and neck squamous carcinoma, proinflammatory cytokines such as IL-6 and GM-CSF are highly expressed (Chen et al., 1999). IL-6 plays an important role in thrombocytosis and in
the storage of VEGF in thrombocytes (Suzuki et al., 1992). IL-6 has been shown to upregulate VEGF-A load in the platelet aggregate on the tumour endothelium (Chechlinska et al., 2008).

GM-CSF is a pleiotropic cytokine produced by fibroblasts, endothelial cells and various carcinoma cells. GM-CSF takes part in angiogenesis events including differentiation of angioblast into endothelial cells, their migration and proliferation (Kuhlmann et al., 2007). Tumours expressing GM-CSF have been shown to have megakaryocyte potentiating activity due to IL-6 and cause thrombocytosis (Frenkel, 1991). Since thrombocytes release VEGF into the circulation, thereby inhibition of GM-CSF, by *D.hamiltonii* and *S.muricatum* treatment can have a direct effect on the production of VEGF there by regulating angiogenesis.

IL-2 is of clinical value in natural immunity by stimulating natural killer cell and cytotoxic T-lymphocyte production (Boymann et al., 2012). In this study treatment with *D.hamiltonii* and *S.muricatum* shows significantly increased the IL-2 levels, which may contribute to the stimulation of the immune system against tumour growth.

The Phagocytic index was carried out to assess the effect of drugs on the reticulo-endothelial system (RES). The phagocytic cells in this system mainly comprise of mononuclear phagocyte system (MPS). Cells of the RES and MPS are
known to be important in the clearance of particles from the bloodstream. In this experiment, the increment of phagocytic index as seen from the carbon clearance test after administration with *D. hamiltonii* and *S. muricatum* supports its stimulation of the immune system.

NF-κB proteins have been implicated as playing a major role in the cellular transformation by either providing a role in the cellular transformation by either providing a continued positive cell growth stimuli such as that mediated by cytokines, or through inhibition of apoptotic pathways (Mantovani, 2010). NF-kB over expression is required for the development of tumour invasiveness in melanoma (Madonna et al., 2012). NF-kB is a heterodimer composed of p50 and p65 subunits. In the present study we found that *D. hamiltonii* and *S. muricatum* could inhibit the activation and nuclear translocation of transcription factors such as NF-kB p65 and NF-kB p50 and could regulate the cytokine profile thereby inhibiting tumour metastasis.