CHAPTER - 7

INVESTIGATION OF THE ANTI-METASTATIC EFFECT OF RHIZOPHORA APICULATA

7. Aim

To evaluate the effect of R.apiculata on the inhibition of tumor metastasis

7.1 Introduction

Cancer is a term used for disease in which abnormal cells proliferate without control and invade other tissues. Cancer is one of leading cause of death worldwide. The main reason for such high mortality from cancer is due to the highly invasive behavior of cancer cells, which usually results in metastasis (Khan and Mukhtar, 2010). Metastasis is a process by which cancer cells spread to other parts of the body through blood circulation and lymphatic system. A tumor formed by metastatic cancer cell is called as metastatic tumor (Klein, 2008). Many metastatic tumors develop at the first area of blood vessels where cancer cells arrive after leaving the primary tumor. After leaving the primary tumor, the lungs are one of the first sites where metastatic cells are carried by the bloodstream (Hyoudou et al., 2004). The other common site for the cancer cells to metastasize includes brain, bones, liver, adrenal gland, lymph nodes, peritoneum, skin and other organs.

Metastasis is an extremely complex process that remains to be a major problem in the management of cancer (Hunter et al., 2008). The metastatic process involves tumor cell invasion from the primary tumor, intravasation, arrest and extravasation of the circulatory system to form small tumors known as micrometastasis that stimulate
angiogenesis. Tumor cells break away from the primary tumor site and degrade proteins that make up the surrounding extracellular matrix (ECM) that separates tumor from neighboring tissues. Cancer cells degrade the protein, breach the ECM and metastasize to form secondary tumor at distant organs (Nguyen and Massagué, 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 tumor is known as metastatic breast cancer, not lung cancer (Talmadge and Fidler, 2010). However, metastatic cancer cells and cells of the primary cancer usually have some molecular features in common such as the expression of certain proteins or the presence of specific chromosome changes. The competence of metastasize cells depends on the host’s immune cells at the niche, blood circulation and capillary beds, but most of the cancer cells are trapped by these barriers, but only few cancer cells prevail over these barriers and metastasize. Sometimes metastasized cancer cells can be dormant at distance niche for many years and could redevelop in later stage (Luzzi et al., 1998; Aragon Ching and Zujewski, 2007). More over there are many molecules involved during metastasis which includes adhesion molecules, proteases, cell mobility, ECM, growth factors, oncogenes, signal transductions and transcription factors.

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.

Natural products and their derivatives contribute more than 50% of all the drugs in clinical use of the world. Almost 60% of drugs approved for cancer treatment are of natural origin (Sithranga Boopathy and Kathiresan, 2010). Many experimental studies and clinical trials showed that many natural plants played an important role in blocking
of lung metastasis from primary tumors (Schantz et al., 1987; Leyon and Kuttan, 2004; Leyon et al., 2005; Thejass and Kuttan, 2006).

Marine flora constitutes more than 90% of oceanic biomass that offer a great scope for discovery of new drugs. It is recognized that ocean contains a large number of natural products and novel chemical entities with unique biological activities that may be useful in finding the potential drugs with greater efficacy and specificity for the treatment of human diseases (Sithranga Boopathy and Kathiresan, 2010). The marine flora may contain novel compounds to withstand extreme variations in pressure, salinity, temperature and the chemicals produced are unique in diversity, structural, and functional features.

Mangroves have long been used in folk medicine to treat diseases and very few mangrove plants are reported for possible source of anticancer drugs, based on traditional knowledge and preliminary scientific work (Sithranga Boopathy and Kathiresan, 2010). Mangrove, *Rhizophora apiculata* (*R. apiculata*) (Family: Rhizophoraceae) is a halophytic mangrove used as folk medicine, based to on the fact that use of its root, leaf or stem extracts to a greater extent imparts an inhibitory effect on the growth of bacterial, viral and fungal pathogens (Premanathan et al., 1999; Antony et al., 2011). *R. apiculata* have a high content of flavanoids, tannins, catachin, anthroquinone, pyroligneous acid and syringol. The phytochemical analysis of methanolic extract of *R. apiculata* by GC/MS and LC/MS analysis (Chapter 3) showed the presence of pyrazole (alkaloid), ketone, thiazolidinediones and 4-pyrrolidinyl. In the present study we have evaluated the anti-metastatic activity *R. apiculata* in B16F-10 melanoma cell induced lung metastasis in C57BL/6 mice.
7.2 Material and Methods

7.2.1 Plant collection

*R. apiculata* (Vernacular name - Surapunnai in Tamil), whole plant were collected from Pichavaram mangrove forest which is located in Cuddalore District, Tamil Nadu, India. The plants materials were authenticated by an eminent taxonomist and a voucher specimen (Rhiz-018) were deposited in the department of Botany, M.E.S. Kalladi College, Mannarkkad, India.

7.2.2 Experimental animals

C57BL/6 male mice weighing (20–25g) were purchased from Sri Venkateshwara Enterprises Laboratory animals, Bangalore, India. The animals were kept in a pathogen-free air-controlled room maintained at 24°C with a 50% relative humidity and 12-hr light/dark cycle, and fed with normal mice chow (Sai Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were performed after getting approval from Institutional Animal Ethics Committee, Karunya University.

7.2.3 Cell lines

B16F-10 melanoma cells were obtained from the National Centre for Cell Sciences (Pune, India) and maintained in DMEM (Hi Media, Mumbai, India) containing 10% fetal bovine serum (FBS; Hi Media) and 1% antibiotic/antimycotic solution. The cells were incubated at 37 °C with 5% CO₂.
7.2.4 Chemicals and kits

Gum acacia was purchased from Hi-Media, Formaldehyde solution was procured from Universal Laboratories Pvt. Ltd. (Hyderabad, India). GGT kit was purchased from Span diagnostics, Surat, India. All chemicals used were of analytical or reagent grade.

7.2.5 Extract preparation

The plant material was dried at 45°C and then powdered using a polarizer. Ten gram of the material was stirred overnight in 70% methanol (100 ml), and then centrifuged at 10,000 rpm for 10 min at 4°C. The resultant supernatant was collected and the methanol was removed by evaporation. The yield of the extract was found to be 12% [w/w]. For in vivo experiments the extract was administered via intraperitoneal (i.p) injection at a concentration of 10 mg/kg b.wt daily, for 10 consecutive days.

7.2.6 Experimental design

C57BL/6 mice (n=19) were separated into two groups (9 nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting B16F-10 melanoma cells (1x10^6 cells/animal) via lateral tail vein. Group I was kept as metastasis control. Group II were treated with R.apiculata (10 mg/kg b.wt. (i.p.) for ten consecutive days. The animals were euthanized by cervical dislocation and blood samples were collected from cardiac puncture at different day intervals (day 7, day 11 and day 21). The serum samples after centrifugation (5000 rpm, 10 minutes) were used for the estimation of NO and GGT (Green et al., 1982; Tate and Meister, 1974).
7.2.7 Determination of the effect of *R. apiculata* on the serum NO level during metastasis

The serum samples obtained from the above mentioned groups were used for the quantification of NO (Green et al., 1982). On day (7th, 11th, and 21st day) serum NO level was estimated by the use of a Griess reaction. The reaction mixture containing sodium nitroprusside and serum was incubated at 25ºC for 150 min. After incubation, 1ml of the reaction mixture mixed with equal volume of Griess reagent and allowed to stand for 15 min at room temperature. The absorbance of the pink colored chromophore formed was measured at 546 nm against the corresponding blank solutions and expressed in μM.

7.2.8 Determination of the effect of *R. apiculata* on the serum GGT level during metastasis

The serum GGT level was estimated by measuring the release of p-nitroaniline from gamma glutamyl p-nitroaniline in the presence of glycyl glycine. The GGT content was determined from the graph plotted using p-nitroaniline as the standard (Tate and Meister, 1974).

7.2.9 Determination of the effect of *R. apiculata* on the serum sialic acid level during metastasis

C57BL/6 mice were separated into two groups (9 nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting metastatic B16F-10 melanoma cells (1x10⁶ cells/animal) via lateral tail vein. Group I was kept as metastasis control. Group II were treated with *R. apiculata* (i.p.) for ten consecutive days. Blood samples were collected by cardiac puncture (day 21) and serum was separated and used
for the estimation of protein bound sialic acid (Skoza and Mohos, 1976; Bhavanandan et al., 1981). The serum sample was hydrolyzed using 0.2 N sulphuric acid. The hydrolysate was oxidized with periodic acid and incubated at 37 °C for 1 min. After terminating oxidation using sodium arsenate, 6% thiobarbituric acid was added. Sialic acid was estimated at 549 nm with reference to 532 nm after adding DMSO. Sialic acid content was determined from the standard graph plotted using n-acetyl neuraminic acid.

7.2.10 Determination of the effect of *R. apiculata* on lung collagen hydroxyproline, hexosamine and uronic acid during metastasis

C57BL/6 mice were separated into two groups (9 nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting highly metastatic B16F-10 melanoma cells (1x10^6 cells/animal) via lateral tail vein. Group I was kept as metastasis control. Group II were treated with *R. apiculata* (i.p.) for ten consecutive days. Animals from each group were euthanized by cervical dislocation on day 21 (final day of experiment) of tumor induction and the lungs were excised and used for the estimation of lung hydroxyproline, hexosamine and uronic acid (Elson and Morgan, 1933; Bitter and Muir, 1962; Bergman and Loxley, 1970).

Lung collagen hydroxyproline was determined by the method of Bergman and Loxley (1970). The lungs were homogenized and protein precipitated with TCA were hydrolyzed for 24 hr at 110 °C in sealed glass tubes using 6 N HCl. The HCl was evaporated and the remaining hydrolysate residue was allowed to dryness. The residue was dissolved in distilled water and assay by chloramine -T method. The absorbance was measured at 560nm. Standard graph was plotted using reagent hydroxyproline standard.
The hexosamine content present in the lung tissue was estimated by the method of Elson and Morgan (1933). Lyophilized tissue samples were hydrolyzed with 2 N HCl at 100 °C for 6 hr in sealed glass tubes. After hydrolysis, the HCl was evaporated and the remaining hydrolysate residue was allowed to dryness. The residue was dissolved in distilled water and treated with 2% acetyl acetone. The hexosamine level was determined in the presence of Ehrlich’s reagent at 530 nm. Standard graph was plotted by using glucosamine standard.

The uronic acid content present in the lungs of metastatic tumor bearing animals was estimated by the carbazole reaction (Bitter and Muir, 1962). The lungs were digested with crude papain and were hydrolyzed at 100 °C for 20 min in a sealed glass tubes. After hydrolysis, the hydrolysate was allowed to treat with sulfuric acid. The uronic acid level was determined by using carbazole reagent at 530 nm. Standard graph was plotted using by glucuronic acid lactone.

7.2.11 Determination of the effect of *R. apiculata* on the survival rate of metastatic tumor bearing animals

The remaining were set as mentioned above. Three animals from each group were observed for survival rate. The mortality rate of each animal were observed and the percentage increase in life span (% ILS) was calculated using the formula % ILS=T-C/ C×100. Where ‘T’ represents the number of survival days of treated animals and ‘C’ represents the number of survival days of control animals.

7.2.12 Histopathological examination

A portion of excised lung tissue were fixed in 10% formalin, cut into 5-μm thickness, stained using H&E (hematoxylin and eosin) and then examined for
histopathological changes. The stained sections of lung tissue were examined for metastatic nodules, hyperchromatic nucleus and pleomorphism.

7.2.13 Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dennett’s test using Graph pad Instat Version 3.0 for Windows 95 (Graph Pad Software, San Diego, California, USA). *p* <0.05 were considered to be statistically significant.

7.3 Results

7.3.1 Effect of *R.apiculata* on the serum NO and GGT level during metastasis

Effect of *R.apiculata* on serum NO and GGT level during metastasis is shown in Table 7.1. The administration of *R.apiculata* significantly (*p* < 0.01) reduced the serum NO level (27.20 ± 0.31µM) on 21st day when compared with metastasis control (34.29 ± 1.13µM) on the same day. The administration of *R.apiculata* also significantly (*p*<0.01) decreased the serum GGT level (44.30 ± 1.4 nmol *p*-nitroaniline/ml) on 21st day when compared with metastasis control (81.44 ± 4.0 nmol *p*-nitroaniline/ml) on the same day.
<table>
<thead>
<tr>
<th>NO (µM)</th>
<th>Day</th>
<th>7</th>
<th>11</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis control</td>
<td>26.58 ± 0.38</td>
<td>29.74 ± 0.33</td>
<td>34.29 ± 1.13</td>
<td></td>
</tr>
<tr>
<td>Metastasis+ R. apiculata</td>
<td>25.83 ± 0.31*</td>
<td>27.08 ± 0.25**</td>
<td>27.20 ± 0.31**</td>
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</table>

<table>
<thead>
<tr>
<th>GGT (nmol p-nitroaniline/ml)</th>
<th>Day</th>
<th>7</th>
<th>11</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis control</td>
<td>9.64 ± 0.6</td>
<td>29.71 ± 1.7</td>
<td>81.44 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Metastasis+ R. apiculata</td>
<td>7.71 ± 1.3</td>
<td>20.80 ± 2.0**</td>
<td>44.30 ± 1.4**</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1 Effect of *R. apiculata* on serum NO and GGT level during metastasis
Blood samples were collected by tail vein on 7th, 11th and 21st day. Serum samples were isolated to determine NO and GGT level. Values are expressed as mean ± SD. Value is significantly different from metastasis control (*p < 0.05; **p < 0.01).
7.3.2 Effect of *R. apiculata* on serum sialic acid, lung hydroxyproline, hexoamine and uronic acid during metastasis

The effect of *R. apiculata* on serum sialic acid, lung hydroxyproline, hexoamine and uronic acid during metastasis is shown in Table 7.2. The administration of *R. apiculata* significantly (*p* < 0.01) decreased the serum sialic acid level (35.51 ± 0.42 µg/ml) when compared to metastasis control (105.33 ± 0.91 µg/ml). The *R. apiculata* significantly (*p* < 0.01) reduced the lung hydroxyproline level (5.34± 0.17 µg/mg protein) when compared with metastatic control (20.25±1.12 µg/mg protein).

The lung hexoamine level of *R. apiculata* treated animals were significantly (*p* < 0.01) decreased (1.35±0.14 mg/100 mg tissue dry weight) when compared with metastatic control (3.17±0.17 mg/100 mg tissue dry weight). After the treatment of *R. apiculata* the lung uronic acid level significantly (*p* < 0.01) reduced (93.20±2.30 µg/100 mg tissue wet weight) when compared with metastatic control animals (238.30±4.25 µg/100 mg tissue wet weight).
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Serum Sialic acid (µg/ml)</th>
<th>Hydroxyproline (µg/mg protein)</th>
<th>Hexosamine (mg/100 mg tissue dry weight)</th>
<th>Uronic acid (µg/100 mg tissue wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis control</td>
<td>105.33 ± 0.91</td>
<td>20.25 ± 1.12</td>
<td>3.17 ± 0.17</td>
<td>238.30 ± 4.25</td>
</tr>
<tr>
<td>Metastasis+ R. apiculata</td>
<td>35.51 ± 0.42**</td>
<td>5.34 ± 0.17**</td>
<td>1.35 ± 0.14**</td>
<td>93.20 ± 2.30**</td>
</tr>
</tbody>
</table>

Table 7.2 Effect of *R. apiculata* on the serum sialic acid, lung hydroxyproline, hexoamine and uronic acid during metastasis Blood samples were collected 21st day by cardiac puncture and serum samples were isolated. On the same day lungs were excised. Values are expressed as mean ± SD. Values is significantly different from metastasis control (**p < 0.01).
7.3.3 Effect of *R. apiculata* on the lung nodule formation and survival of animals

Effect of *R. apiculata* on the lung nodule formation and survival rate of animals is shown in Table 7.3. Injection of B16F-10 melanoma cells via tail vein resulted in the formation of lung tumor nodules. Treatment with *R. apiculata* significantly (*p < 0.01*) reduced the number of colonies to (26.6 ± 8.1) compared with metastatic control group (45.2 ± 9.0). The percentage decrease in lung nodule formation by *R. apiculata* treatment was found to be 41.1%.

The survival rate of *R. apiculata* treated animals was increased when compared to metastatic control animals. The survival rate was increased to 81 days by *R. apiculata* treatment compared to metastatic control animals which survived up to only 39 days (Table 7.3). 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. The percentage increase in life span by *R. apiculata* treatment was found to be 107.3%.

7.3.4 Histopathological analysis

Histopathological analysis of the lungs of the experimental animals is shown in Figure 7.1. Pictures shown are from representative lung samples collected at the end of the experimental period (i.e., Day 21). Metastatic control animals shown prominent large metastatic tumor nodules and hyperchromatic nucleus as well as necrosis (Figure 7.1A). *R. apiculata* treatment showed massive reduction in the lung nodule formation as well as decreased the fibrosis compared with the metastasis control group (Figure 7.1 B).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of tumor nodules</th>
<th>No of days survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis control (C) (n=3)</td>
<td>45.2 ± 9.0</td>
<td>39 ± 2.1</td>
</tr>
<tr>
<td>Metastasis + R. apiculata (T) (n=3)</td>
<td>26.6 ± 8.1**</td>
<td>81 ± 3.2**</td>
</tr>
</tbody>
</table>

Table 7.3 Effect of *R. apiculata* on the lung nodule formation and survival rate of animals during metastasis

Metastasis was induced by injecting (B16F-10 cell line 1x 10⁶ cells / animal) via lateral tail vein. Group I served as metastasis control where Group II were treated with *R. apiculata* (10 mg/kg b.wt) for 10 consecutive days. On day 21 animals were euthanized and lungs were dissected out and metastatic nodules were counted. The percentage increase in life span was calculated by (T - C / C x100), where T and C are the number of days survived by the treated animals and metastatic control animals respectively. Values are expressed as mean ± SD. Values is significantly different from metastasis control (**p < 0.01).
Figure 7.1 Histopathology of lung metastasis. A portion of the excised lung tissue were fixed in 10% formalin, cut into 5-μm thickness, stained using H&E (hematoxylin and eosin) and observed under microscope (45x). (A) Metastatic control (B) Metastasis + *R. apiculata*. 
7.4 Discussion

Metastasis is one of the hallmarks of malignant neoplasm or cancer which is the leading cause of death in many cancer patients (Nonaka et al., 1993; Lee et al., 2008). The degree of ability to spread varies between different types of tumors. In most cases, cancer patients with localized tumors have a better chance of survival than those with metastatic tumors. The metastatic capacity of a tumor cell is reliant on several factors. Tumor cells invade the tissues adjacent of primary tumor, increase their motility and migrates into the blood circulation (intravasate). Then the tumor cells evade the host defense mechanisms and adhere to a suitable niche. Consequently the tumor cells (extravasate) and invade into the secondary niche and evade apoptosis, regulates proliferation and angiogenesis (Chambers et al., 2002; Herzig and Christofori, 2002). Among the metastasis cancers, the lung is the first organ to be encountered by the tumor cells making it a major site for tumor metastasis (Hyoudou et al., 2004). The survival rate for patients with metastatic melanoma is less than 10% (Bhatia et al., 2009). Tumor metastasis are treated with surgery, radiotherapy, chemotherapy and hormone therapy or "multimodal therapy" (Wang et al., 2012). The metastatic treatment depends on, type of primary cancer, size and location of the metastasis. Therefore, metastasis is a major target of cancer therapy and it is complicated to treat metastasis effectively with the available treatment with lesser survival rate (Bhatia et al., 2009). Many plants serve as novel drugs throughout the world. World Health Organization (WHO) reveals around 80% of the world population depends on plant derived medicine for their little of no adverse effects (WHO, 2008). Therefore in this study we have investigated the anti-metastatic potential of R.apiculata on B16F-10 metastatic lung cancer cell line in C57BL/6 mice.

Nitric oxide (NO) is a pleiotropic regulator with numerous biological processes, including vasodilatation, neurotransmission, macrophage-mediated immunity and
immune defenses (Nathan, 1992). Concurrently NO also play an important role in tumor progression causing DNA damage, metastasis and angiogenesis (Shi et al., 1999; Fukumura and Kashiwagi, 2006). Increased NO generation in cancer cells has direct correlation with circulating tumor cells for their survival, high metastatic ability and angiogenesis by up-regulating vascular endothelial growth factor (VEGF) and VEGF-induced neovascularization (Edwards et al., 1996; Ambs et al., 1998). NO regulates some of the cellular adaptive responses to hypoxia which are associated with increased metastatic potential (Branco-Price et al., 2012). In the present study R.apiculata significantly reduced the NO production level preventing high metastatic ability of tumor cells in experimental animals. The R.apiculata treatment showed significant reduction in lung tumor nodules (41.1% inhibition) compared to the metastatic control. This reduction in tumor nodules can be correlated with an increase in the life span (107.6 %) of the metastatic tumor bearing animals after R.apiculata treatment..

γ-glutamyl transpeptidase (GGT) is the only known enzyme that cleaves the γ-glutamyl-cysteine peptide bond in GSH and other γ-glutamyl compounds. GSH is synthesized intracellularly which provides energy to the tumor cells through gamma glutamyl cycle. GGT catalyzes glutathione (GSH) breakdown. An increase of GGT levels is a common finding in human tumors and as an important aspect of the tumor cell phenotype. In tumor cells, GGT over expression and its effects on GSH metabolism may affect several specific functions. It has been reported that variations in GGT expression and cellular levels of GSH is associated with modulation of metastatic properties of several tumors (Carretero et al., 1999). During tumor progression the serum GGT level increases due to cellular proliferation and therefore serum GGT level can be used as marker for cellular proliferation (Pradeep and Kuttan, 2002). The administration of R.apiculata significantly reduced the serum GGT level in metastatic tumor bearing animals which is directly correlated with decrease in tumor cell proliferation and metastasis.
Sialic acid is a derivative of neuraminic acid which occurs as a terminal component of carbohydrate chain of glycoproteins. Sialic acid at the terminal position is involved in cellular adhesion (components of many cell surface receptors) and have ability to cellular recognition sites during invading foreign cells including cancer cells (Schauer, 1985). Metastatic cancer cells often express a high density of sialic acid-rich glycoproteins. Total serum sialic acid level has been recognized as a valuable non-specific monitor of tumor burden in melanomas. The increase in the sialic acid level on the surface of malignant cells creates a negative charge on cell membrane which creates electrostatic repulsion between cells that initiates the malignant cells to metastasize through blood circulation (Fuster et al., 2005). The amount of sialic acid on the surface of malignant cells is directly correlated with the metastatic ability of the tumor cells (Vedolva and Borovansky, 1994). The treatment of *R. apiculata* significantly decreased the serum sialic acid level in metastatic tumor bearing animals. The *R. apiculata* prevented the expression of sialic acid on the surface of malignant cells preventing negative charge and electrostatic repulsion thus inhibiting metastasis. The decreased serum sialic acid level can be directly correlated with the reduction in lung metastasis.

Hydroxyproline is a major component of the protein collagen. Hydroxyproline and proline play key roles for collagen stability (Nelson and Cox, 2005). In the lung, collagen is found associated with bronchi, with blood vessels and with the alveolar interstitium. They plays an important role in the maintenance of lung structure and function. During lung metastasis there will be enormous accumulation of fibrosis in lungs resulting in substantial deposit of collagen (hydroxyproline) in the alveoli of lungs (Pradeep and Kuttan, 2002). Elevated level of collagen in the lung is associated with pulmonary fibrosis resulting in the inability of the lung to function in normal gaseous exchange. Therefore lung hydroxyproline level has been used as an indicator to determine collagen amount and lung fibrosis (Voet and Voet, 1995). In the present
study the administration of *R. apiculata* significantly reduced the lung hydroxyproline level in metastatic tumor bearing animals. The *R. apiculata* inhibited excessive accumulation of collagen in lungs preventing pulmonary fibrosis during lung metastasis.

Hexosamine is the acidic and basic modification of monosaccharide yielding uronic acids (glucoronic acid) and amino sugars (hexosamine). Hexosamine plays an important role in the production of N-acetyl neuraminic acid (sialic acid) which is present on the surface of malignant cells responsible for metastasis. Hexosamine, an integral part of many structural polysaccharides and glycosaminoglycans (GAG) found in the ground surface of ECM which promotes metastasis by opening the spaces for malignant cells to migrate. The excess hexosamine level is directly correlated with the active growth and proliferation of malignant cells (West et al., 1985). Therefore it is a known marker and promoter of metastasis (Lipponen et al., 2001). In the study administration of *R. apiculata* resulted in significant reduction in lung hexosamine level during metastasis. The *R. apiculata* significantly inhibited the excess synthesis of hexosamine by blocking the ECM thus preventing malignant cells metastasis and lung fibrosis.

Malignant cells yield uronic acid by the oxidation of the primary alcohol group of aldoses (monosaccharide) sugar derivatives. This leads to the formation of glucuronic acid lactone which is an essential form of uronic acid. In the present study the *R. apiculata* significantly decreased the lung uronic acid level in metastatic tumor bearing animals. The histopathological analysis of the lungs of *R. apiculata* treated animals also shows consonant results as above by reduction in the lung tumor nodules. Therefore, the above experimental evidences substantiate the anti-metastasis activity of the *R. apiculata* on experimentally induced lung metastasis in C57BL/6 mice.
In conclusion, this study is the first of its kind to report on the anti-metastatic activity of *R. apiculata* in metastatic tumor bearing animals. The inhibitory effect exhibited by *R. apiculata* could be attributed to the high content of pyrazole, 4-pyrrolidinyl, ketone derivatives and thiazolidinediones (Chapter 3). Therefore *R. apiculata* may be used as a therapeutic target to inhibit metastasis during tumor progression. Further investigations are required to trace out the exact mechanisms involved in the anti-metastatic property of *R. apiculata*. 