CHAPTER - 6

INVESTIGATION OF THE ANTI-TUMOR ACTIVITY OF *RHIZOPHORA APICULATA*

6. Aim

To evaluate the inhibitory effect of *R. apiculata* on tumor progression

6.1 Introduction

Cancer is one of the most life threatening disease in both developed and developing countries. Cancer is a condition where cells in a specific part of the body grow and proliferate uncontrollably. The cancerous cells can invade and destroy surrounding healthy tissue, including organs (Kuper et al., 2000; Balkwill and Mantovani, 2001). Cancer remained as the largest cause of mortality worldwide and the number of individuals living with cancer is steadily expanding. Hence, a major portion of the current pharmacological research is involved with the anticancer drug design (Balkwill and Mantovani, 2001). The treatment of cancer with the available anticancer drugs is often unsatisfactory due to their cytotoxicity to the normal cell and with diverse adverse effects like mucosal, vomiting, nausea, ulceration, pulmonary fibrosis, alopecia, cardiac and hepatic toxicity (Guruvayoorappan and Kuttan, 2007). Drugs that could mitigate these adverse effects will be useful in cancer therapy. Plants have been used as folk remedies and ethno-botanical literature have described the usage of plants extracts to treat human diseases since time immemorial (Patel et al., 2010). There are several medicinal plant that are considered to possess anti-tumor properties. Some of the plants with such activity include *Rubia cordifolia*, *Biophytum sensitivum*, *Convolvulus arvensis*, *Dysoxylum malabaricum*, *Combretum caffrum*, *Oldenlandia diffusa*, *Indigofera aspalathoides* and *Piper longum* (Meng et al., 2002;
Mangrove *R. apiculata*, (Family *Rhizophoraceae*), is an important plant used in traditional medicines by many people in Asia and Africa. The healing properties attributed to *R. apiculata* tree in folk medicine are based, to a great extent, on the fact that use of root, leaf, and/or stem extracts imparts an inhibitory effect on the growth of human bacterial, viral and fungal pathogens (Hernandez-Perez and Banos, 1978). Through several plants from mangroves are extensively used in traditional medicine, only some have been assessed for biological activities (Agogramoorthy et al., 2008). In the present study we investigated the anti-tumor efficacy of *R. apiculata* using experimental tumor mice model.

6.2 Materials and Methods

6.2.1 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.

6.2.2 Animals

Male BALB/c mice (4-6 weeks old) were purchased from the Pasteur institute of India, Coonoor, Tamil Nadu, 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.

6.2.3 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.

6.2.4 Chemicals

Gum acacia was purchased from Hi-Media, Drabkin’s solution from Nice
Chemicals Pvt. Ltd. (Cochi, India). Formaldehyde solution was procured from
Universal Laboratories Pvt. Ltd. (Hyderabad, India). All chemicals used were
analytical or reagent grade.

6.2.5 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.
6.2.6 Determination of the effect of *R. apiculata* on hematological parameters and survival rate during solid tumor development

The animals were divided in to two groups (n=6 / group). All the animals received (B16F-10 cell line 1x10^6) cells via intra muscular. Six animals were kept as tumor control (Group I) were as the remaining six animals were treated with *R.apiculata* (Group II). Blood was collected from all the animals every third day starting from day 0 to day 30. The total white blood cell (WBC) counts were performed using a haemocytometer counting. Hemoglobin levels were estimated using cyanomethemoglobin method. Similarly another set of animals (n=12) were divided in to two groups as mentioned above. The survival rate was observed and the percentage increased of life span (ILS%) was calculated according to the formula ILS% = (T-C)/C x 100, where T represents mean survival time of treated animals and C represents mean survival time of control group. According to UN National Cancer Institute criteria, ILS exceeding 25% indicate that the drug has significant anti-tumor activity (Plowman et al., 1997).

6.2.7 Determination of the effect of *R. apiculata* on the tumor volume during solid tumor development

Male BALB/c mice (n=12) were divided in to two groups as mentioned above. Tumor induction and *R.apiculata* treatment were same as explained previously. The tumor volume was measured using vernier caliper for 30 days at an interval of three days starting from day 0. Tumor volume was calculated by using equation \( V = \frac{4}{3} \pi r_1^2 r_2 \), where ‘r_1’ and ‘r_2’, represent the major and minor diameters respectively, of the measured mass (Atia and Weiss, 1966). At the end of the experiment, the animals were sacrificed and the tumor mass was dissected out and fixed in 10% formaldehyde and subjected to histopathological analysis.
6.2.8 Determination of the effect of *R. apiculata* on serum γ-glutamyl transpeptidase (GGT), glutathione (GSH), nitric oxide (NO) level and TNF-α level during solid tumor development

The animals were divided into two groups (n=6/group). All the animals received (B16F-10 cell line 1x10^6) cells via intramuscular. Six animals were kept as tumor control (Group I) as the remaining six animals were treated with *R. apiculata* (Group II). Blood was collected by tail vein bleeding on day 5, day 10 and day 15 and used for the estimation of GGT, GSH, and NO (Szasz 1976; Moran et al., 1979; Green et al., 1982). The level of serum TNF-α were determined using a commercially available ELISA kit (USCN Life Sciences Inc, Houston, USA) according to the manufacturer’s instruction.

6.2.9 Effect of *R. apiculata* on the body weight during solid tumor development

The animals were divided into two groups (n=6/group). All the animals received (B16F-10 cell line 1x10^6) cells via intramuscular. Six animals were kept as tumor control (Group I) as the remaining six animals were treated with *R. apiculata* (Group II). The body weight of all the animals were monitored every third day till day 30.

6.2.10 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.
6.3 Result

6.3.1 Effect of *R. apiculata* on the hematological parameters and survival rate during solid tumor development

Administration of *R. apiculata* increased the total WBC count during tumor progression compared with tumor control Figure 6.1. The WBC count in *R. apiculata* treated mice was significantly \((p<0.001)\) increased to \((9250 \pm 821 \text{mm}^3)\) on day 30 compared to tumor control \((4158 \pm 310 \text{mm}^3)\). Hemoglobin content in the *R. apiculata* treated mice was also significantly \((p<0.01)\) higher \((16.9 \pm 0.7 \text{g/dl})\) (day 30) compared with tumor control (Table 6.1.) The *R. apiculata* increased the live span of the tumor bearing animals to \((\text{ILS} = 91.6 \%)\) (Table 6.2)
Figure 6.1 Effect of *R. apiculata* on the total WBC counts during tumor development  Blood was collected from all the animals every third day starting from day 0 to day 30. The total white blood cell (WBC) counts were performed using a haemocytometer counting. Values are expressed as mean ± SD. Value is significantly different from tumor control (*p* < 0.05; **p** < 0.001)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
<th>Day 15</th>
<th>Day 18</th>
<th>Day 21</th>
<th>Day 24</th>
<th>Day 27</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor control</td>
<td>15.2</td>
<td>± 0.8</td>
<td>± 1.1</td>
<td>± 1.0</td>
<td>± 0.9</td>
<td>± 0.8</td>
<td>± 0.5</td>
<td>± 0.4</td>
<td>± 0.4</td>
<td>± 0.4</td>
<td>± 0.2</td>
</tr>
<tr>
<td>Tumor + R. apiculata</td>
<td>15.8</td>
<td>± 0.8</td>
<td>± 0.8</td>
<td>± 0.8</td>
<td>± 0.8</td>
<td>± 1.3</td>
<td>± 0.8</td>
<td>± 0.4</td>
<td>± 0.9</td>
<td>± 0.8</td>
<td>± 0.7*</td>
</tr>
</tbody>
</table>

Table 6.1 **Effect of R. apiculata on the hemoglobin content during solid tumor progression** Blood samples was collected from all the animals every third day starting from day 0 to day 30. Hemoglobin levels were estimated using cyanomethemoglobin method units = (g/dl). Values are expressed as mean ± SD. Value is significantly different from tumor control (*p < 0.05).
Table 6.2 Effect of *R. apiculata* on the survival rate during tumor development

The percentage increase in life span was calculated by \((T-C)/Cx100\), where T and C are the number of days survived by the treated animals and tumor control animals respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of days</th>
<th>Increase in life span %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor control (C)</td>
<td>48 ± 3.1</td>
<td>-</td>
</tr>
<tr>
<td>Tumor +<em>R. apiculata</em> (T)</td>
<td>92 ± 4.2</td>
<td>91.6</td>
</tr>
</tbody>
</table>
6.3.2 Effect of *R. apiculata* on the tumor volume during solid tumor development

The results indicated that there was a significant ‘reduction’ (relative to control values) in the tumor volumes after *R. apiculata* treatment (Figure 6.2). On Day 30, the tumor volume of control animals were found to be (2.31 ± 1.26 mm$^3$) whereas treatment with *R. apiculata* could significantly ($p<0.05$) reduce the tumor volume to (1.52 ± 1.21 mm$^3$). Histopathological analysis of the solid tumor mass also shows the anti-tumor efficacy of the plant extract (Figure 6.3).

The tumor section from the tumor control group displayed hyper-melanin pigmentation whereas the incidence of such pigmentation was markedly lower after *R. apiculata* treatment.
Figure 6.2 Effect of *R*. *apiculata* on the tumor volume during solid tumor development

The tumor volume was measured using vernier caliper for 30 days at an interval of three days starting from day 0. Tumor volume was calculated by using equation $V = \frac{4}{3} \pi r_1^2 r_2$, where ‘$r_1$’ and ‘$r_2$’, represent the major and minor diameters of tumor respectively. Values are expressed as mean ± SD. Value is significantly different from tumor control (*$p < 0.05$).
Figure 6.3 Effect of *R. apiculata* on tumor histopathology  
A) Tumor control.  
B) Tumor + *R. apiculata*. 
6.3.3 Effect of *R. apiculata* on body weight during solid development

The tumor control group animals showed a gradual increase in body weight and on day 30 the body weight was found to be 29.0 g. Treatment with *R. apiculata* showed significant decreased in body weight and on day 30 the body weight was found to be 26.0 g (Figure 6.4).
Figure 6.4 Effect of *R. apiculata* on the body weight during solid tumor development The body weight of all the animals were monitored every third day till day 30 and expressed in (gm). Values are expressed as mean ± SD. Value is significantly different from tumor control (*p* < 0.05).
6.3.4 Effect of *R. apiculata* on serum GGT, NO, GSH and TNF-α level during solid tumor development

Effects of *R. apiculata* on serum GGT, NO, and GSH levels during solid tumor development is presented in Table 6.3. On the 15\(^{th}\) day after the tumor challenge, serum GGT levels in tumor control animals were found to be 142.8 ± 2.3 nmol *p*-nitroaniline / ml. These values were significantly lowered to 55 ± 1.6 nmol *p*-nitroaniline/ml as a result of *R. apiculata* administration. Similarly, serum NO levels was found to be higher (34.8 ± 1.9 µM). In the tumor control mice were significantly decreased to 27.1 ± 0.6 µM by *R. apiculata* treatment. The serum GSH level in tumor bearing control mice on day 15 was found to be 15.6 ± 0.6 µM where as *R. apiculata* treated mice showed significantly decreased the level of serum GSH to 8.0 ± 0.4 µM. The serum TNF-α in tumor bearing control mice on day 15 was found to be 107.3±3.8 pg/ml where as *R. apiculata* treated mice showed significantly decreased the level of TNF-α to 46.7±3.4 pg/ml.
Table 6.3 Effect of *R. apiculata* on serum GGT, NO, GSH and TNF-α level during solid tumor development

Blood samples were collected on day 5, 10 and 15 respectively and the serum obtained after centrifugation were used for biochemical estimation. Values are expressed as mean ± SD. Value is significantly different from tumor control (**p < 0.01).
6.4 Discussion

The role of the immune system during cancer development is complex involving extensive reciprocal interactions between genetically altered cells, adaptive and innate immune cells (Van Kempen et al., 2006). Plants have been used as folk medicine to treat cancer. Low cost and easy availability has generated a renewed interest in plant medicine in the last decade; in addition, plant-based drugs have drawn greater attention due to the findings of little-to-no adverse effects. The medicinal properties of \textit{R. apiculata} provide a wide domain for medicinal uses. The literature has indicated that \textit{R. apiculata} contains flavanoids, tannin, catechin, anthroquinone, and phenolic groups (Ravikumar et al., 2011). Of these, catechin was seen earlier to be the predominant constituent of mangrove tannins (Rahim et al., 2007a). Anthroquinone has been shown to be a bioactive constituent having \textit{in vitro} anti-tumor effects against four human cancer cell lines (Cichewicz et al., 2004).

Certain plant extracts are reported to enhance the activity of human lymphocyte proliferation and the secretion of interferon (IFN)-\(\gamma\) (Chiang et al., 2003). Naturally-occurring mono-terpenes carvone and perillic acid increase total white blood cells (WBC) levels in BALB/c mice (Raphael and Kuttan, 2003). Mangrove tannins are phenolic polymers that contain flavan-3-ol units linked together through C\(_4\)-C\(_6\) or C\(_4\)-C\(_8\) bonds. These tannins have strong and effective antioxidant activity (Rahim et al., 2007a). Flavonoids have important effects in plant biochemistry and physiology (as anti-oxidants, enzyme inhibitors, of toxic substance precursors) and are known to possess anti-inflammatory, anti-oxidant, anti-allergic and anti-carcinogenic properties (Harborne et al., 1975; Havsteen 1984). Active flavonoids (i.e.) tannin, quercetin, catachin) have the ability to inhibit (NO) synthase (all three isoforms) activity, as well as that of serum GGT and to affect the levels of GSH (Chiesi and Schwaller, 1995; Soliman and Mazzio, 1998; Rehman et al., 2009). Certain members of flavonoid
compounds display a variable extend of biochemical and pharmacological action that affect the function of immune system and inflammatory cells (Middleton and Kandaswami, 1992). Many flavonoids specifically affect the function of enzymes involved in generation of inflammatory processes (i.e.) tyrosine and serine-threonine protein kinases involved in signal transduction/cell activation processes (Hunter 1995; Nishizuka 1998). (i.e.), normally mast cells release pro-inflammatory tumor necrosis factor (TNF-α) upon activation. TNFα can, in turn, induce endothelial leukocyte adhesion molecule-1 (ELAM-1), a cell surface glycoprotein expressed by cytokine-activated endothelium that mediates adhesion of blood neutrophils. Thus, flavonoids - ubiquitous in plants used in traditional medicine to treat diseases - seem to have a role in inhibiting various enzymes and potentially tumor progression.

In the present study, the anti-tumor activity of *R. apiculata*, an important plant in indigenous medicinal practices, was examined. Administration of *R. apiculata* extract was found to increase total white blood cells (WBC) and hemoglobin count in melanoma-bearing BALB/c mice. These are fortuitous outcomes in that often during cancer chemotherapy a major problem seen in patients is myelosuppression and anemia (Price and Greenfield., 1958; Maseki et al., 1981). In many cases, the anemia that appears in tumor-bearing hosts is mainly due to reductions in red blood corpuscle (RBC) levels or hemoglobin content - each of which could be due to an iron deficiency or a hemolytic/myelopathic condition (Gupta et al., 2004). Treatment with the *R. apiculata* yielded relative increases in hemoglobin and WBC counts in melanoma-injected mice. This may indicate that the *R. apiculata* could provide some protective action in the hematopoietic system, an important finding in light of the above-noted ‘by-stander’ effects seen during chemotherapy.

Administration of *R. apiculata* significantly inhibited the growth of solid tumors induced by the B16F-10 melanoma cells in the BALB/c mice. A change in tumor
volume during treatment is a basic measure of a therapeutic response to the extract (or for that fact, any novel product). Analysis of tumor growth curves provide important theoretical and practical information for clinical and experimental oncologists. As changes in DNA synthesis rates precede tumor cell proliferation increases which result in the increase of tumor volume (Zhou et al., 2001).

Glutathione (GSH) represents the most prevalent intracellular thiol tripeptide and an agent considered crucial for tumor cell metabolism and (ultimately, the ability to proliferate) - was reduced in the serum of melanoma-injected mice as a result of the administration of the *R. apiculata* extract. The extract also reduced (relative to those in tumor-bearing non-extract-treated counterparts) levels of serum gamma-glutamyl transpeptidase (GGT), an enzyme that catalyses the transfer of gamma-glutamyl moieties from glutathione to other amino acids and dipeptides (Meister and Tate, 1976; Kachadourian and Leitner, 2007), in these hosts. Moreover, administration of extract was found to inhibit nitric oxide (NO) production in these animals. Taken together, these outcomes suggest that the *R. apiculata* could serve as a good anti-oxidant and could potentially help to deprive tumor cells of key cell products critical for their survival/ability to proliferate. Further studies are needed to clarify more precisely whether the changes in NO, GSH, and/or GGT that were seen are due to the presence of the extract *per se*, related to changes in host-tumor status (which itself imparts effects on the measured outcomes), or a combination of these two factors.

In conclusion, this study is the first of its kind to report on the anti-tumor activity possessed by the mangrove plant *R. apiculata*. These activities could be attributed to the high content of pyrazole, 4-pyrrolidinyl, ketone derivatives, and thiazolidinediones found in the *R. apiculata* methanolic extract (Chapter 3). The results of the present work indicate that *R. apiculata* could potentially be useful as a (natural) anti-tumor agent.