CHAPTER – 4

INVESTIGATION OF THE ANTI-INFLAMMATORY ACTIVITY OF

RHIZOPHORA APICULATA

4 Aim

To evaluate the anti-inflammatory activity of *R. apiculata* methanolic extract using inflammatory animals models

4.1 Introduction

Inflammation is defined as a primary defense mechanism that helps the body to protect itself against allergens, infection, burns and toxic chemicals (Ferrero-Miliani et al., 2007). Immune cells are invited to the site through the blood stream and the blood vessels near the site will become warm and red due to the increased blood flow. But sometimes inflammation may be act as etiologic factor for all degenerative disease. It is a major response of the immune system to tissue damage and infection, though in most cases it doesn’t lead to inflammation. Recent studies reported that unchecked inflammations could lead to cancer development (Coussens and Werb, 2002). Inflammation can be acute or chronic where acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. Chronic inflammation is defined as a progressive shift in the type of cells present at the site of the inflammation and it is characterized by simultaneous destruction and recovery of the tissue from the inflammatory process. Acute inflammation has therapeutic consequence whereas chronic inflammation leads to harmful diseases like cancer, alzheimer’s disease, diabetes, arthritis and autoimmune diseases (Van Kempen et al., 2006). Inflammation
is a critical component of tumor progression and it is now becoming clear that the tumor microenvironment which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration (Coussens and Werb, 2002).

The relationship between inflammation, innate immunity and cancer is more widely accepted however, many of the molecular and cellular mechanisms mediating this relationship remain unresolved (Coussens and Werb, 2002). Cancer related inflammations include presence of inflammatory cells and inflammatory mediators (chemokines, cytokines and prostaglandins) in new blood vessels and tumor tissues (Van Kempen et al., 2006). The inflammatory component of a developing neoplasm may include a diverse leukocyte population – i.e. neutrophils, dendritic cells, macrophages, eosinophils, mast cells as well as lymphocytes - all of which are capable of producing an assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine protease, cysteine protease, membrane-perforating agents, and soluble mediators of cell killing, such as TNF-α, interleukins and interferons (IFNs) (Wahl and Kleinman, 1998; Kuper et al., 2000).

The strongest association of chronic inflammation with malignant diseases is in colon carcinogenesis arising in individuals with inflammatory bowel diseases i.e. chronic ulcerative colitis and crohn’s disease (Ernst and Gold, 2000). An inflammatory cell plays an important role in tumor promotion and producing an attractive environment for tumor growth, facilitating genomic instability and promotes angiogenesis. The inflammatory cells, chemokines and cytokines influence the whole tumor organ by regulating the growth, migration and differentiation of all cell types in the tumor microenvironment including neoplastic cells, fibroblasts and endothelial cells (Coussens and Werb, 2002).
Inflammation is an important host response to external challenge or cellular injury, that are mediated by a variety of cell signaling pathways to balance the restoration of tissue structure and function results in the activation of these signaling pathways, such as nitric oxide (NO) and prostaglandins, which are generated by inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Abnormal production of these mediators results in severe tissue damage, systemic inflammatory syndrome, septic shock and atherosclerosis (Choi et al., 2007).

Plants have been used as folk medicine to treat inflammation and cancer. Low cost and easy availability has generated new interest in plant medicine which have greater attention due to its little to no side effects. Mangrove plants are widely used in medicine to treat variety of diseases. Some mangrove plants such as *R. mucronata* and *R. mangle* have been screened and known to have anti-bacterial, anti-viral, anti-ulcer and anti-inflammatory properties (Agogramoorthy et al., 2008; Rahim et al., 2007). Though several plants from mangrove are extensively used in traditional medicine only some have been assessed for biological activities. In the present study we have investigated the anti-inflammatory efficacy of *R. apiculata* using inflammatory animal model.

### 4.2 Materials and methods

#### 4.2.1 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.
4.2.2 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.

4.2.3 Chemicals

Gum acacia was purchased from Hi-Media, Carrageenan from Hi- Pure chemical Industries (Chennai, India). Formaldehyde solution was procured from Universal Laboratories Pvt. Ltd. (Hyderabad, India). All chemicals used were analytical or reagent grade.

4.2.4 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.
4.2.5 Evaluation of anti-inflammatory effect of *R. apiculata* against carrageenan and formalin induced paw edema

4.2.6 Carrageenan regimen

Male BALB/c mice were divided into two groups (n = 6/group). Group I was kept as normal untreated control; mice in Group II received the *R. apiculata* resuspended in 1% gum acacia that was injected i.p. (10 mg/kg b.wt) on 10 consecutive days; the last dose was provided 60 min before induction of inflammation. Subsequently, all mice received a subcutaneous injection of 0.1 ml of a 1% (w/v) carrageenan solution in the plantar region of their right hind paw to induce edema. The paw volume was measured initially and then at 30 min intervals for up to 8 hr after the injection using a vernier caliper.

4.2.7 Formalin regimen

Male BALB/c mice was divided into two groups (n = 6/group). Group I was kept as normal untreated control; mice in Group II received the *R. apiculata* resuspended in 1% gum acacia that was injected i.p.(10 mg/kg b.wt) on 10 consecutive days; the last dose was provided 60 min before induction of inflammation. Subsequently, all mice received a subcutaneous injection of 0.1 ml of a 2% (v/v) formalin solution in the dorsal surface of their right hind paw. Diameters of the hind paw were first measured to obtain the baseline value before the injection; thereafter, measures of dorsal plantar foot thickness (at metatarsal level) were performed on five consecutive days using a vernier caliper.
4.2.8 Determination the effect of *R. apiculata* on serum iNOS, cyclooxygenase-2 and prostaglandin E-2 during carrageenan and formalin induced inflammation

The levels of serum iNOS, cyclooxygenase-2 (COX-2) and prostaglandin E-2 during carrageenan and formalin induced inflammation were determined using a commercially available ELISA kit (USCN Life Sciences Inc, Houston, USA and BLUE GEN BIOTECH, Shanghai, China) according to the manufacturer’s instruction.

4.2.9 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.

4.3 Results

4.3.1 Anti-inflammatory activity of *R. apiculata* on carrageenan and formalin induced paw edema

4.3.2 Carrageenan model

Effect of *R. apiculata* during carrageenan induced paw edema is shown in Figure 4.1. The *R. apiculata* treatment could significantly decreased the paw edema induced by carrageenan. On hour 4 after carrageenan injection the control group showed the maximum paw edema of (0.47 ± 0.03 cm) where as *R. apiculata* treated group showed a significantly reduced value of (0.39 ± 0.04 cm).
4.3.3 Formalin model

Effect of the *R. apiculata* during formalin-induced paw edema is shown in Figure 4.2. Administration of *R. apiculata* showed significant reduction in paw size during at day 4 post-formalin injections (0.37 ± 0.03 cm) compared with the control animals (0.43 ± 0.01 cm).
Figure 4.1 Effect of *R. apiculata* during carrageenan-induced paw edema
The paw volume was measured initially and then at 30 min intervals using a vernier caliper. Values are expressed as mean ± SD. Value is significantly different from carrageenan control (*p* < 0.05; **p** < 0.01; ***p*** < 0.001) (n=6/group)
Figure 4.2 Effect of *R. apiculata* during formalin-induced paw edema

The paw volumes were measured initially and for five consecutive days after using a vernier caliper. Values are expressed as mean ± SD. Value is significantly different from formalin control (*p < 0.05; **p < 0.01) (n=6/group)
4.3.4 Effect of *R. apiculata* on serum prostaglandin E-2 during carrageenan induced inflammation

Effect of *R. apiculata* on serum prostaglandin E-2 during carrageenan induced inflammation is shown in Figure 4.3. The *R. apiculata* treated group significantly (**p<0.01**) decreased the serum prostaglandin E-2 level in fourth hour (66.5 ± 7.2 pg/ml) when compared with carrageenan control (95.5 ± 5.3 pg/ml).

4.3.5 Effect of *R. apiculata* on serum prostaglandin E-2 during formalin induced inflammation

Effect of *R. apiculata* on serum prostaglandin E-2 during formalin model is shown in Figure 4.4. The *R. apiculata* treated group significantly (**p<0.01**) decreased the serum prostaglandin E-2 level (72.3 ± 2.6 pg/ml) when compared with formalin control (89.7± 2.0 pg/ml).

4.3.6 Effect of *R. apiculata* on serum COX-2 during carrageenan induced inflammation

Effect of *R. apiculata* on serum COX-2 level during carrageenan model is shown in Figure 4.5. The *R. apiculata* treated group significantly (**p<0.01**) decreased the serum COX-2 level in the second hour (11.23 ± 1.5 ng/ml) when compared with carrageenan control (14.34 ± 1.3 ng/ml).
4.3.7 Effect of *R.apiculata* on serum COX-2 during formalin induced inflammation

Effect of *R.apiculata* on serum COX-2 level during formalin model is shown in Figure 4.6. The *R.apiculata* treated group significantly (**p<0.01) decreased the serum COX-2 level from day 1 (10.20 ± 1.5 ng/ml) when compared with formalin control (15.37 ± 1.9 ng/ml) on the same day.
Figure 4.3 Effect of *R. apiculata* on serum prostaglandin E-2 during carrageenan induced inflammation The blood samples were collected at different time intervals by tail vein bleeding and serum were separated and used for prostaglandin E-2 quantification. Values are expressed as mean ± SD. Value is significantly different from carrageenan control (**p < 0.01).
Figure 4.4 Effect of *R. apiculata* on serum prostaglandin E-2 during formalin induced inflammation. The blood samples were collected at different days intervals by tail vein bleeding and serum were separated and used for prostaglandin E-2 quantification. Values are expressed as mean ± SD. Value is significantly different from formalin control (**p < 0.01).
Figure 4.5 Effect of *R. apiculata* on serum COX-2 during carrageenan induced inflammation  
The blood samples were collected at different time intervals by tail vein bleeding and serum were separated and used for COX-2 quantification. Values are expressed as mean ± SD. Value is significantly different from carrageenan control (*p < 0.05; **p < 0.01).
Figure 4.6 Effect of *R. apiculata* on serum COX-2 level during formalin induced inflammation

The blood samples were collected at different days intervals by tail vein bleeding and serum were separated and used for COX-2 quantification. Values are expressed as mean ± SD. Value is significantly different from formalin control (***p < 0.01).
4.3.8 Effect of *R. apiculata* on serum iNOS during carrageenan induced inflammation

Effect of *R. apiculata* on serum iNOS level during carrageenan induced inflammation is shown in Figure 4.7. The *R. apiculata* treated group significantly (**p<0.01**) decreased the serum iNOS level in fourth hour (3.7 ± 0.43 ng/ml) when compared with carrageenan control (6.9 ± 0.53 ng/ml).

4.3.9 Effect of *R. apiculata* on serum iNOS during formalin induced inflammation

Effect of *R. apiculata* on serum iNOS level during formalin model is shown in Figure 4.8. The *R. apiculata* treated group significantly (**p<0.01**) decreased the serum iNOS level on day two (3.5 ± 0.27 ng/ml) when compared with formalin control (5.3 ± 0.63 ng/ml).
Figure 4.7 Effect of \textit{R. apiculata} on serum iNOS during carrageenan induced inflammation The blood samples were collected at different time intervals by tail vein bleeding and serum were separated and used for iNOS quantification. Values are expressed as mean ± SD. Value is significantly different from carrageenan control (*$p < 0.05$, **$p < 0.01$).
Figure 4.8 Effect of *R. apiculata* on serum iNOS during formalin induced inflammation. The blood samples were collected at different days intervals by tail vein bleeding and serum were separated and used for iNOS quantification. Values are expressed as mean ± SD. Value is significantly different from formalin control (*p < 0.05; **p < 0.01).
4.4 Discussion

Inflammation is the tissue reaction due to infection, irritation or foreign substance. It is a part of the host defense mechanisms that are known to be involved in the inflammatory reactions such as release of histamine, bradykinin and prostaglandins. The early inflammation changes in damaged tissues releases variety of active materials from polymorph nuclear leukocytes and lysosomal enzymes. The vascular effects are mediated by kinins, prostaglandins and vaso-active amines i.e. histamine, released by mast cells leading to increased vascular permeability resulting in plasma exudation.

Prostaglandins are involved in several physiological and pathological processes, including vasodilation or vasoconstriction, contraction and relaxation of bronchial or uterine musculature, hypotension, ovulation, bone metabolism, increase in renal blood flow etc. Prostaglandins also have pathophysiologic effects such as erythema and increased local blood flow, hyperalgesia, probably due to sensitization of pain receptors, and increased body temperature at the hypothalamus through cytokine stimulation. When prostaglandin production is increased, there is increased sensitivity to pain and fever and increased inflammatory response. During damage to the cell membrane, which is basically made up of phospholipids, the enzyme phospholipase A2, that is present in leukocytes and platelets, is activated by proinflammatory cytokines, such as interleukin (IL)-1. This enzyme leads to the degradation of phospholipids, resulting in production of arachidonic acid. This, when metabolized, forms leukotrienes through the action of the enzyme lipoxygenase, and prostaglandins, prostacyclins and thromboxanes, through the action of the cyclooxygenase enzyme (COX) (Brune and Hinz, 2004)
COX is the first enzyme involved in producing prostaglandins from arachidonic acid. It converts, by oxygenation, the arachidonic acid into two unstable components: prostaglandin G2 and prostaglandin H2. These prostaglandins are later transformed by isomerases into prostacyclin, thromboxane A2, prostaglandins D2, E2 and F2. Prostaglandin E2 is important because of its pyrogenic action responsible for inflammation and in increasing sensitivity to pain. Whereas COX 1 has 17 amino acids at the terminal amino section, COX 2 has 18 amino acids at the terminal carboxyl section. Although they are very similar in terms of their protein structure, these enzymes are coded by different genes. Genetically, COX 1 and COX 2 are approximately 60% homologous and their genes are located at chromosomes 9 and 1, respectively. COX 2 is present at the site of inflammation, and because of this is defined as an inducible enzyme. It is primarily expressed by cells that are involved in the inflammatory process, such as macrophages and monocytes. Nevertheless, it is known that COX 2 can also be found in other tissues and organs, such as kidneys, brain, ovaries, uterus, cartilage, bones and vascular endothelium. COX 2 is induced by cytokines (IL-1, IL-2 and tumor necrosis factor [TNF]) and other mediators (such as growth factor and endotoxins) at the site of inflammation (Cronstein, 2002).

Nitric Oxide has both inflammatory and anti-inflammatory properties depending on when, where, and how much nitric oxide (NO) is produced. Under inflammatory conditions, expression of Inducible Nitric Oxide Synthase (iNOS) is induced in response to inflammatory cytokines (i.e., IL-1β, interferon-γ, and tumor necrosis factor-α). During oxidative environment there will be high output of iNOS that produce more NO that reacts with superoxide leading to peroxynitrite formation and cell toxicity. The rapid release of NO production from iNOS induces vascular permeability and leukocyte infiltration to the inflamed tissues results in inflammation at the site.
COX has constitutive and inducible isoforms of which is triggered by those cytokines which also induce iNOS. Cells such as i.e, macrophages, endothelium, chondrocytes produce NO and prostaglandins simultaneously in response to cytokines. The synergetic effects of these molecules are often similar and include the capacity to relax smooth muscle, inhibit platelet and neutrophils adhesion and inhibit neutrophil oxidant production. Therefore the two pathways works closely where NO can stimulate COX activity through reaction with the heme component which binds to the active site of the COX enzyme (Clancy and Abramson, 1995). Furthermore, NOS inhibitors decrease IL-1 induced release of PGE-2 in rat mesangial cells that indicate that endogenous NO production enhances COX level in the cells. Conversely, endogenous PGE, reduces the IL-1-stimulated iNOS mRNA induction in these same studies. Therefore, NO and prostaglandin pathways appear to interact as synergetic effect regulators of a variety of physiological and inflammatory processes.

The present clinically used anti-inflammatory drugs have variety of adverse effects and high cost of treatment. Alternative to these drugs are traditional medicines and natural products provides an alternative to conventional therapy in preventing the cells of the body from performing some function that has become hyperactive and a great hope in the identification of bioactive compounds and their development into drugs for treating inflammatory diseases (Gautam and Jachak, 2009).

Natural products have long been recognized as an important source of therapeutic and effective medicines. In a study by Benoit et al., 1976), 163 species of plants and fungi were tested to determine their anti-inflammatory activity. Of these, 17 led to a 30-39% reduction in inflammation, 21 between 40-49%, 15 between 50-59%, 4 between 60-69%, and 2 > 70%. The medicinal properties of R. apiculata provide a wide domain for medicinal uses. Chemical compounds released by plants may have broad medicinal application such as suppressing new tissues and various inhibitory
properties. The literature has indicated that *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 *in vitro* anti-tumor effects against four human cancer cell lines (Cichewicz et al., 2004).

In the present study, the anti-inflammatory activity of *R. apiculata* was examined. In the carrageenan induced mice paw edema model, *R. apiculata* showed significant inhibitory effect on the edema formation. This effect started from the second hour (early phase) and fourth hour (late phase) and was maintained in all the inflammatory phases, suggesting that the main mechanism of action of the tested may involve interfering in histamine and prostaglandin biosynthesis pathway that may influence other mediators of inflammation. Inflammation induced by formalin results from cell damage which provokes the production of endogenous mediators such as, histamine, serotonin, prostaglandins and bradykinin. It is then be known from these results that *R. apiculata* prevented formalin-induced paw edema in a dose dependent manner showing significant anti-inflammatory effect on late phase by interference of inflammatory mediators such as prostaglandins and bradykinin thus prevents chronic inflammation. This *R. apiculata*, anti-inflammatory properties were further supported by showing significant decrease in the serum COX-2, iNOS, and prostaglandin E-2 levels during carrageenan and formalin induced inflammation. *R. apiculata* significantly inhibited the level of NO, COX and prostaglandin E-2 that may be considered that the synergetic effect of these proinflammatory cytokines has been inhibited that prevented the adhesion of neutrophils, platelets and macrophages results in prevention of inflammatory process during carrageenan and formalin induced inflammation. In conclusion, this study is the first of its kind to report on the anti-inflammatory activity possessed by the mangrove plant *R. apiculata*. These protective
activities could be attributed to the high content of 4-pyrroldinyl, pyrazole, ketone derivatives and thiazolidinediones found in R. apiculata. The results of the present work indicate that R.apiculata could potentially be useful as a (natural) anti-inflammatory agent.