CHAPTER - 9

EFFECT OF RHIZOPHORA APICULATA AGAINST ACETIC ACID INDUCED ULCERATIVE COLITIS

9. Aim

To evaluate the effect of *R. apiculata* against acetic acid induced ulcerative colitis

9.1 Introduction

Ulcerative colitis (UC) is a common inflammatory bowel disease (IBD) with chronic inflammatory process in the digestive mucosa that causes inflammation and ulcers in the lining of the rectum and colon (García Callejo et al., 2005). The etiology of IBD still remains unclear but environmental factor and genetic susceptibility could be the major cause of involvement in the initiation of UC (Medhi et al., 2008). The prevalence of IBD rapidly increased in United States, Europe and in Asia (Martins and Peppercorn, 2004; Ray 2011). UC affects approximately 500,000 to 2 million people in the United States and they are most common during adolescence and early adulthood. The symptoms includes abdominal pain, diarrhea with bloody and/or mucus diarrhea, dehydration, abdominal cramps, fever, anemia, weight loss and psychosocial consequences (Casati et al., 2000). Patients with severe and recurrent UC have an elevated risk of developing colorectal cancer (CRC). CRC is the second leading causes of death in United States and of around 108,000 people were diagnosed with CRC every year (Medhi et al., 2008).

CRC is a feared complication of chronic UC and inflammation which appears to be an important biological risk factor for the development of CRC (Cosnes et al., 2001; Rutter et al., 2004). Chronic inflammation increases oxidative stress, regeneration,
repair and dysplasia in colon that transform into an invasive CRC (Itzkowitz, 2003). Consumption of alcohol increases the risk of development of ulcer. Excessive ethanol ingestion results in gastritis characterized by mucosal edema, subepithelial hemorrhages, cellular exfoliation and inflammation cell infiltration.

To scavenge reactive oxygen species (ROS), gastric cells have several enzymatic and non enzymatic anti-oxidants including superoxide dismutase (SOD) reduced glutathione (GSH) and catalase (CAT) but excessive generation of ROS enhance lipid peroxides (LPO) (oxidative stress) and could deplete these antioxidants enzymes (Cadirci et al., 2007). Oxidative stress could be a major contributing factor to the tissue injury and fibrosis that characterize IBD. The colon mucosa of UC secretes high amount of pro-inflammatory cytokines and inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, nitric oxide (NO), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) that seem to play an important role in UC in relevant experimental models (Reimund et al., 1996; Rogler et al., 1998). UC is associated with neutrophils infiltration which is directly correlated with increase in myeloperoxidase (MPO) level that produces hypochlorous acid from hydrogen peroxide and chloride anion that are cytotoxic (Garrity-Park et al., 2012). Increased level of superoxide and NO during UC increases peroxynitrite formation that mediates oxidation of lipids, proteins and DNA.

Therapeutic strategies for treating IBD now focus on use of anti-inflammatory agents such as sulfasalazine the most widely prescribed drug. Sulfasalazine is converted to 5-ASA in intestine that disturbs the prostaglandins and leukotrienes pathway. The adverse effects of sulfasalazine includes vomiting, hypospermia, hepatitis, pneumonitis, hemolytic anemia, chronic nephrotic and encephalitis (DeMichele et al., 2012; Halmos et al., 2004). In rare cases, sulfasalazine can exacerbate the colitis, resulting in diarrhea, abdominal cramps and discomfort.
In the present study we investigate the effect of *R. apiculata* in an experimental model of colitis induced by administration of acetic acid in mice.

### 9.2 Materials and methods

#### 9.2.1 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.

#### 9.2.2 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.
9.2.3 Chemicals

All chemicals used in these studies were of analytical or reagent grade. Gum acacia was purchased from Hi-Media (Mumbai, India), Formaldehyde solution was procured from Universal Laboratories Pvt. Ltd. (Hyderabad, India). Sulfasalazine was purchased from Wallace Pharmaceutical Pvt. Ltd. Goa. India.

9.2.4 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 7 consecutive days.

9.2.5 Induction of colitis to the experimental animals

Animals were divided into four groups of six mice each (n=6): Group I served as normal untreated and received the vehicle (1% (w/v) gum acacia; Group II served as a ulcerative colitis control and received vehicle along with the acetic acid; Group III mice received R. apiculata (10 mg/kg b.wt) and acetic acid; Group IV mice received standard drug sulfasalazine (100 mg/kg b.wt) and the acetic acid (Thippeswamy et al., 2011). Each vehicle/drug treatment was given daily for seven days via (i.p.) injection. On Day 6 of the regimen, the animals were fasted (post-injection) overnight but had ad libitum access to water. On day 7, 1 hr after the final injection of vehicle/drug, the mice was anesthetized by ether inhalation and a polypropylene tube with 2 mm diameter was
inserted through the rectum into the colon to a distance of 4 cm. For the mice in Groups II, III and IV, a solution of 2 ml of acetic acid (3%, v/v) in 0.9% saline (intrarectal) was instilled into the lumen of the colon. The mice were then maintained in a supine Trendelenburg position for 30 sec to prevent the leakage of the intra-colonic instillate. Group I mice received only a saline. After 24 hours of single dose administration of acetic acid (Day 8), the animals were anaesthetized with ether and blood samples were collected into non-coated tubes for isolation of serum for analysis (Thippeswamy et al., 2011). The mice were then euthanized by cervical dislocation and their colons recovered, washed gently with saline and weighed. The colon samples were cut opened longitudinally for macroscopic scoring, histopathology analysis and biochemical measures.

9.2.6 Assessment of colitis

The severity of colitis in each mouse was evaluated by an independent observer blinded to the treatments. The intact colon samples of each mouse was removed and gently cleaned with physiological saline to remove fecal residue and weighed. Macroscopic inflammation scores were assigned based on clinical features; thereafter the tissue was frozen at -80 °C for later measures of biochemical parameters (Jagtap et al., 2004). Macroscopic scoring pattern employed for evaluation of disease severity is presented in Table 9.1.
<table>
<thead>
<tr>
<th>Pattern for evaluation of disease activity index</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>No visible damage</td>
<td>0</td>
</tr>
<tr>
<td>Focal hyperemia (water oozes out)</td>
<td>1</td>
</tr>
<tr>
<td>Ulcerization without hyperemia or bowel wall thickness</td>
<td>2</td>
</tr>
<tr>
<td>Ulcerization with inflammation at one site</td>
<td>3</td>
</tr>
<tr>
<td>Ulcerization with inflammation at two sites</td>
<td>4</td>
</tr>
<tr>
<td>Major sites of inflammation &gt; 1 cm along the organ with redness</td>
<td>5</td>
</tr>
<tr>
<td>Major sites of inflammation &gt; 2 cm along the organ with redness</td>
<td>6</td>
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<tr>
<td>Major sites of inflammation &gt; 3 cm along the organ with redness</td>
<td>7</td>
</tr>
<tr>
<td>Major sites of inflammation &gt; 4 cm along the organ with redness</td>
<td>8</td>
</tr>
<tr>
<td>Major sites of inflammation &gt; 5 cm along the organ with redness and bleeding</td>
<td>9</td>
</tr>
<tr>
<td>Major sites of inflammation &gt; 6 cm along the organ with redness, swelling and bleeding</td>
<td>10</td>
</tr>
</tbody>
</table>

**Table 9.1 Macroscopic scoring pattern for evaluation of disease severity index of colitis**
9.2.7 Biochemical assays

Biochemical assays were performed on colonic tissue samples of the mice. The blood samples that were collected in non-coated tubes were allowed to clot and serum was isolated. Then the serum was used for measures of NO, COX-2 and lactate dehydrogenase (LDH) content. A portion of isolated colonic tissue from each mouse was homogenized in 10% (w/v) Tris-HCL buffer (pH 7.0) for use in measurements of SOD, LPO, MPO, TNF-α, iNOS and reduced GSH.

9.2.8 Determination of the effect of *R. apiculata* on colonic SOD level during ulcerative colitis

SOD level in colonic tissues was determined based on the ability of the enzyme to inhibit nitroblue tetrazolium (NBT) reduction by superoxide (Kakkar et al., 1984). Incubation medium containing 0.1ml of test sample, 2.55 ml phosphate buffer, 0.2 ml EDTA/NaCN, 0.1 ml NBT and 0.05 ml riboflavin (total volume of 3 ml). The tubes then received uniform illumination for 15 min and the absorbance was then measured at 560 nm in spectrophotometer (ELICO, Hyderabad, AP). One unit of enzyme activity was defined as the amount of enzyme giving 50% inhibition of reduction of NBT and expressed as U/mg protein. Enzymatic activity was calculated from inhibition of reduction of NBT using standard curve constructed by varying amount of the test samples.

9.2.9 Determination of the effect of *R. apiculata* on colonic GSH content during ulcerative colitis

GSH levels in colonic tissue were determined by the method of (Beutler and Kelly (1963). GSH was measured by its reaction with 5,5’-dithionitrobenzoic acid
(DTNB). For the reactions, 0.125 ml of 25% (w/v) trichloroacetic acid solution (TCA) was added to 0.5 ml of colonic tissue homogenate. The tubes were placed on ice for 5 min and then further diluted with 0.6 ml 5% TCA. Each sample was then centrifuged (5000 rpm, 4°C, 10 min) and resultant supernatant taken for GSH estimation. A volume of aliquot (0.3 ml) was combined with 0.7 ml of 0.2 M phosphate buffer, and then 2 ml of 0.6 mM DTNB was added to the tubes and the intensity of the resulting yellow color were measured at 412 nm. Values are expressed as nanomole/mg of protein.

9.2.10 Determination of the effect of *R*. *apiculata* on serum NO activity during ulcerative colitis

NO radical inhibition can be estimated by the use of a Griess reaction. The reaction mixture containing sodium nitroprusside and serum was incubated at 25°C for 150 min. After incubation, 1 ml of the reaction mixture mixed with equal volume of Griess reagent and allowed to stand for 15 min at room temperature. The absorbance of pink colored chromophore formed was measured at 546 nm against the corresponding blank solutions and expressed in µm (Green et al., 1982).

9.2.11 Determination of the effect of *R*. *apiculata* on colonic LPO concentration during ulcerative colitis

The tissue homogenate (0.1 ml) was treated with 200 µl sodium dodecyl sulfate (SDS-8%) and 1.5 ml thiobarbutric acid (TBA). The mixture was kept in water bath for 1 hr at room temperature and cooled by adding 1 ml of distilled water followed by 5 ml of a mixture of *n*-butanol and pyridine (15:1, v/v) and centrifuged. The supernatant were taken and measured the optimal intensity at 532 nm. The levels of LPO were
expressed as nmole/mg protein. The protein level was estimated by the Lowry method (Lowry et al., 1951).

9.2.12 Determination of the effect of *R. apiculata* on colonic MPO, TNF-α and iNOS activity during ulcerative colitis

The levels of colonic MPO, TNF-α and iNOS were determined using a commercially available ELISA kit (USCN Life Sciences Inc, Houston, USA) according to the manufacturer’s instruction.

9.2.13 Determination of the effect of *R. apiculata* on serum LDH and COX-2 activity during ulcerative colitis

The levels of serum LDH and COX-2 were determined using a commercially available ELISA Kit (BIOVISION, California, USA and BLUE GEN BIOTECH, Shanghai, CHINA) according to the manufacturer’s instruction.

9.2.14 Histopathology analysis

A portion (2 cm) of the colonic tissue specimen from each mice (n = 6) was fixed in 5% formalin, cut into 5-µm thickness, stained using H&E (hematoxylin and eosin) and then examined for histopathological changes. The stained sections of colon samples were examined for inflammatory changes like infiltration of the cells, necrotic foci, damage to payer’s patches and nucleus.
9.2.15 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.

9.3 Results

9.3.1 Clinical and macroscopic assessment of colitis by scoring

Macroscopic assessments of colitis in colonic tissues of the experimental mice is shown in Figure 9.1
Figure 9.1 Macroscopic assessment of colitis in colonic tissue of the experimental animals (A) Normal untreated (B) ulcerative colitis (C) ulcerative colitis + *R. apiculata* (D) ulcerative colitis + sulfasalazine.
Effect of *R. apiculata* on wet colon weight during UC is presented in Figure 9.2. *R. apiculata* treatment (10 mg/kg b.wt) significantly (*p* < 0.01) decrease the wet colon weight (0.52 ± 0.02 mg/cm) (Group III) when compared with UC control (0.61 ± 0.03 mg/cm) (Group II). Sulfasalazine treatment (100 mg/kg b.wt) also significantly (*p* < 0.01) decreased the colon weight (0.50 ± 0.02 mg/cm) when compared with UC control (Group II). Normal colon weight was (0.47 ± 0.04 mg/cm).

Acetic acid caused severe macroscopic edematous inflammation in the colons. The clinical lesions (n = 6) in the hosts received a maximum score of 9 (major sites of inflammation > 5 cm along organ) (Figure 9.3). In contrast, in hosts that were pretreated with the *R. apiculata*, the maximum score was 3 (ulceration with inflammation at one side) and in those that received sulfasalazine, the maximum score was 2 (ulcerization without hyperemia).

### 9.3.2 Effect of *R. apiculata* on colonic tissue SOD level during ulcerative colitis

Effect of the *R. apiculata* on colonic tissue SOD level during UC is shown in Figure 9.4. Treatments with the *R. apiculata* significantly (*p* < 0.05) increased colonic tissue SOD activity level (8.9 ± 1.8 U/mg protein) (Group III) when compared with UC control (5.4 ± 2.8 U/mg protein) (Group II). The sulfasalazine treated hosts also displayed (*p* < 0.05) relative increases in colonic tissue SOD level (9.3 ± 1.5 U/mg protein) (Group IV) when compared with UC control (Group II).

### 9.3.3 Effect of *R. apiculata* on serum NO level during ulcerative colitis

Effect of *R. apiculata* on serum NO levels during UC are presented in Figure 9.5. The *R. apiculata* significantly (*p* < 0.01) reduced the activity of serum NO level (29.9 ± 3.3 µM) (Group III) when compared with UC control (88.5 ± 3.8 µM) (Group II).
II). The standard sulfasalazine drug also shown significant ($p < 0.01$) reduction in the activity of serum NO in UC mice ($32.0 \pm 2.0 \mu M$) (Group IV) when compared with UC control (Group II).
Figure 9.2 Effect of *R. apiculata* on wet colon weight during ulcerative colitis

The animals were euthanized by cervical dislocation and the intact colon was dissected out, flushed gently with saline and weighed. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
Figure 9.3 Effect of *R. apiculata* on ulcerative colitis colonic morphology

Colonic morphology of the experimental animals was observed on day 8 during ulcerative colitis. Values are expressed as mean ± SD (6 mice/group).
Figure 9.4 Effect of *R. apiculata* on SOD level during ulcerative colitis
A portion of colonic tissue recovered from each mouse was used to determine SOD level. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (*p < 0.05) (n=6 / group)
Figure 9.5 Effect of *R. apiculata* on NO level during ulcerative colitis

Blood samples were collected from the tail vein and assayed for serum nitric oxide. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group)
9.3.4 Effect of *R. apiculata* on reduced GSH level during ulcerative colitis

Effect *R. apiculata* on GSH level colonic tissue during UC is presented in Figure 9.6. *R. apiculata* significantly (*p* < 0.01) increased the level of GSH in colonic tissue (45.5 ± 1.25 µg/min/mg protein) (Group III) when compared with UC control (30.5 ± 0.87 µg/min/mg protein) (Group II). The standard sulfasalazine drug also shown significant (*p* < 0.01) increase in GSH level in UC mice (47.2 ± 2.15 µg/min/mg protein) (Group IV) when compared with UC control (Group II).

9.3.5 Effect of *R. apiculata* on LPO level during ulcerative colitis

Effect of *R. apiculata* on colonic tissue LPO level during UC is presented in Figure 9.7. *R. apiculata* significantly (*p* < 0.01) reduced in the level of tissue LPO (10.2 ± 3.2 n mole/mg protein) (Group III) when compared with UC control (20.4 ±3.6 n moles/mg protein) (Group II). The standard sulfasalazine drug also shown significant (*p* < 0.01) reduction in tissue LPO level in UC mice (8.3 ± 2.5 n mole/mg protein) (Group IV) when compared with UC control (Group II).

9.3.6 Effect of *R. apiculata* on MPO activities during ulcerative colitis

Effect of *R. apiculata* on colonic MPO activities during UC is shown in Figure 9.8. *R. apiculata* significantly (*p* < 0.01) decreased colonic MPO level to (29.34 ± 3.2 U/gm tissue) (Group III) when compared with UC control (74.45 ± 7.8 U/gm tissue) (Group II). The standard sulfasalazine treated host also displayed relative decrease in colonic MPO level (24.47±2.8 U/gm tissue) (Group IV) when compared with UC control (Group II).
Figure 9.6 Effect of *R. apiculata* on reduced GSH level during ulcerative colitis
A portion of colonic tissue recovered from each mouse was used to determine colonic reduced GSH levels. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
Figure 9.7 Effect of *R. apiculata* on LPO level during ulcerative colitis
A portion of colonic tissue recovered from each mouse was used to determine colonic LPO level. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
Figure 9.8 Effect of *R. apiculata* on MPO activities during ulcerative colitis

A portion of colonic tissue recovered from each mouse was used to determine colonic MPO activities level. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
9.3.7 Effect of *R. apiculata* on TNF-α level during ulcerative colitis

*R. apiculata* on colonic TNF- α level during UC is shown in Figure 9.9. Treatments with *R.apiculata* the significantly (*p* < 0.01) decreased colonic TNF- α level to (29.4 ± 4.5 pg/mg tissue) (Group III) compared with UC control (73.5 ± 3.7 pg/mg tissue) (Group II). The standard sulfasalazine treated host also displayed significant (*p* < 0.01) decrease in colonic TNF- α level (26.2 ± 2.3 pg/mg tissue) (Group IV) when compared with UC control (Group II).

9.3.8 Effect of *R. apiculata* on iNOS level during ulcerative colitis

*R. apiculata* on colonic iNOS activities during UC is shown in Figure 9.10. Treatments with *R.apiculata* significantly (*p* < 0.01) decreased colonic tissue iNOS activity level (22.5 ± 1.93 ng/mg tissue) (Group III) when compared with UC control (53.4 ± 3.9 ng/mg tissue) (Group II). The standard sulfasalazine treated host also displaced relative decrease in colonic tissue iNOS level (19.6 ± 1.80 ng/mg tissue) (Group IV) when compared with UC control (Group II).

9.3.9 Effect of *R. apiculata* on serum LDH activity during ulcerative colitis

*R. apiculata* on serum LDH activities during UC is shown in Figure 9.11. *R. apiculata* significantly (*p* < 0.01) decreased serum LDH activity level (545.2 ± 45 U/L) (Group III) when compared with UC control (1643.5 ± 185 U/L) (Group II). The standard sulfasalazine treated hosts also displayed relative decrease in serum LPO (497.4 ± 37 U/L) (Group IV) when compared with UC control (Group II).
Figure 9.9 Effect of *R. apiculata* on TNF-α level during ulcerative colitis

A portion of colonic tissue recovered from each mouse was used to determine colonic TNF-α level. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
Figure 9.10 Effect of *R. apiculata* on iNOS level during ulcerative colitis

A portion of colonic tissue recovered from each mouse was used to determine colonic iNOS level. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group).
Blood samples were collected from the tail vein and assayed for serum LDH activity. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group)

Figure 9.11 Effect of R. apiculata on LDH activity during colitis

Blood samples were collected from the tail vein and assayed for serum LDH activity. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (**p < 0.01) (n=6 / group)
9.3.10 Effect of *R. apiculata* on serum COX-2 during ulcerative colitis

*R. apiculata* on serum COX-2 activities during UC is shown in Figure 9.12. *R. apiculata* significantly (*p* < 0.01) decreased serum COX-2 level (23.42 ± 1.9 ng/ml) (Group III) when compared with UC control (54.67 ± 3.1 ng/ml) (Group II). The standard sulfasalazine treated hosts also displayed significant (*p* < 0.05) decrease in serum COX-2 level (20.14 ± 1.2 ng/ml) (Group IV) when compared with UC control (Group II).

9.3.11 Histopathology analysis

Effect of *R. apiculata* on UC colons histopathology is shown in Figure 9.13. Pictures shown are from representative colon samples collected at the end of the experimental period (i.e., Day 8). (Figure 9.13A) As expected, normal mouse had no remarkable changes to their tissues. (Figure 9.13B) In contrast, the non-drug-treated colitis control group showed severe necrotic destruction of the epithelium and evidence of massive hemorrhaging along the lining. (Figure 9.13C) *R. apiculata* treated mouse colonic histopathology revealed minimal damage to the mucosa, with just slight submucosal edema and mild inflammatory cell infiltration and (Figure 9.13D) shows standard sulfasalazine also protects the host from colitis by showing minimal damage of colonic mucosa.
Figure 9.12 Effect of *R. apiculata* on COX-2 activities during ulcerative colitis

Blood samples were collected from the tail vein and assayed for serum COX-2 activities. Values are expressed as mean ± SD. Value is significantly different from ulcerative colitis control (*p* < 0.05; **p** < 0.01) (n=6 / group)
Figure 9.13 Histopathology analysis of colonic tissues from experimental animals
Representative tissue samples from within each treatment group are shown. (A) normal untreated, mouse showing colon with normal mucosa (B) ulcerative colitis control showing acetic acid induced colitis; note massive necrotic destruction of epithelium, areas of hemorrhage, sub-mucosal edema and inflammatory cellular infiltration (C) ulcerative colitis + *R. apiculata*, showing minimal damage of mucosa with slight sub-mucosal edema and mild inflammatory cell infiltration (D) ulcerative colitis + sulfasalazine, showing minimal damage of mucosa, lymphoid tissue and muscularis propria (40x).
9.4 Discussion

UC is a leading health related disease that affects quality of life of the people, also affecting the economic and social cost of patients and their families. Although many anti-inflammatory therapeutic agents are available today has efficient protective role but exhibits many adverse effects. Therefore an alternative therapeutic option plant source (natural) with greater therapeutic efficacy for UC is essential along with less or no adverse effects.

Acetic acid induced experimental colitis has been proved to be an efficient UC animal model to study inflammatory mechanism that affects colons structure, destruction of colons mucosa, increased in inflammatory cytokines, inflammatory mediators and promotion of fibrin hydrolysis (Thippeswamy et al., 2011). Therefore in the present investigation the effect of *R.apiculata* in acetic acid induced colitis in experimental mouse model was evaluated. The wet weight of the inflamed colons tissue is reliable and sensitive indicator to demonstrate the extent of the inflammatory response (Bozkurt et al., 2003). The pretreatment with *R.apiculata* significantly reduced the wet weight of colons and lesion score compared with colitis control, indicating its protective effect from UC.

Oxidative stress associated cellular damage play an important role in the pathogenesis of the colitis. ROS and reactive nitrogen species (RNS) from the inflammatory cells, free radicals and prooxidant molecules during the inflammatory process are involved in the development of colitis (Hofseth et al., 2003). Excessive production of ROS in mucosal cells which is induced by immune responses and inflammatory cells damages the intestinal epithelial cells, mucosal integrity and inflammatory signaling cascade that lead to severe UC. The key mechanism behind the pathogenesis of colitis is due to expression of adhesion molecules on endothelial cells,
including vascular cell adhesion molecule 1 (VCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and intercellular adhesion molecule 1 (ICAM-1), that allows the adhesion of circulating leukocytes filtration into intestinal endothelium during the development of colitis (Seguí et al., 2003).

SOD catalyzes dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified by catalase. To regulate ROS levels, the intestinal mucosa possesses a complex of antioxidant systems where SOD is the primary anti-oxidant enzymes in converting superoxide anion to $H_2O_2$. SOD has powerful anti-inflammatory activity and is highly effective in treatment of colonic inflammation in experimental colitis by reducing ROS generation and oxidative stress (Kruidenier et al., 2003). SOD can also reduce peroxidation reactions during colitis by restraining the adhesion molecules such as VCAM-1, MAdCAM-1 and (ICAM-1) which are responsible for leukocyte infiltration and mucosal damage in colonic venules during colitis (Seguí et al., 2003). The pretreatment of *R. apiculata* significantly increased the colonic tissue SOD level that reduces oxidative stress and ROS generation during UC which indicates their protective role over the colonic ulcer. SOD also inhibits endothelial activation, adhesion molecule expressions and leukocyte-endothelial interactions that play an important role in regulation of IBD (Seguí et al., 2004).

NO regulates variety of processes in the gastrointestinal tract, including blood flow, leukocyte recruitment, vascular permeability, immune regulation, mucosal defense, fluid secretion and intestinal motility. NO is largely produced by three isoforms of NO synthase (NOS): neuronal (nNOS, also called as NOS1), Inducible (iNOS, or NOS2) and endothelial (eNOS or NOS3). These isoforms are further divided in to NOS type I and type III. iNO regulates multifarious processes in the gastrointestinal tract (Chhanalal et al., 2011). NO produced through iNOS, by a number of inflammatory cells such as (neutrophils, monocytes, macrophages) as well
as epithelial cells into colonic lumen of patients during IBD provides evidence for the hypothesis that the enzyme, iNOS is the sources of excess NO production (Chhanalal et al., 2011). But the exact role of NO in intestinal inflammation is still a mystery. The NO level up regulates in both UC and CD also in animal models of colitis. There have been conflicting reports on whether NO protects or exacerbates injury in colitis or as a simply a marker of inflammation (Beck et al., 2004). The higher level of NO production have direct role over the regulating inflammation, carcinogenesis and angiogenesis. In the present study, the administration of *R. apiculata* was found to down regulate iNOS level and thereby NO production in colitis induced mice preventing the recruitment of inflammatory cells such as neutrophils, macrophages and monocytes to the inflamed colons site and decreases the peroxynitrite formation thus preventing oxidation of lipids, protein, DNA breakdown, ATP depletion, tissue damage, intestinal wall thickening during colonic inflammation under UC condition.

GSH, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate and cysteine. GSH can directly neutralize ROS such as LPO and protects the body from the inflammation of gastritis, stomach ulcers, pancreatitis and IBD including UC and Crohn's disease (Sido et al., 1998). In a study it was reported that decreased enzyme activity is accompanied by a decreased availability of cysteine for GSH production contribute to mucosal GSH deficiency in IBD (Sido et al., 1998; Dief et al., 2008). This implies that declining GSH level may actually contribute due to down regulation of mucosal γ-glutamyl cysteinyln synthase (γ-GCS) activity which is the enzyme essential for GSH synthesis (Sido et al 1998). GSH deficiency induced the enlargement of lymphoid aggregates in intestine by recruiting lymphocytes from peripheral circulation resulting in intestinal inflammation of IBD. Therefore GSH as a master anti-oxidant play an important role in preventing IBD. The pre-treatment with *R. apiculata* mitigated the depletion of GSH level in treated hosts closer to the normal hosts than to the control with induced ulcers. Increase in the reduced GSH level
neutralizes the ROS and LPO preventing from inflammation during UC. Increased GSH level trigger the down regulation of pro-inflammatory signaling molecules such as leukotrienes and eicosanoid which prevents the pathogenesis of UC (Kanodia et al., 2011).

Superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, secreted by neutrophils and phagocytes accumulating in the inflammatory lesion, lead to impairment of cellular membrane stability and cell death by leading lipid peroxidation. Oxygen-free radicals and LPO (oxidative stress) are highly reactive factor to the tissue injury and fibrosis during IBD. The increased formation of LPO that occur in colonic tissues involves various cycles that generate enormous reactive metabolites that ultimately exhaust cellular anti-oxidants including vitamins E and C; in turn, this leads to increases in severity of inflammation and ulceration (Kuroki et al., 1993). It was reported that IBD patients have high level of LPO due to the inbalance between prooxidant and anti-oxidant mechanism that accumulate oxidative modified proteins and lipids (Reifen et al., 2000). The pretreatment of *R.apiculata* significantly inhibits the increasing LPO level in colonic tissue. These outcomes could reflect an overall protective role against colitis by reducing the oxygen-free radicals and preventing injury of cellular membrane stability and cell death during UC.

Neutrophils accumulation in the inflamed intestinal mucosa is a prominent feature in UC. Neutrophils granulocytes contain MPO that combat against bacteria. These MPO are proteolytic enzymes, released from the activated neutrophils that are recruited at inflamed colon tissue during colitis. During UC mesangial cells and neutrophils release interleukin-8, which promotes neutrophil migration to the inflamed tissue and activate neutrophils results in release of MPO that produces hypochlorous acid from hydrogen peroxide that lead to the colitis-induced oxidative injury (Heinecke et al., 1993; Donnahoo et al., 1999). The activity of MPO from the inflamed tissue is
directly proportional to the number of neutrophils seen in histologic sections (Krawisz et al., 1984). It was reported that the MPO level increased several fold in the patients with UC compared with healthy controls correlating the neutrophil activity (Raab et al., 1992). Therefore, measurement of MPO activity has been used as quantitative and sensitive assay for acute intestinal inflammation and also increased MPO activity has been reported to be an index of neutrophil infiltration and inflammation (Choudhary et al., 2001). *R. apiculata* administration decreased the MPO level during colitis indicates a significant reduction in the neutrophils within the colons mucosa preventing the release of $\text{H}_2\text{O}_2$, oxidative damage, cytotoxicity and colonic inflammation.

TNF-$\alpha$ is not commonly detectable in healthy individuals, but increase in tissue TNF-$\alpha$ levels are found during inflammatory conditions (Nurnberger et al., 1995). The macrophages and lymphocytes remains to be the main key cells for the production of TNF during IBD. TNF-$\alpha$ signal transduction pathway are complex and still not fully understood. Most of the tissues co-express TNF-$\alpha$ receptors. TNF-$\alpha$ receptors are expressed on colons tissues to mediate vascular endothelium and endothelial leukocyte interactions that promotes inflammation of colons tissue by regulating adhesion molecules for leukocytes such as vascular VCAM-1, ICAM-1 that allows infiltration of leukocytes to the inflamed site. TNF-$\alpha$ regulate the expression of COX-2 which is responsible for the production of prostaglandin synthesis which plays an important role during inflammatory process (Bevilacqua et al., 1986; Bradley, 2008). The pretreatment of *R. apiculata* significantly decrease the TNF-$\alpha$ level in the colonic tissue suggesting that the plant could have protective role over inflammation during UC. The *R. apiculata* could able to suppress the TNF-$\alpha$ expression on colonic tissue and thus prevented the leukocytes recruitment, adhesion and interaction to the inflamed site preventing inflammation of colons tissue during colitis.
LDH is found in almost all tissues of the body and can be elevated in many pathological conditions (Faruqi et al., 2012). LDH is used as a marker of tissue breakdown as LDH is abundant in red blood cells (RBC) and can be a marker for cytotoxicity and inflammatory responses respectively since LDH assay has been proved to be one of the best methods for determining the relative amount of cellular lysis (Moran and Schellmann, 1996). High serum LDH levels are common found during CRC and which is the most vital prognostic variables in CRC (Kemeny et al 1989; Watine and Friedberg 2004). In this study the pretreatment of *R. apiculata* significantly decreased the LDH level during UC condition. The *R. apiculata* prevented the breakdown of RBC thereby preventing cellular lysis during colonic inflammation.

COX-1 and COX-2 are cyclooxygenase enzymes that convert arachidonic acid to inflammatory and other physiological mediators, including prostaglandins, thromboxane and prostacyclin responsible for inflammation. COX-2, the inducible isoform of prostaglandin H synthase, has been involved in the growth and progression of CRC. COX-2 over expression occurs early in UC neoplastic progression. Increased COX-2 expression are found in colons cancer tissues which modulate the production of angiogenic factors by colon cancer cells by multiple signaling pathway (Takeda et al., 2003; Einspahr et al., 2003). COX-2 plays an important role in sporadic colorectal neoplasia, based on it’s over expression in colonic adenomas and carcinomas that reveals to understand the role of COX-2 important for its therapeutic implications. Increased COX-2 level during IBD, accelerate genetic damage through increased production of malondialdehyde, a byproduct of COX-mediated prostaglandin synthesis and lipid peroxidation (Basu and Marnett, 1983). Therefore it is necessary to inhibit the over expression of COX-2 enzyme that could inhibit the progression of inflammation during IBD. In our study the *R. apiculata* decrease the COX-2 level during UC signifying the defensive activity of *R. apiculata* in preventing inflammatory mediator’s production: prostaglandins, thromboxane and prostacyclin thus preventing the
progression of inflammation during UC. *R. apiculata* in inhibiting ulcerative colitis by enhancing antioxidants is shown in Figure 9.14. Taken together, the present study data suggest that the pre-treatment of methanolic of *R.apiculata* prevents acetic acid induced colitis in experimental mouse model and this protective effect could be due to its anti-oxidant and anti-inflammatory action.
Figure 9.14 *R. apiculata* inhibiting ulcerative colitis by enhancing anti-oxidant level
In conclusion, this study is the first of its kind to report on the protective role played by the *R. apiculata* against colitis in mice. The active role responsible for these activities could be attributed to the high content of pyrazole, 4-pyrrolidinyl, ketone derivatives and thiazolidinediones found in the *R. apiculata* (Chapter 3). The results of the current study suggest that *R. apiculata* could potentially be useful as a (natural) therapy for IBD. However, further research is essential to evaluate those similar protective roles in other experimental colitis animal models that could imitate human IBD.