CHAPTER – 8
EFFECT OF *Decalepis hamiltonii* AND *Solanum muricatum* ON AGAINST ACETIC ACID INDUCED ULCERATIVE COLITIS

8.1 Introduction

Inflammatory bowel disease (IBD) is a major intestinal disease that comprises of Ulcerative colitis (UC) and Crohn’s disease (CD). IBD is characterized by diffused mucosal inflammation of colon and ulcers in the lining of the rectum and colon (Baugmart and Carding 2007; Patil et al., 2012). IBD is associated with neutrophil infiltration which in turn produces reactive oxygen species there by contributing to oxidative stress at the site of inflammation. (Seril et al., 2003). Excessive production of reactive oxygen metabolites such as superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives, such as N-chloramines has been reported during IBD (Keshavarzian et al., 1992). The antioxidant defense mechanism of the host regulates the oxidative damage during IBD and scavenges the free radicals generated during inflammation (Blau et al., 1999). There will be an excessive production of proinflammatory cytokines and these proinflammatory cytokines has been shown to play a significant role in the progression of IBD (Rogler and Andus, 1998; Pastorelli et al., 2011). The increased production of nitrite in the inflammatory site contributes to the progression of IBD. The production of nitrite is mediated by the iNOS and COX-2 genes at the site of inflammation (Ardizzone and Porro, 2005).
The treatment procedure varies with the severity of ulcerative colitis. The current treatment procedure involves use of synthetic drugs such as glucocorticosteroids, 5-aminosalicylic acid and mesalazine (Baumgart and Sandborn 2007; Kornbluth and Sachar 2004; Travis et al., 2008). These synthetic drugs have been reported to suppress the immune system and have been associated with toxic side effects (Shanahan et al., 2001; Toruner et al., 2008). Plant based drugs have been reported to be safe and less toxic compared with synthetic drugs to treat several diseases including inflammation and cancer.

In the present investigation we had evaluated the therapeutic potential of *D.hamiltonii* and *S.muricatum* and its protective effect on the inhibition of ulcerative colitis.

### 8.2 Materials and Methods

#### 8.2.1 Preparation and administration of plant extract

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

Male Wistar rats (200-250g) (n=6) were purchased from Kerala Veterinary and Animal Science University, Mannuthy and used for the present study. The animals were maintained under controlled condition as explained in chapter 4. The animal experiments were conducted after getting approval from Institutional Animal Ethics Committee, Karunya University (IAEC/KU/BT/12/017).

8.2.3 Chemicals

Lactate dehydrogenase kit was obtained from Bio vision, California, Sulfasalazine was purchased from Wallace pharmaceuticals, Goa, India. USA. Myeloperoxide (MPO), Inducible Nitric Oxide Synthase (iNOS) Tumour Necrosis Factor (TNF-α) were procured from USCN Life science Inc, Houston, USA. Cyclooxygenase 2 (COX-2) was procured from Bluegen Biotech, Shanghai, China. Transcription factor profiling kits for p65 and p50 were procured from Cayman (Ann Arbor, MI). All other chemicals used were analytical reagent grade.

8.2.4 Experimental setup

The animals were divided into 5 groups (n=6 each group). Group I: normal group. Group II: Ulcerative colitis alone; Group III: Ulcer + sulfasalazine
(100mg/kg. B.wt.); Group IV: Ulcer + *D.hamiltonii* (20 mg/kg B.wt.) and Group V: Ulcer + *S.muricatum* (20 mg/kg B.wt.).

**8.2.5 Induction of colitis**

All animals (except group I) were kept fasting overnight, giving water *ad libitum* and anesthetized using ether before induction of colitis. 2 ml of acetic acid (3%, v/v) was infused of the colon. The animals were killed after 24h by cervical dislocation. Blood was collected and the serum was separated. The colonic specimen was collected and washed using ice cold phosphate buffered saline (pH 7.2) and the colon were homogenized using 10 mmol Tris-HCl buffer (pH 7.4). A portion of the colon was kept in 10% formaldehyde and used for histopathology and immunohistochemistry studies.

**8.2.6 Assessment of colitis**

**8.2.6.1 Macroscopic scoring**

The distal 10 cm portion of the colon was dissected out, and macroscopic inflammation scoring was done (Millar et al., 1996).
8.2.6.2 Biochemical Studies

The serum and colon tissue homogenate was used for the assessment of LPO (Ohkawa et al. 1979), GSH (Moron et al. 1979), GPx (Paglia and Valentine, 1967), SOD (Marklund and Marklund, 1974), NO (Green et al. 1982), TNF-α, iNOS, COX-2, MPO and LDH [ELISA] (USCN life science Inc™, Houston, USA). Nuclear extract for prepared and NF-kB p65 and NF-kB p50 was done according to method of Dignam et al., 1983 and was quantified by kits (Cayman™, Michigan, USA).

8.2.6.3 Histopathological study

The colon tissues were examined microscopically for mucosal damage by histopathological analysis. The tissues were observed under microscope at 40x magnification for interpreting the results.

8.2.6.4 Immunohistochemical study

Immunohistochemical studies were performed using iNOS and COX-2 antibodies as explained previously (He et al., 2011). The tissue specimens were observed under inverted microscope [EVOS] under 40x magnifications for interpreting the results.
8.2.7 Statistical analysis

For all the in vivo studies statistical analysis were performed according to the details given in chapter 4.

8.3 Results:

8.3.1 Macroscopical results

The effect of *D.hamiltonii* and *S.muricatum* on colon morphology is shown in Figure 8.1. Colitis control group showed severe colon inflammation (Figure 8.1b). The macroscopic scoring and wet colon weight for colitis control group was found to be 3.90 and 172 mg/g respectively. Treatment with *D.hamiltonii* or *S.muricatum* significantly decreased the macroscopic scoring to 1.69 and 1.63 respectively. Similarly the wet colon weight was also significantly decreased to 123 and 121 mg/g respectively after *D.hamiltonii* or *S.muricatum* treatment (Table 8.1).

8.3.2 Colon LPO level

The effect of *D.hamiltonii* and *S.muricatum* on the colon LPO level is shown in Figure 8.2. Treatment with *D.hamiltonii* and *S.muricatum* significantly decreased the colon LPO to 3.68 nmol and 3.29 nmol/mg proteins respectively compared with colitis control group (6.43 nmol/mg protein). In normal the LPO level was found to be 2.36 nmol/mg protein. (Figure 8.2).
A) Normal   B) Ulcerative colitis control   C) UC + sulfasalazine (100 mg/kg B.wt.)
D) UC + D.hamiltonii (20 mg/kg B.wt).   E) UC + S.muricatum (10 mg/kg B.wt).

Figure 8.1: Effect of D.hamiltonii and S.muricatum on colon morphology during ulcerative co experimental colitis
Table 8.1: Effect of *D. hamiltonii* and *S. muricatum* on the macroscopic scoring and wet weight during experimental colitis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Macroscopic Score</th>
<th>% Reduction</th>
<th>Wet colon weight (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>70.5 ± 4.1</td>
</tr>
<tr>
<td>Colitis control</td>
<td>3.90 ± 0.10</td>
<td>0</td>
<td>172 ± 11.4</td>
</tr>
<tr>
<td>Colitis+ Sulfasalazine</td>
<td>2.25 ± 0.25 **</td>
<td>42.30</td>
<td>145 ± 9.5 **</td>
</tr>
<tr>
<td>Colitis + <em>D. hamiltonii</em></td>
<td>1.69 ± 0.21 **</td>
<td>56.66</td>
<td>123 ± 10.3 **</td>
</tr>
<tr>
<td>Colitis + <em>S. muricatum</em></td>
<td>1.63 ± 0.29 **</td>
<td>58.20</td>
<td>121 ± 8.5 **</td>
</tr>
</tbody>
</table>

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

Figure 8.2: Effect of *D.hamiltonii* and *S.muricatum* on colon lipid peroxidation (LPO) level during experimental colitis.
8.3.3 Colon GSH, SOD and GPx level

The decreased GSH level in the colitis control group (33.91 nmol/mg protein) was found to be significantly \( p<0.05 \) increased to 42.43 nmol/mg protein and 46.77 nmol/mg protein respectively after \( D. hamiltonii \) and \( S. muricatum \) treatment. The decreased SOD level (943.01 nmol/mg protein) in the colitis control group was significantly increased to 1222.45 nmol/mg protein and 1217.64 nmol/mg protein after \( D. hamiltonii \) and \( S. muricatum \) treatment. Similarly the decreased GPx level (27.08 µg of GSH utilized/min/mg protein) in the colitis control group was significantly increased to 37.20 and 41.60 µg of GSH utilized/min/mg protein after \( D. hamiltonii \) and \( S. muricatum \) treatment respectively (Table 8.2).

8.3.4 Colon NO, iNOS and COX-2 quantification

Treatment with \( D. hamiltonii \) and \( S. muricatum \) significantly inhibited acetic acid induced NO production (colon and serum) and iNOS level in colon (Figure 8.3 and 8.4). NO level was increased in the colon tissue (211.4 µM) whereas treatment with \( D. hamiltonii \) and \( S. muricatum \) significantly \( p<0.01 \) reduced the NO production to 182.40 µM and 179.16 µM respectively (Figure 8.3). The iNOS level showed significant inhibition after treatment with \( D. hamiltonii \) and \( S. muricatum \) (Figure 8.5). The colonic COX-2 level in colitis control group (74.6 ng/g tissue)
were significantly increased compared with normal animal (18.4 ng/g tissue). Treatment with sulfasalazine (43.6 ng/g tissue) *D.hamiltonii* (39.2 ng/g tissue) or *S.muricatum* (37.3 ng/g tissue) significantly (*p*<0.01) decreased the colon COX-2 level compared with colitis control group.

### 8.3.5 Colon TNF-α, MPO and LDH level

Effect of *S.muricatum* on colon TNF-α, MPO and LDH level is shown in Figure 8.7, 8.8 and 8.9 respectively. The increased TNF-α level in colitis control group (135.41 pg/mg tissue) was found to be significantly decreased by *D.hamiltonii* and *S.muricatum* treatment (51.30 pg/mg tissue and 46.28 pg/mg tissue respectively). Similarly the MPO level (30.0 ng/g tissue and 26.8 ng/g tissue) and LDH (812.10 U/L and 779.31 U/L) in colitis control animals was also found to be decreased after *D.hamiltonii* and *S.muricatum* treatment. The colitis control group of MPO and LDH was found to be 92.18 ng/g tissue and 2046.67 U/L respectively.

### 8.3.6 Translocation of Transcription Factor NF-κB (p65/p50)

The DNA-bound transcription factor NF-κB (p65/p50) was detected using the primary antibody. The effect of *D.hamiltonii* and *S.muricatum* on the activation and nuclear translocation of transcription factor during ulcerative colitis is shown in
Table 8.3. *D.hamiltonii* and *S.muricatum* treatment could inhibit the transcription factor NF-κB subunits, p65 (75.52% for *D.hamiltonii* and 74.30% for *S.muricatum*), p50 (61.80% for *D.hamiltonii* and 60.75% for *S.muricatum*), whereas sulfasalazine exposure inhibited p65 and p50 subunits to 71.52% and 58.19% respectively (Table 8.3).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH (nmol/mg protein)</th>
<th>SOD (n mol/mg protein)</th>
<th>GPx (µg of GSH utilized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>60.53 ± 7.60</td>
<td>1239.20 ± 155.90</td>
<td>59.24 ± 3.80</td>
</tr>
<tr>
<td>Colitis alone</td>
<td>33.91 ± 6.10</td>
<td>943.01 ± 120.20</td>
<td>27.08 ± 1.80</td>
</tr>
<tr>
<td>Colitis+ Sulfasalazine</td>
<td>56.10 ± 8.20 **</td>
<td>1184.00 ± 76.10 *</td>
<td>41.40 ± 0.90 **</td>
</tr>
<tr>
<td>Colitis+ D. hamiltonii</td>
<td>42.43 ± 3.50</td>
<td>1222.45 ± 134.10 **</td>
<td>37.20 ± 1.6 **</td>
</tr>
<tr>
<td>Colitis + S. muricatum</td>
<td>46.77 ± 6.1 **</td>
<td>1217.64 ± 197.61 **</td>
<td>41.60 ± 2.8 **</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *p<0.05, **p<0.01 compared with colitis alone group

**Table 8.2: Effect of D. hamiltonii and S. muricatum on colon GSH, SOD and GPx level during experimental colitis.**
Values are expressed as mean ± S.D. **$p<0.01$ compared with colitis control group.

**Figure 8.3:** Effect of *D. hamiltonii* and *S. muricatum* on the tissue NO level during experimental colitis.
Values are expressed as mean ± S.D. *p<0.05, **p<0.01 compared with colitis control group.

Figure 8.4: Effect of *D.hamiltonii* and *S.muricatum* on the Serum NO level during experimental colitis.
Values are expressed as mean ± S.D. **p<0.01 compared with colitis control group.

**Figure 8.5: Effect of *D. hamiltonii* and *S. muricatum* on the iNOS level during experimental colitis.**
Values are expressed as mean ± S.D. **p<0.01 compared with colitis control group.

Figure 8.6: Effect of *D.hamiltonii* and *S.muricatum* on the COX-2 level during experimental colitis.
Values are expressed as mean ± S.D. **p<0.01 compared with colitis control group.

**Figure 8.7:** Effect of *D. hamiltonii* and *S. muricatum* on the TNF-α during experimental colitis.
Values are expressed as mean ± S.D. **p<0.01 compared with colitis control group.

Figure 8.8: Effect of *D.hamiltonii* and *S.muricatum* on the MPO during experimental colitis.
Values are expressed as mean ± S.D. **p<0.01 compared with colitis control group.

Figure 8.9: Effect of *D.hamiltonii* and *S.muricatum* on the LDH during experimental colitis.
Values are expressed as mean ± S.D. **p<0.01 compared with colitis alone group.

Table 8.3: Effect of *D. hamiltonii* and *S. muricatum* on the Translocation of Transcription Factors (NF-κB) during experimental colitis

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Colitis alone (OD)</th>
<th>Ulcerative colitis + Sulfasalazine (OD)</th>
<th>Ulcerative colitis + <em>D. hamiltonii</em> (OD)</th>
<th>Ulcerative colitis + <em>S. muricatum</em> (OD)</th>
<th>Percentage inhibition by Sulfasalazine Treatment</th>
<th>Percentage inhibition by <em>D. hamiltonii</em> Treatment</th>
<th>Percentage inhibition by <em>S. muricatum</em> Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB p65</td>
<td>0.576±0.023</td>
<td>0.164±0.010 **</td>
<td>0.141±0.011**</td>
<td>0.148±0.012**</td>
<td>71.52</td>
<td>75.52</td>
<td>74.30</td>
</tr>
<tr>
<td>NF-κB p50</td>
<td>0.665±0.025</td>
<td>0.278±0.029 **</td>
<td>0.254±0.020**</td>
<td>0.261±0.022**</td>
<td>58.19</td>
<td>61.80</td>
<td>60.75</td>
</tr>
</tbody>
</table>
8.3.7 Histopathological analysis

Effect of *D. hamiltonii* and *S. muricatum* on histopathological changes during colitis is shown in Figure 8.10. The normal mucosa (Figure 8.10a) showed no necrosis or inflammation. In contrast the colitis control animal (Figure 8.10b) showed severe destruction of the epithelium, transmutable necrosis in all layers of the bowel wall and the diffusion of inflammatory cells in the mucosa. The crypts were also found to be distorted in the control group tissue specimen. Intraperitoneal administration of *D. hamiltonii* (Figure 8.10d) or *S. muricatum* (Figure 8.10e) markedly attenuated the histological signs of cellular damage, reduction in mucosal injury, edema and reduced the infiltration of the necrotic cells in the lamina propriety. Similarly standard sulfasalazine (Figure 8.10c) also protected the host from colitis by showing minimal damage in the colonic mucosa.

8.3.8 Immunostaining of iNOS and COX-2

Expression of iNOS by immunostaining was primarily showed on neutrophils and smooth muscle cells with a distribution in the epithelial cells in negative control (Figure 8.11). iNOS was over expressed in acetic acid-induced colitis group and was localized in the inflammatory cells in comparison with the normal group. At the same time treatment with *D. hamiltonii, S. muricatum* and sulfasalazine reduced the over expression of iNOS induced by acetic acid treatment.
The expression of COX-2 was found to be high in cells of surface epithelium and cells of the inflammatory infiltrate in the acetic acid-induced ulcerative colitis group. Animals treated with *D. hamiltonii*, *S. muricatum* and sulfasalazine displayed lowered level of COX-2 expression in colitis control group (Figure 8.12).
A) Normal colon. B) Control specimen from acetic acid induced host showing colitis with large necrotic destruction of epithelial cells, areas of hemorrhage, submucosal edema and inflammatory cellular infiltration. C) Colitis + sulfasalazine (100 mg/kg B.wt.). D) Colitis + *D.hamiltonii* (20 mg/kg B.wt.) E) Colitis + *S.muricatum* (10 mg/kg B.wt.) (40× magnification).

**Figure 8.10:** Effect of *D.hamiltonii* and *S.muricatum* on colon histopathology analysis.
Immunohistochemical localization of iNOS A) Normal control B) Ulcerative colitis alone C) Colitis + sulfasalazine (100 mg/kg B.wt.). D) Colitis + *D.hamiltonii* (20 mg/kg B.wt.) E) Colitis + *S.muricatum* (10 mg/kg B.wt.).

Figure 8.11: Effect of *D.hamiltonii* and *S.muricatum* on expression of iNOS in colon tissue
Immunohistochemical localization of COX-2 in normal control B) Ulcerative colitis alone C) Colitis + sulfasalazine (100 mg/kg B.wt.). D) Colitis + *D.hamiltonii* (20 mg/kg B.wt.) E) Colitis + *S.muricatum* (10 mg/kg B.wt.).

**Figure 8.12: Effect of *D.hamiltonii* and *S.muricatum* on expression of COX-2 in colon tissue**
8.4 Discussion

Ulcerative colitis is an inflammatory bowel disease (IBD) causing severe damage to the intestinal tract and can lead to colon cancer. There is support for an intense local immune response and has been associated with lymphocytes infiltration and macrophage flow into the mucosa followed by release of cytokines (Sunkara et al., 2011). Although many anti-inflammatory therapeutic agents are available today that has efficient protective roles but exhibit many adverse effects. Therefore an alternative therapeutic approach with higher therapeutic efficacy is essential along with less or no side effects. Natural drugs have been reported to fewer side effects compared with synthetic drugs. The present study shows the protective effect of *D. hamiltonii* and *S. muricatum* against acetic acid induced experimental ulcerative colitis.

Free radicals serve as an important cause for the damage of tissues. In IBD, oxidative stress plays on critical role in infection, initiation and progression (Kruidenier and Verspaget, 2002). In the present study the *D. hamiltonii* and *S. muricatum* treated group reduced the colon wet weight and macroscopical scoring compared to ulcerative colitis control group. Histopathology examination also supported that *D. hamiltonii* and *S. muricatum* treatment could reduce the colitis in experimental animals.
In this study, acetic acid induced ROS formation was evidenced by elevated LPO level associated with decreased SOD, GSH and GPx activity. GSH, GPx is involved in the repair mechanism and inhibits free radical damage. Therefore, GSH play an important role in protecting the intestinal cells and as a defense mechanism against tissue inflammation (Boyd et al., 1979). Treatment with *D. hamiltonii* and *S. muricatum* significantly increased the GSH levels and preventing the manifestation of mucosal injury during ulcerative colitis. SOD plays an important role in catalyzing hydrogen peroxide and Oxygen (Hagar et al., 2007). The H$_2$O$_2$ is converted to water in the presence of Catalase or GPx. These enzyme activity is usually balanced which maintains a steady ROS state. The SOD level was decreased during colitis. Administration of *D. hamiltonii* and *S. muricatum* significantly increased the level of colon GSH, SOD & GPx.

Nitric oxide (NO) plays an important role in the pathogenesis of colitis (Perner and Rask-Madsen, 1999; Dong et al., 2003). NO is produced as three isoforms: neuronal NOS (NOS1), inducible NOS (iNOS or NOS2) and endothelial NOs (NOS3) (Prabhu and Guruvayoorappan, 2010). The over production of iNOS and NO has a direct role in the regulation of disease-specific during colitis (Kolios et al., 2004). Administration of *D. hamiltonii* and *S. muricatum* significantly inhibited the iNOS and NO.
COX has two different isoforms namely COX-1 and COX-2. COX-1 is involved in cellular and metabolic activity where as COX-2 is an inducible enzyme (Morita 2002). COX-2 activation produces the extreme inflammatory mediator which may contribute to the development of colonic inflammation (Itzkowitz 2006). The present study showed that *D.hamiltonii* and *S.muricatum* could inhibit the expression of COX-2 thereby inhibiting inflammation in the colon.

Inflammatory bowel disease has been related with the release of soluble cytokines, inflammatory mediators and enrolment of lymphocytes (Ogata and Hibi, 2003). TNF-α is one of the most important pro-inflammatory cytokine. It is highly expressed in the gut during colitis condition (Nakamura et al., 2006). TNF-α can disrupt the epithelial barrier, and induce epithelial cell necrosis, edema, neutrophil infiltration and can activate the macrophages (Nilsen et al., 1998). Administration of *D.hamiltonii* and *S.muricatum* significantly inhibited the TNF-α production compared with control animals.

MPO is a heme enzyme and also a biochemical marker for neutrophil infiltration in injured tissue. (Kettle et al., 1997). This present investigation showed that oxidative cell damage is related with acetic acid induced ulcerative colitis resulted in an increased MPO activity. *D.hamiltonii* and *S.muricatum* treatment significantly reduced the heme enzyme activity compared with control animals thereby controlled the neutrophil infiltration. LDH is a cytosolic enzyme that
regulates the biochemical reaction in body fluids and tissue. LDH elevation is indicates the increase amount of lactic acid production (Thrippeswamy et al., 2011). The colon mucosa secrete the pro-inflammatory cytokines such as TNF- α, IL-2, and IL-6 (Reimund et al., 1996; Rogler and Andus, 1998). IL-6 causes the pro-inflammatory effect due to over production and activation of signal transducers. In the present study the *D.hamiltonii* and *S.muricatum* pre-treatment altered the colon LDH level induced by acetic acid towards the normal level. The NF-κB is a transcription factor composed of p50 and p65 subunits, which are major components of active NF-κB (Neurath et al., 1998). In summary, our data confirm that administration of *D.hamiltonii* or *S.muricatum* extract shows protective effect in acetic acid-induced ulcerative colitis in rats by modulating the oxidant/antioxidant balance in the colonic tissue, inhibiting the production of TNF-α, IL-1β and IL-6 (pro-inflammatory cytokines), iNOS, COX-2 (inflammatory mediators) and NF-κB signal transduction pathways.