4. MATERIALS AND METHODS

4.1 Identification and collection of plant material

Stem bark of *Holarrhena antidysenterica* and tubers of *Cyperus rotundus* were obtained from a commercial supplier in Ahmedabad. To prove that the plant material used for the study was authentic, it was studied for its characteristics features. The plant materials were identified and authenticated by Dr. Geetha, Sr. Scientist, Directorate of Medicinal and aromatic plants research, Boriavi, Anand, Gujarat and Dr. Satyabrata Maiti, Director, Directorate of Medicinal and aromatic plants research, Boriavi, Anand, Gujarat respectively. Voucher specimens (No. APC/2011-2012/SAJ/CR/01 and APC/2011-2012/SAJ/HA/02) of the plant materials were deposited in the herbarium of Department of Pharmacognosy, Anand Pharmacy College, Anand.

4.2 Phytochemical tests and preliminary pharmacological activities of hydromethanolic whole extracts of *Holarrhena antidysenterica* and *Cyperus rotundus*

4.2.1 Drugs and Chemicals:

2,4-Dinitro benzene sulfonic acid (DNBS) was purchased from Cyno Chem Company, Ahmedabad. 5-amino salicylic acid was purchased from Sigma. All chemicals and solvents used in the study were of analytical grade.

4.2.2 Preparation of hydromethanolic whole extracts of *Holarrhena antidysenterica* and *Cyperus rotundus* and Phytochemical tests:

The stem bark of *Holarrhena antidysenterica* and tubers of *Cyperus rotundus* were ground to a coarse powder separately and stored at room temperature. For each extraction procedure, 250gm of powdered plant material was extracted with 1000ml of Methanol (70%) in Soxhlet Apparatus for 5-7 hours at 65°C. Then the organic extracts were concentrated by evaporation below 45°C and then further dried at ambient temperature for 24 hr to obtain dry extract. All dried extracts were stored at −20 °C. These extracts were used for studying the biological activity in DNBS induced inflammatory bowel disease model. hydromethanolic whole extract of
Holarrhena antidysenterica was labeled as WMEHA and hydromethanolic whole extract of Cyperus rotundus was labeled as WMECR.

Both extracts were subjected to qualitative chemical tests to find out the presence or absence of phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins and flavonoids. (Mukherjee, 2002; Khandelwal, 2006; Kokate, 2009)

4.2.3 Animals:

Healthy Male Sprague Dawley (SD) rats (250-300gm, 10-11 weeks age) were housed in cages with free access to standard rat chow (diet) and water ad libitum and acclimatized to the surroundings for one week prior to the experiment. Animals were maintained on a light/dark cycle (12/12hr) at a constant temperature (22º±1ºC) and humidity (55±1). The experimental protocol (Protocol No. 9012 dated 26th Dec 2009) was approved by Institutional Animal Ethical Committee of Anand Pharmacy College as per the guidance of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

4.2.4 Grouping and drug administration:

Holarrhena antidysenterica is reported in literature for its antidiarrhoeal and anti amoebic activity (Dhar et al, 1968; Premnath Shenoy et al, 2008; Bhutani, 1988). Studies in Cyperus rotundus have reported for its anticonvulsant activity (Porwal et al, 2011), antidysmenorrhoea effect (Yuan Hao et al, 2006) along with antispasmodic effect (Shamkuwar et al, 2012) and its use in abdominal problems. Based on preclinical and clinical studies, doses were calculated and three doses (200mg/kg, 400mg/kg and 600mg/kg) of hydromethanolic extract of Holarrhena antidysenterica and three doses (300mg/kg, 500mg/kg and 800mg/kg) of hydromethanolic extract of Cyperus rotundus were selected.

Treatment period was of 18 days. Rats were randomly allocated to 10 groups containing three animals each. Animals in all groups were fasted for 24 hrs prior to study, given access to water ad libitum. Group I served as the normal control group. Group II served as vehicle control group which received 50% ethanol intracolonically on 11th day of the study. Group III served as Model control. Animals of Group IV to
X were given the Standard drug (5-ASA 100mg/kg), WMEHA (600 mg/kg), WMEHA (400 mg/kg), WMEHA (200 mg/kg), WMECR (800 mg/kg), WMECR (500 mg/kg) and WMECR (300 mg/kg) respectively for 18 days once a day orally. On the 11th day of the study, colitis was induced with DNBS in animals of Group III-X.

4.2.5 Induction of Colitis by DNBS in Rats:
Rats were kept for overnight fasting. A flexible plastic catheter with an outer diameter of 2mm was inserted intracolonically into the colon (via anus) with the aim to place the catheter tip 8cm proximal to the anus (i.e. until approximately reaches the splenic flexure). Colitis was induced by intracolonic instillation of DNBS (120mg/kg prepared in 50% ethanol) (Cuzzocrea et al, 2004; Wallace et al, 1995). Animal were kept for 5 minutes in a trendelenburg position to avoid reflex. The rats were inspected for the presence of diarrhea.

4.2.6 Parameters Observed:
Stool consistency was measured for each group daily and scored from 0 to 2 (Normal stool-0, Soft stool-1, and Liquid stool-2), the average score was calculated from above data (Michele et al, 2007). On 18th day, the animals were weighed and anaesthetized with chloroform and the abdomen was opened by a midline incision. The colon was removed, made free from surrounding tissues, rinsed and length and weight of it was measured. Colon was opened along the anti mesenteric border, fixed on a wax block and scored macroscopically for Colon Mucosa Damage Index (CMDI) (Wei-Guo Dong et al, 2003). The scoring formula for assessment of CMDI is briefly described below.

The scores were calculated for all specimens from each group. These were then added to obtain the total score, which was then divided by the number of rat in each group to obtain the average score of induced colitis for the group.
Table 4.1: Colon Mucosa Damage Index

<table>
<thead>
<tr>
<th>Score</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Mild hyperemia, no erosion or ulcer on the mucosal surface</td>
</tr>
<tr>
<td>2</td>
<td>Moderate hyperemia, erosion appearing on the mucosal surface</td>
</tr>
<tr>
<td>3</td>
<td>Severe hyperemia, necrosis and ulcer on the mucosal surface with the</td>
</tr>
<tr>
<td></td>
<td>major ulcerative area extending &lt; 40%</td>
</tr>
<tr>
<td>4</td>
<td>Severe hyperemia, necrosis and ulcer on the mucosal surface with the</td>
</tr>
<tr>
<td></td>
<td>major ulcerative area extending &gt; 40%.</td>
</tr>
</tbody>
</table>

4.3 Phytochemical tests and pharmacological activities of extracts of *Holarrhena antidysenterica* and *Cyperus rotundus* obtained successively using various solvents

4.3.1. Drugs and Chemicals:

Drugs and chemicals were same as 4.2.1

4.3.2 Successive Solvent Extraction:

The air dried powdered stem bark of *Holarrhena antidysenterica* and tubers of *Cyperus rotundus* powder were successively extracted with the solvents of increasing polarity in a soxhlet apparatus i.e. petroleum ether, hexane, chloroform, acetone and methanol-water (70%) mixture for 48 h respectively, filtered and filtrate was evaporated under reduced pressure to obtain dry extract (Trease and Evans, 2002; Mukherjee, 2002; Kokate, 2009). Fresh powder was used for each extraction. The extracts were stored in cool and dry place. The petroleum ether extract, hexane, chloroform, acetone and hydromethanolic extracts of *Holarrhena antidysenterica* were labeled as PEHA, HEHA, CHHA, ACHA and MEHA respectively. The petroleum ether extract, hexane, chloroform, acetone and hydromethanolic extracts of *Cyperus rotundus* were labeled as PECR, HECR, CHCR, ACCR and MECR respectively.
4.3.3 Phytochemical Tests:

Each extract was subjected to qualitative chemical tests to find out the presence or absence of phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins and flavonoids (Mukherjee, 2002; Khandelwal, 2006; Kokate, 2009).

4.3.4 Grouping and drug administration:

Animals were maintained as mentioned in 4.2.3. Treatment period was of 18 days. Rats were randomly allocated to 12 groups containing three animals each. Since it was again preliminary studies to explore the efficacy of various extracts, three animals were taken in each group. Animals in all groups were fasted for 24 hrs prior to study, given access to water ad libitum. Group I served as the normal control group throughout 18 days study period. Group II served as Model control. Animals of Group III to XII were given PEHA (600 mg/kg), HEHA (600 mg/kg), CHHA (600 mg/kg), ACHA (600 mg/kg), MEHA (600 mg/kg), PECR (800 mg/kg), HECR (800 mg/kg), CHCR (800 mg/kg), ACCR (800 mg/kg) and MECR (800 mg/kg) respectively for 18 days once a day orally. On the 11th day of the study, colitis was induced with DNBS in animals of Group II-XII. High dose of both the plants showing efficacy was administered in this study. Colitis was induced as mentioned in 4.2.5. Parameters observed were same as 4.2.6.

4.4 Subacute Toxicity Studies of MEHA and CHCR

4.4.1 Drugs and Chemicals:

All chemicals and solvents used in the study were of analytical grade.

4.4.2 Preparation of Extracts:

 Preliminary studies were done with whole hydromethanolic extracts which did not show any toxicity. Before conducting main efficacy studies, we obtained the extracts successively using various solvents and checked the efficacy of each extract in experimental IBD. The constituents present in the successively obtained extracts were less compared to earlier used whole hydromethanolic extracts. Hence less toxicity was expected in main studies. Based on preliminary investigations with hydromethanolic whole extracts and extracts using various solvents of both plants, the most efficacious
extracts were chosen for the toxicity studies. Dried methanolic and chloroform extracts of *Holarrhena antidysenterica* (MEHA) and *Cyperus rotundus* (CHCR) respectively were used. The extracts were prepared by same method as mentioned in 4.3.2 and stored in cool dry place.

4.4.3 Animals:

Healthy male and female Sprague Dawley rats (250-350gm, 11-13 weeks age) were housed in cages with free access to standard rat chow (diet) and water *ad libitum* and acclimatized to the surroundings for one week prior to the experiment.

4.4.4 Grouping and drug administration:

Subacute oral toxicity studies were conducted by following OECD guidelines- 407 (OECD guidelines, 2001) to evaluate the safety of herbal extracts. The rats were divided into 7 groups of 10 rats (5 males and 5 females) where group I served as control group and received distilled water only. Groups II to VII received MEHA (Low dose-150 mg/kg), MEHA (Intermediate dose-450 mg/kg), MEHA (High dose-1350 mg/kg), CHCR (Low dose-200 mg/kg), CHCR (Intermediate dose-600 mg/kg) and CHCR (High dose-1800 mg/kg) respectively daily for 28 days. The intermediate dose of most efficacious extract of both the plants were chosen and 1/3rd dose of same was given as low dose and 3 times its dose was given as high dose. It was calculated and given in divided doses in a day. Maximum oral volume fed was not >10 ml/kg.

4.4.5 Methodology and Parameters observed:

4.4.5.1 Body weight, Food and water intake:

During study period body weight was also recorded at every week. Total food intake and water intake of each group was measured daily.

4.4.5.2 Mortality, clinical signs and assessment of motor and sensory activity:

In subacute toxicity studies, animals were observed with intervals for the first 4 h, afterwards every 6 h for the next 24 h. Second day onwards animals were observed once a daily for any changes in general behavior and physiological activities up to 28 days. The visual observations included mobility, aggressiveness, sensitivity to sound
and pain, as well as respiratory movements. During the four-week dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 4 h after dosing. The number of survivors was noted. Changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, and unusual respiratory pattern) were observed. Also changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self mutilation, walking backwards) were recorded (IPCS, Environmental Health Criteria, 1986). In the fourth exposure week sensory reactivity to stimuli of different types (IPCS, Environmental Health Criteria, 1986) (e.g. auditory, visual and proprioceptive stimuli) (Tupper and Wallace, 1980; Gad, 1982; Moser et al, 1991), assessment of grip strength (Meyer et al, 1979) and motor activity assessment (Crofton et al, 1991) were also conducted. Attention was also directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

### 4.4.5.3 Hematological and Biochemical parameters:

On 28th day after completion of treatment, rats were kept for overnight fasting. Next day, blood was collected from retro-orbital plexus into heparinized and non-heparinized centrifuge tubes for the estimation of haematological (total red blood cells (RBC), hemoglobin (Hb) content, various types of white blood cells (WBC) and platelet count) and biochemical (glucose, serum creatinine, blood urea nitrogen (BUN), cholesterol, triglyceride (TG), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) parameters respectively. For Complete Blood Count, non-heparinized blood samples were run on fully automated hematology analyzer PCE 210. Serum glucose was estimated by GOD-POD method and all biochemical parameters were estimated using Biochemistry analyzer.

### 4.4.5.4 Gross pathology and Relative organ weight:

After collection of blood samples, the rats were sacrificed using overdose of anesthesia and their liver, heart, lung and kidney were excised, rinsed in ice-cold normal saline, were observed for any macroscopic changes and then weighed. The relative organ weight of each animal was then calculated as follows:
Materials and Methods

The external appearance of sacrificed animals and appearance of other visceral organs like stomach, uterus, testicles, spleen etc were carefully noted.

4.4.5.5 Microscopic examination:

From each group one specimen of each organ (liver, heart, lung and kidney) from each group was stored in the formalin (10%) and was used for histopathology study. Histopathological examination was done by H & E staining and mounted on DPX.

4.5 Main Efficacy Studies

4.5.1 Drugs and Chemicals:

Drugs and chemicals were same as 4.2.1

4.5.2 Preparation of extracts:

Based on preliminary investigations with hydromethanolic whole extracts and extracts using various solvents of both plants, the most efficacious extracts were chosen for the main studies. Dried methanolic and chloroform extracts of Holarrhena antidysenterica (MEHA) and Cyperus rotundus (CHCR) respectively were used. The extracts were prepared by same method as mentioned in 4.3.2 and stored in cool dry place.

4.5.3 Grouping and drug administration:

Animals were maintained as mentioned in 4.2.3. Treatment period was of 18 days. Rats were randomly allocated to 9 groups containing six animals each. Animals in all groups were fasted for 24 hrs prior to study, given access to water ad libitum. Group I served as the normal control group throughout 18 days study period. Group II served as vehicle (used to dissolve DNBS) control group which received 50% ethanol intracolonically on 11th day of the study. Group III served as Model control. Animals of Group IV to IX were given the Standard drug (5-ASA 100mg/kg), MEHA (450 mg/kg), MEHA (600 mg/kg), CHCR (600 mg/kg), CHCR (800 mg/kg) and CMC suspension respectively for 18 days once a day orally. The extracts were dissolved in

<table>
<thead>
<tr>
<th>Relative Organ Weight =</th>
<th>Absolute organ weight (g)</th>
<th>X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight of rat on sacrifice day (g)</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

0.5% CMC suspension. On the 11th day of the study, colitis was induced with DNBS in animals of Group III-IX. Colitis was induced as mentioned in 4.2.5.

4.5.4 Methodology and parameters observed:

During the study total food intake, water intake and body weight of each group was measured daily. Stool consistency was measured for each group daily and scored as described in 4.2.6.

Blood collection for serum cortisol:

On 18th day, Blood samples were taken between 8 and 9 am from retro-orbital plexus of rats and rapidly stored at -80°C then serum was separated by using centrifuge at 15000 rpm, 4°C for 20minutes. The animals were weighed and sacrificed. Colon length and weight was measured. Colon was opened and scored for macroscopic parameter CMDI. The scoring formula for assessment of CMDI is described in 4.2.6. After scoring, colon homogenate was prepared. From each group one colon of randomly selected animal was stored in the formalin (10%) and was used for histopathology scores.

4.5.4.1 Preparation of tissue homogenate (Cuzzocrea et al, 2003):

The colon was dissected out and homogenized (50gm/L) in 50mmol/L ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% of hexa decyl trimethyl ammonium bromide. The homogenate was freezeed and thawed thrice, then centrifuged at 4000 rpm for 20 min at 4°C for the measurement of myeloperoxidase activity. For the determination of malondialdehyde, superoxide dismutase and nitric oxide activity, colon (50gm/L) was homogenized in ice-cold potassium phosphate buffer (pH 7.4) and centrifuge at 3000 rpm for 10 min at 4°C. The resulting supernatant was used for analysis of various biochemical parameters.

Estimation of Biochemical parameters:

The parameters measured using tissue homogenate were Malondialdehyde (MDA) level (Wei-Guo Dong et al, 2003; Cuzzocrea et al, 2003), Nitric oxide (NO) level (Wei-Guo Dong et al, 2003; Cuzzocrea et al, 2003), Myeloperoxidase (MPO) activity
(Wei-Guo Dong et al., 2003; Cuzzocrea et al., 2003), Superoxide dismutase (SOD) activity (Misra, 1972) and using serum was Cortisol level (Rainer et al., 1998).

**A) Malondialdehyde level** (Cuzzocrea et al., 2003; Wei-Guo et al., 2003)

**Procedure:** 1.0 ml of homogenate sample was mixed with 0.2 ml 4% w/v sodium dodecyl sulfate, 1.5 ml 20% acetic acid in 0.27M hydrochloric acid (pH 3.5) and 1.5 ml of 0.8% Thiobarbituric acid (TBA) in test tube. The mixture was heated in water bath at 85°C for 1hr. The intensity of developed pink color was read against a reagent blank at 532 nm. Malondialdehyde was calculated using molar extinction coefficient 1.56x10^5 M^-1 cm^-1 and was reported as nmol/gm.

**B) Nitric oxide level:** (Cuzzocrea et al., 2003; Wei-Guo et al., 2003)

**Composition of solution:**
- NED solution: 0.1% N-1napthylethylenediaminedihydrochloride in water
- Sulfanilamide solution: 1% sulfanilamide in 5% phosphoric acid
- Nitrite standard: 0.001M sodium nitrite in water

**Procedure:** The sulfanilamide solution and NED solution were allowed to equilibrate at room temperature (15-30 min). 1ml of homogenate sample was added in a test tube. Then 1ml of the Sulfanilamide Solution was added into that test tube and incubated for 5-10 minutes at room temperature & protected from light. Then 1ml of NED Solution was added into the test tube. Then again the same was incubated for 5-10 minutes at room temperature, protected from light. A purple/magenta color was developed and absorbance readings were taken within 30 min at 540nm. The concentration was calculated from the standard curve and reported as nmol/gm.

**C) Myeloperoxidase activity** (Cuzzocrea et al., 2003; Wei-Guo et al., 2003)

**Procedure:** MPO, a marker of neutrophil migration was estimated by measuring H_2O_2 dependent oxidation of O-dianisidine. 0.1ml homogenate sample, 2.9ml 0.1M potassium phosphate buffer (pH 6.2), 0.15ml 2mM hydrogen peroxide (H_2O_2) and 0.2ml 0.53M O-dianisidine were mixed and incubated at 37°C for 15 min. in test tube. The reaction was stopped by adding 0.2ml of 0.153 M sodium azide. Absorbance of the orange-brown color complex was measured at 460 nm for 5 min.
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Myeloperoxidase activity was expressed as the amount of enzyme necessary to produce a change in absorbance per minute per mg of protein (U/gm). MPO activity was calculated by molar extinction coefficient for O-dianisidine (11300 M⁻¹ cm⁻¹).

D) Superoxide dismutase activity (Misra et al, 1972)

Procedure: 0.1ml of homogenate sample was mixed with 0.1ml EDTA (1x10⁻⁴M), 0.5ml of carbonate buffer (pH 9.7) and 1.0ml of epinephrine (3x10⁻³M) in test tube. The optical density of formed adrenochrome was noted at 480nm for 3 min at interval of 30 sec and results were expressed as U/gm of tissue.

E) Serum Cortisol level (Rainer et al, 1998)

Quantitative value of Cortisol was measured by Fluorescence Polarization Immuno Assay at Dr. Sanjay Laboratory, Anand, Gujarat.

4.5.4.2 Histopathology of Colon (Uritski, 2004; Wei-Guо et al, 2003):

The scoring formula for assessment of Disease Activity Index was according to below.

<table>
<thead>
<tr>
<th>Score</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Intact crypt</td>
</tr>
<tr>
<td>1</td>
<td>Loss of 1/3 of the crypt</td>
</tr>
<tr>
<td>2</td>
<td>Loss of 2/3 of the crypt</td>
</tr>
<tr>
<td>3</td>
<td>Loss of entire crypt with the surface epithelium remains intact</td>
</tr>
<tr>
<td>4</td>
<td>Loss of entire crypt and surface epithelium both.</td>
</tr>
</tbody>
</table>

Colon tissues were fixed in 10% formalin for 24 hrs and washed in running water for 24 hrs. They were processed with an automate tissue processor (Shandon Citadel Model 2000). Samples were dehydrated with alcohol in an Autotechnician. & then cleared in benzene to remove absolute alcohol embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a
cubical block of paraffin made by the “C” moulds following dehydration & embedding section were cut at 3µm with a rotary microtome, stained with hematoxylin & eosin and examined microscopically, by pathologist Dr. Brahmbhatt (M.D.Pathologist). The histologic scoring of induced colitis was determined by examining each specimen for the following features and allocating increasing points according to the severity of the findings:

A) Degree of Necrosis:

<table>
<thead>
<tr>
<th>Score</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Necrosis in mucosal only</td>
</tr>
<tr>
<td>2</td>
<td>Necrosis in mucosal and submucosal</td>
</tr>
<tr>
<td>3</td>
<td>Necrosis in mucosal, submucosal, and muscularis propria</td>
</tr>
<tr>
<td>4</td>
<td>full thickness tissue Necrosis</td>
</tr>
</tbody>
</table>

**Table 4.3: Degree of Necrosis**

B) Degree of Inflammation:

<table>
<thead>
<tr>
<th>Score</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Inflammation in mucosa only</td>
</tr>
<tr>
<td>2</td>
<td>Inflammation in mucosal and submucosal</td>
</tr>
<tr>
<td>3</td>
<td>Inflammation in mucosal, submucosal, and muscularis propria</td>
</tr>
<tr>
<td>4</td>
<td>full thickness tissue Inflammation</td>
</tr>
</tbody>
</table>

**Table 4.4 Degree of Inflammation**
C) Degree of Fibrosis:

<table>
<thead>
<tr>
<th>Score</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Fibrosis in mucosa only</td>
</tr>
<tr>
<td>2</td>
<td>Fibrosis in mucosal and submucosal</td>
</tr>
<tr>
<td>3</td>
<td>Fibrosis in mucosal, submucosal, and muscularis propria</td>
</tr>
<tr>
<td>4</td>
<td>full thickness tissue Fibrosis</td>
</tr>
</tbody>
</table>

Table 4.5: Degree of Fibrosis

4.6 Analysis of Gene Expression by quantitative Real Time-PCR (qPCR)

4.6.1 Drugs and Chemicals:

Drugs and chemicals were same as 4.2.1

4.6.2 Preparation of Extracts:

Dried methanolic and chloroform extracts of *Holarrhena antidysenterica* (MEHA) and *Cyperus rotundus* (CHCR) respectively were used. The extracts were prepared by same method as mentioned in 4.3.2 and stored in cool dry place.

4.6.3 Grouping and drug administration:

Animals were maintained as mentioned in 4.2.3. Treatment period was of 18 days. Male SD rats (250-300gm, 12-14 weeks age) were randomly allocated to 6 groups containing three animals each. Animals in all groups were fasted for 24 hrs prior to study, given access to water *ad libitum*. Group I served as the normal control group throughout 18 days study period. Group II served as vehicle control group which received 50% ethanol intracolonically on 11th day of the study. Group III served as Model control. Animals of Group IV to VI were given the Standard drug (5-ASA 100mg/kg), MEHA (600 mg/kg) and CHCR (800 mg/kg) respectively for 18 days
once a day orally. On the 11\textsuperscript{th} day of the study, colitis was induced with DNBS in animals of Group III-VI. Colitis was induced as mentioned in 4.2.5.

4.6.4 Methodology and Parameters observed:

4.6.4.1 Collection of Tissue:

4.6.4.1.1 Requirements:

Phosphate Buffer Solution (PBS) (pH 7), RNA later, 2 ml screw cap vial (sterile-preferably with chloroform treatment), Whatman filter paper and blotting paper, Gloves, forceps, scissors (sterile), Liquid Nitrogen (LN2), Tip box with tips of 1 ml, Plastic bags for autoclaving. All the chemicals and instruments used were autoclaved. RNA later was purchased from Ambion.

4.6.4.1.2 Method:

At the end of treatment, fasted rats were euthanized with ether and sacrificed. Colon tissues were removed aseptically, washed directly with PBS (pH 7) and immediately cut in small pieces. The samples were placed in 2 ml. screw cap vials containing 1 ml. RNA later solution and immediately transferred in liquid nitrogen container and till further estimations.

4.6.4.2 RNA Extraction:

4.6.4.2.1 Requirements:

TRI reagent, LN2, Mortar pestle, Chloroform, Isopropanol, 75\% ethanol, DEPC water, Microcentrifuge tubes of 2 and 1.55 microlitre, Micropipettes and tips. TRI reagent was bought from Sigma.

4.6.4.2.2 Method:

Total RNA was extracted from tissue samples following TRI reagent based protocol (Chomczynski, 1987). Mortar pestle were autoclaved in oven at 240 °C overnight. Frozen tissue sample weighing 100 gm was taken in pre-chilled mortar. It was then powdered by grinding in mortar with the help of pestle and occasionally adding liquid nitrogen in the mortar to prevent thawing. Pulverizing the tissue into a powder, while
keeping the tissue completely frozen, was the key to isolate intact total RNA. Once the tissue was ground to a fine powder, 1 ml of TRI-reagent per 100 mg of tissue was added (taking care that the tissue volume should not exceed 10% of the TRI-reagent volume) and the semi frozen mixture was stirred. The mixture was thawed and transferred in 2 ml round bottom microcentrifuge tube. The homogenized sample was then incubated at room temperature for 10 minutes and then centrifuged at 13,000 rpm for 10 minutes at 4 °C in Refrigerated centrifuge (Eppendorf – 5804R). This settles the tissue debris. 200 microlitres chloroform was added per 700 microlitre of TRI-reagent used and mixed vigorously for 15 seconds. Sample was centrifuged at 13000 rpm for 15 minutes at 4 °C. Upper aqueous phase was transferred to a fresh 1.5 ml microcentrifuge tube and 0.5 ml of isopropanol was added per 700 microlitre of TRI-reagent used and mixed by inverting tubes. The mixture was kept in refrigerator at -10 °C for 20 minutes to 45 minutes and centrifuged at 13000 rpm for 15 minutes at 4 °C. The RNA formed a pellet at the bottom of the tube. Supernatant was removed carefully and pellet was washed twice with 75% ethanol, adding atleast 1ml/ml of TRI-reagent used, followed by vortexing and centrifugation at 11000 rpm for 15 min. at 4 °C. Supernatant was removed and RNA pellet was air dried. It was then re-suspended in 30 microlitre DEPC treated Milli-Q water and slightly tapped for few times to dissolve the RNA pellet completely.

**4.6.4.3 Assessment of quantity and quality of isolated RNA:**

Total 18 RNA samples were extracted from dissected colon tissues of 3 animals of each group of six groups and checked for quality and quantity. The U.V. absorbance was checked at 260 and 280 nm for determination of sample concentration and purity. Purity of RNA was done on the basis of Optical Density (O.D) ratio at 260: 280. The salt concentration as well as other impurities of RNA solution was done on the basis of 260: 230. The samples with ratio 1.8 to 2.0 were acceptable and processed for cDNA synthesis. RNA was quantified using ND-1000 spectrophotometer (Nano Drop Technologies, Inc. USA). After pooling all the samples of same group upto concentration of 1000 ng/ microlitre, they were mixed and then cDNA was prepared using reverse transcriptase PCR method.
4.6.4.4 **DNase treatment:**

To remove contaminating DNA, samples were treated with DNase 1 purchased from Fermentas. DNase treatment cycle was as follow:

Prepare the tubes containing following component (in µL):

1. RNA sample 25.0
2. DNase 1 5.0
3. Dnase 1 buffer ( 10 X) 15.0
4. DEPC treated 5.0
Total 50

Put it on the PCR machine (Minicycler) set the protocol: 37 °C for 60 min. After 60 min 1 microlitre of EDTA (25 mM) was added. Continue cycle on 65 °C for 10 min further.

4.6.4.5 **First strand cDNA synthesis:**

Template RNA solution was thawed on ice while the primer solutions , 5x Buffer, Revert aid RT enzyme purchased from Fermentas , dNTP Mix, and RNase free water thawed at room temperature. They were then stored on ice immediately. Each solution is mixed by vortexing and centrifuged briefly to collect residual liquid from the sides of the tubes. Protocol was as follows:

Add into sterile, nuclease free tube on ice in the indicated order:

Template 100 ng-5 µg
Primer ( Oligo-(dT)) 0.5 µg ( 100 pmol)
DEPC treated water upto 12.5 µL
Now add following component in the indicated order :

5x reaction buffer 4 µL
Ribolock RNase inhibitor 0.5 µL
dNTP Mix 2 µL
Revert Aid M-M microlitre V Reverse 1 µL transcriptase

Total Volume 20 µL

Mix gently and centrifuge briefly. Incubate 60 min at 42 ºC reverse transcriptase was carried out in Minicycler Thermal cycler (Bio rad). Terminate the reaction by heating at 70 ºC for 10 min.

4.6.4.6 Real Time PCR Assay:

After cDNA synthesis, amplification of cDNA was done using REAL TIME PCR to measure the fold of expression of specific genes in each group. Real Time PCR reactions were performed in triplicate with specific oligoprimery pairs using the Quantitect SYBR Green PCR kit purchased from Qiagen according to the manufacturer’s recommendations in optical 96 well plates. The primers were commercially synthesized by Ocimum Biosolutions, Hyderabad from a published reference. The specificity of the primers was checked by NCBI blast software (http://www.ncbi.nlm.nih.gov/BLAST/). The amplification was carried out in a final reaction volume of 50 microlitre containing 2X Quanti Tect SYBR Green PCR master mix, 5 pmol of each gene specific primer and 1 microlitre of cDNA template. The PCR amplification profile was as follows: 95 degree C for 5 min followed by 50 cycles of denaturation at 95 ºC / 30 s, gene specific annealing temperature / 30 s, and extension at 72 ºC/35 s and final extension of 72 ºC / 5 min. The data was collected at step 3 of stage 2. Annealing temperatures per primer set were determined empirically. A melting curve was generated after each run to verify the specificity of the primers, shown by the presence of the single peak and no primer-dimer artifacts. Real time analysis of PCR amplification was performed with an Applied Biosystems 7500 and data generated by Sequence Detection software (SDS V. 1.3.1). Fold of expression for all the samples were normalized with endogenous control gene glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) and calibrator used was Model control. Sequence detection software was used to calculate fold of expression.

$C_T$ cycle means: Cycle when amplification starts
$dC_T= C_T$ cycle of test sample-$C_T$ cycle of endogenous control or $C_T$ target-$C_T$ endo
ddC_{T} = \text{difference between Avg. } dC_{T} \text{ value of a target sample and the average } dC_{T} \text{ value for the corresponding calibrator sample or } ddC_{T} = \text{Avg. } dC_{T} \text{ (test or target) - avg. } dC_{T} \text{ (Calibrator sample)}

This value is used to calculate expression fold value. Expression fold value = 2^{-ddC_{T}}

Dissociation curve was generated after each run to verify the specificity of the primers, shown by the presence of a single peak and no primer-dimer artifacts, thus ensured the quality of amplified product. So each gene study records amplification cycle graphs used for ddC_{T} study and dissociation curve to confirm primers specificity. These experiments were repeated several times for optimization of REAL TIME PCR conditions for best amplification results.

Table 4.6: Primer Sequences of selected Genes

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4F</td>
<td>CCCTGTTCCTGCTTTTCATA</td>
</tr>
<tr>
<td>IL-4R</td>
<td>CCGAGAACCCCAAGACTTGGT</td>
</tr>
<tr>
<td>IL-6F</td>
<td>GCCAGAGTCATTCAAGCAATCTG</td>
</tr>
<tr>
<td>IL-6R</td>
<td>TTGGGATATCAGGTTCGATGG</td>
</tr>
<tr>
<td>IL-12F</td>
<td>GGGTCCGGTTTGATGATGTCCTG</td>
</tr>
<tr>
<td>IL-12R</td>
<td>GGAGAAACGGTGACCCCTACCT</td>
</tr>
<tr>
<td>IFN- gamma F</td>
<td>TATGGAAGGAAGACAGCCTCC</td>
</tr>
<tr>
<td>IFN- gamma R</td>
<td>TCTGTGGGTTGTCACCTCG</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>CCGAGTCAACGGATTTGGTCGTAT</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>AGCCTTCTCCATGGTGGAAGAC</td>
</tr>
</tbody>
</table>
Table 4.7: Annealing Temperature per Primer Set

<table>
<thead>
<tr>
<th>Name Of Primer</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>58 ºC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>53 ºC</td>
</tr>
<tr>
<td>IL-6</td>
<td>52 ºC</td>
</tr>
<tr>
<td>IL-12</td>
<td>54 ºC</td>
</tr>
<tr>
<td>IFN gamma</td>
<td>53 ºC</td>
</tr>
</tbody>
</table>

4.7 Clinical Studies

4.7.1 Preparation of Monoherbal formulation using MEHA Extract:

Tablet was prepared in WHO certified cGMP manufacturing unit Pharmanza Herbals Pvt. Ltd. Each tablet weighing 750 mg was prepared using necessary excipients in the following composition (per 900 gm approx.):

Table 4.8: Composition of Tablet

<table>
<thead>
<tr>
<th>No.</th>
<th>Ingredients</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Holarrhena antidysenterica</em> extract</td>
<td>600 gm</td>
</tr>
<tr>
<td>2</td>
<td>Base granules (starch, Microcrystalline cellulose powder, Dicalcium phospahte)</td>
<td>270 gm</td>
</tr>
<tr>
<td>3</td>
<td>Sodium starch glycolate</td>
<td>9 gm</td>
</tr>
<tr>
<td>4</td>
<td>Magnesium Stearate</td>
<td>9 gm</td>
</tr>
<tr>
<td>5</td>
<td>Talcum</td>
<td>6 gm</td>
</tr>
<tr>
<td>6</td>
<td>Croscarmellose Sodium</td>
<td>10 gm</td>
</tr>
</tbody>
</table>

Disintegration time of the tablet was 20 minutes. It was tested using Veego Digital tablet disintegration test apparatus.
4.7.2 Subject Recruitment Procedure:

Clinical study was done to compare the efficacy of monoherbal formulation containing *Holarrhena antidysenterica* with Modern (Allopathic) treatment in patients with chronic ulcerative colitis. The study was randomized, parallel group and single blind design. Randomization was done to do unbiased study. Parallel group design was selected because since it is efficacy study, at the end of the treatment we cannot go for crossover study. The protocol was approved by Institutional Human Research Ethics Committee of Anand Pharmacy College on 23rd Jan 2013. Patients were enrolled from S.G.Patel Ayurveda Hospital and Maternity Home, G. J. Patel Ayurveda college and Research Centre, New V.V.nagar, Anand, Gujarat and Jivandeep Hospital, Station Road, Anand.

4.7.3 Inclusion Criteria:

1) Patients of either sex and above 18 years of age.
2) Ability to understand and the willingness to sign a written informed consent document at the screening visit before any protocol-specific procedure are performed.
3) Patients meeting the criteria for ulcerative colitis confirmed by clinical diagnosis, stool test, blood tests along with established colonoscopic evidences of ulcerative colitis. Clinical diagnosis was done on basis of signs and symptoms, patient history and scoring pattern (*Annexure I*). Stool test was done for presence of occult blood and infections if any. Blood tests were done for routine CBC, Erythrocyte sedimentation rate, Hemoglobin and blood group (In case of severe bleeding, blood transfusion may be required).

4.7.4 Exclusion criteria:

1. Severe CVS disease
2. Renal or hepatic disease, gall stones, pancreatitis, diabetes mellitus, sepsis, infection, pneumonia
3. Pregnant or nursing women
4. Patients who had undergone surgeries
5. Patients who had complications like anal fistula
6. Patients with any other associated disease
7. Patients with acute ulcerative colitis
8. Patients with history of advanced Sulpha reactions
Severe CVS disease refers to severity of CVS disease as per guidelines recommended by American heart Association and American College of Cardiology. Patients with any other associated disease refers to extra-intestinal complications associated with IBD like pyoderma gangrenosum, erythema nodosum, mouth ulcers, arthritis, episcleritic, uveitis etc.

4.7.5 Methodology of the study:

Patients were allocated to groups (10 in each group) by using simple randomization method. The trial was monitored and balanced at the end with number of subjects on each treatment over time. In clinical studies, the desired power is 0.8. Taking into consideration all dependent and independent variables, to have power of 0.914 (> 0.8), sample size required was 8. The calculation was done using G Power engine software. Taking into consideration dropout and missing values in future, we selected sample size of 10 per group for our studies.

The dosage was based on marketed preparations and preclinical studies done.

Group I Standard Allopathic formulation Mesalamine (Mesalazine)
   2 tabs orally per day (1 after lunch, 1 after dinner)

Group II Monoherbal test formulation
   2 tabs orally per day (1 before lunch, 1 before dinner)

Group III Standard Allopathic formulation + Monoherbal test formulation
   1 tab of each orally per day (1 after lunch, 1 before dinner)

4.7.6 Duration of Treatment and Evaluation Schedule:

Duration of treatment was of 4 weeks. Follow up visits were scheduled on 2nd and 4th week during treatment and then on 2nd and 4th week after completion of treatment.

4.7.7 Investigation Parameters:

4.7.7.1 Baseline Evaluation:

a) Prior screening, the patients were explained the study procedures.

b) Informed consent was taken from them. (In English: Annexure II, in vernacular language: Annexure III)
c) At baseline visit the medical history; general and physical examination, clinical history, signs and symptoms, details of previous therapy against IBD and other concomitant medication were recorded in Case Record Form (CRF) (Annexure IV).

Clinical examination and diagnostic tests were done twice i.e. before and treatment, while only clinical examination was done during both follow up visits after completion of the treatment.

4.7.7.2 Assessment of Efficacy:

Primary endpoint:-

The improvement in the patients was assessed on the basis of relief in the symptoms and signs of the disease together with laboratory investigations. All the symptoms and signs were given grade scores and assessed before, during as well as after treatment. Changes in body weight etc were also recorded.

Secondary endpoint:-

Patients were monitored for their compliance to the therapy. They were also instructed to report if any adverse drug reaction are produced during treatment and complications developed during or after the treatment.

4.8 Statistical Analysis:

Results were expressed as mean ± standard error of the mean (SEM). Data was analyzed using one-way analysis of variance (ANOVA) followed by the Dunnett’s post hoc test. In case of nonparametric data, significance between the mean values was tested using Kruskal Wallis Test followed by the Dunn’s post hoc test. 95% level of significance (p<0.05) was used for the statistical analysis. For toxicity studies, all the parameters were analyzed statistically using ANOVA, followed by Tukey’s Multiple Comparison Test post hoc test. For gene expression studies data, statistical analysis was performed using primer statistical software. Clinical data was evaluated using paired t test, Wilcoxon test, Mann Whitney U test, Kruskal Wallis test and ANOVA, wherever applicable, for finding statistical significance. All the tests were done using GraphPad Prism 6.