3. MATERIAL AND METHODS

3.1. PHARMACOGNOSTICAL INVESTIGATIONS

3.1.1. Plants material and extract preparations

3.1.1.1. Aconitum heterophyllum

3.1.1.1.1. Plant material

Roots of *Aconitum heterophyllum* were purchased from A. Amratlal & Co. Pydhonie, Mumbai. Authentified and submitted at Pharmacognosy department, Maliba Pharmacy College, Uka Tarsadia University, Tarsadi (Voucher specimen no. is UTU/MPC/2009/15).

3.1.1.1.2. Extract preparation

Dried roots of *Aconitum heterophyllum* were powdered and extracted by maceration with water for 7 days. Dried extract was dissolved in distilled water (AH_{aq}) and administered in different doses.

3.1.1.2. Oroxylum indicum

3.1.1.2.1. Plant material

Root bark of *Oroxylum indicum* was collected from Bardoli region. Authentified by Dr. Minoo Parabia, Department of Bioscience, VNSGU, Gujarat, India. The plant was submitted in the herbarium of the Pharmacognosy department of Maliba Pharmacy College (Voucher specimen no. is UTU/MPC/2009/04).

3.1.1.2.2. Extraction and fractionation

Root bark of *Oroxylum indicum* was dried, powdered and extracted by maceration with water for 7 days. Dried extract (OI-A) was dissolved in distilled water (OI_{aq}) and administered in different doses.

OI-A was percolated in pet ether at room temperature for 24 hrs to remove lower polarity fractions. Dried and extracted separately in various solvents like Ethyl acetate (dried extract after extraction denoted as OI-B), Chloroform (dried extract after extraction denoted as OI-C) and n-butanol (dried extract after extraction denoted as OI-D). Solvents were selected on basis of their polarity. Extraction was done using Bioassay guided extraction method.
Fractions OI-B, OI-C and OI-D were evaluated for their preliminary activity against DNBS induced colitis. Comparatively active fraction was selected and processed for further fractionation.

Fraction OI-D was found to be comparatively potent than Fraction OI-B and OI-C. OI-D was subjected to column chromatography using Chloroform: Ethyl acetate: Acetic acid (5:4:1) as solvent system (Solvent system was selected by trial and error basis using TLC plates). Fractions were collected and monitored on TLC. Three different fractions were obtained by column chromatography and identified as OI-E (First fraction obtained), OI-F (Second fraction obtained) and OI-G (Third fraction obtained). Preliminary activity of these compounds were evaluated against experimentally induced colitis and fraction OI-F was found to be more potent than OI-E and OI-G. Each fraction was subjected for phytochemical analysis and reported.

3.1.1.3. Aegle marmelos

3.1.1.3.1. Plant material

4.1. Fruits of *Aegle marmelos* were collected from Bardoli region. The plant was authenticated at and submitted in the herbarium of the Pharmacognosy department, Maliba Pharmacy College, Uka Tarsadia University, Tarsadi (Voucher specimen no. is UTU/MPC/2010/13).

3.1.1.3.2. Extraction and fractionation

Dried powdered fruits of *Aegle marmelos* were extracted with Methanol (70%) for 48 hrs with continuous heating (40º ± 5ºC) and stirring. Filtered with muslin cloth
and dried in air (denoted as Fraction AM-A). This methanolic extract was then percolated in pet ether at room temperature for 24 hrs to remove lower polarity fractions. Dried and extracted separately in various solvents like Ethyl acetate (dried extract after extraction denoted as AM-B), Chloroform (dried extract after extraction denoted as AM-C) and Hexane (dried extract after extraction denoted as AM-D). Solvents were selected on basis of their polarity. Extraction was done using Bioassay guided extraction method.

Fractions AM-B, AM-C and AM-D were evaluated for their preliminary activity against DNBS induced colitis. Comparatively active fraction was selected and processed for Thin Layer Chromatography (TLC).

Fraction AM-C was found to be comparatively potent than Fraction AM-B and AM-D. Fraction AM-C was processed for TLC using TLC aluminum sheets pre-coated with silica gel 60 F 254, thickness 0.2mm, (20×20 cm) (E Merck, Germany) as stationary phase, with Ethyl acetate : Hexane (9:1) as mobile. Two spots were separated at Rf 0.5 (Denoted as fraction AM-E) and 0.1 (Denoted as Fraction AM-F).

Fractions AM-E and AM-F were obtained by column chromatography using Ethyl acetate : Hexane (9:1) as solvent system. Fraction AM-E and not fraction AM-F was found to have protective effect against experimentally induced colitis. Each fraction was subjected for phytochemical analysis and reported.

3.1.2. Standardization of plant material

The quality control of herbal crude drug and bioconstituents is of paramount important for their acceptability in modern system of medicine. One of the major
Material and Methods

problems faced by user in industry is non availability of rigid quality control profile for herbal raw material and their formulation with advanced analytical technique and sophisticated instrument technology; it is possible to suggest a practicable quality assurance profile for a crude drug or its bioactive constituents\(^{[102]}\).

Powdered roots of *Aconitum heterophyllum*, root bark of *Oroxylum indicum* and fruits of *Aegle marmelos* were used to determine following standardization parameters.

3.1.2.1. *Ash value*

3.1.2.1.1. *Determination of Total Ash*

2 to 3 gm accurately weighed powder was placed in silica crucible, previously ignited and weighed. Incinerated by gradually increasing the heat—not exceeding 450\(^\circ\)C until free from carbon, cool and crucible was weighed. Percentage of ash was calculated with reference to air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]

3.1.2.1.2. *Determination of Acid-insoluble Ash*

Ash was boiled for 5 to 10 minutes with 25 ml of dilute hydrochloric acid. Insoluble matter was collected in a dry crucible, washed with hot water, ignited and weighed. Percentage of acid-insoluble ash was calculated with reference to air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]

3.1.2.1.3. *Determination of Water Soluble Ash*

Ash was boiled for five minutes with 25 ml of water. Insoluble matter was collected in a dry crucible, washed with hot water and ignited to constant weight at a low temperature. Weight of insoluble matter was subtracted from weight of ash; difference in weight represents water-soluble ash. Percentage of water-soluble ash was calculated with reference to air-dried drug.

\[
\text{Ash\%} = \frac{\text{Loss in Weight}}{W} \times 100
\]
3.1.2.2. Extractive values:

3.1.2.2.1. Determination of Alcohol-soluble Extractive

Coarsely powdered 5 gm of air-dried drug macerated with 100 ml of alcohol in a conical flask for 24 hours, shaked frequently for six hours and allowed to stand for 18 hours. Filtered rapidly taking precaution against loss of alcohol evaporated 25% of the filtrate to dryness in a tarred bottomed shallow dish dried at 105°C and weighed. Percentage of alcohol soluble extractive was calculated with reference to air-dried drug.

3.1.2.2.2. Determination of Water-Soluble Extractive

Proceeded as directed for determination of alcohol soluble extractive using chloroform water, I.P., instead of alcohol.

3.1.2.2.3. Loss on Drying

About 1.5 g of powder / drug was accurately weighed in petridish and kept in hot air oven maintained at 110°C. After cooling in dessicator, the loss in weight was recorded. This procedure was repeated till constant weight was obtained.

\[
\text{Loss on drying (\%)} = \frac{\text{Loss in weight}}{W} \times 100
\]

Where \( W \) = Weight of the leaf powder in gms.

3.1.2.3. Phytochemical investigations

Phytochemical investigation was carried out to determine presence of alkaloids, amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, proteins, saponins, steroids and triterpenoids \(^{[102]}\).

3.2. PHARMACOLOGICAL EVALUATIONS

3.2.1. Animals

Wistar Strain Albino rat of either sex (200-250 gm), Swiss Albino mice (20-25 gm) and rabbit (crossbreed, 2.2 kg) were obtained from animal house, Department of Pharmacology, Maliba Pharmacy College, Tarsadi. Rats and mice were divided into group of six animals, housed in PVC cages under standard condition (12:12 hour light/ dark cycle at 25±2°C, Humidity 70-75%). The experimental protocols (MPC0905, MPC1005) were approved by Institutional animal ethics committee (Reg. No. 717/02/a/CPCSEA) as per the guidance of Committee for the Purpose of Control
and Supervision on Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Control group animals received same experimental handling as those of test groups except that drug treatment was replaced by administration of appropriate volumes of dosing vehicle.

3.2.1.1. Husbandry Practices
- **Caging**: Polypropylene rat cages covered with stainless steel grid top were Autoclaved clean rice husk was used as the bedding mate
- **Water Bottle**: Each cage was supplied with a polypropylene water bottle with a stainless steel nozzle.
- **Housing**: Single rat per cage
- **Room Sanitation**: Each day, the floor of the experiment room was swept and all work tops and the floor were mopped with a disinfectant solution.

3.2.1.2. Animal Identification
Each animal was uniquely numbered by marking on its tail. Appropriate labels were attached to cages indicating study number, sex of animals, dose of drug administered, type of study, cage number and animal number.

3.2.2. Chemicals
Di-nitro benzene sulphonic acid (DNBS) (Cynochem Inc, Ahmedabad, India), Verapamil (Sigma aldrich), Dextran sulphate sodium (DSS) (Sigma aldrich), Azoxymethane (AOM) (Sigma aldrich), Cyclopiazonic acid (Sigma aldrich), gentamicin (GENTANIK® Lunik Pharma Pvt Ltd, Ahmedabad, India), nystatin (Mystatin-OS® Savorite Pharmaceuticals, Vadodara, India), loperamide (ANDIAL® Liqd, Citadel) were used. ELISA kits for NF-kB and IL-6 were purchased from WUHAN EIAAB SCIENCE CO., LTD., Wuhan, China. All other chemicals used for the study were purchased from Minhas Chemicals, Navsari, India.

3.2.3. Oral toxicity study
Acute and chronic (repeated dose toxicity study for 28 days and 90 days) oral toxicity study of AH_{aq} was performed as per OECD guidelines [103]. In previous literature roots of *A. heterophyllum* was reported to be toxic [104] and hence AH_{aq} was examined for its toxicity before administration. Acute oral toxicity of OL_{aq} was not
Material and Methods

reported previously and evaluated in this study. Toxicity study of methanolic extract of A. marmelos fruit was reported in previous literature\textsuperscript{[105]} and not done here.

3.2.3.1. Acute oral toxicity

Female Wistar Rats were selected by random sampling technique and were acclimatized for 5 days to laboratory conditions prior to dosing. Animals were fasted overnight (food but not water withheld) prior to oral administration of OI\textsubscript{aq} or AH\textsubscript{aq}. Test substance was administered to single animals in a sequential manner following progression slope 2 of OECD-425 throughout study with starting dose of 175 mg of OI\textsubscript{aq} or AH\textsubscript{aq} per kg body weight and observed for 14 days\textsuperscript{[103; 106; 107]}.

3.2.3.2. Repeated dose oral toxicity study

The test substance was orally administered daily for period of 28 days (OECD-407) and 90 days (OECD-408). Limit test with dose 1000 mg/kg/day was done first on both sexes of rats, so as to minimize unnecessary use of animals. During the period of administration animals were observed closely, each day for signs of toxicity and scored as per Functional Observational Battery. Animals which showed mortality or were euthanatized during test were necropsied and at the conclusion of the test surviving animals were euthanatized and necropsied. Organs were separated, weighed and observed for any sign of toxicity or abnormality\textsuperscript{[106; 107]}.

3.2.3.3. Functional Observational Battery

Functional Observational Battery (FOB)\textsuperscript{[108]} is a neurotoxicity screening assay composed of 25–30 descriptive, scalar, binary and continuous endpoints. Binary (B) and descriptive (D) data expressed as percentage of incidence; ranked (R) data expressed as mean score of scale used; continuous (C) data expressed as mean value. Data were converted to corresponding severity scores, on a scale of 1 to 4, as explained in following table (Table 3.1). Continuous data were converted based on distance in standard deviation (SD) units from control mean, while descriptive endpoints were converted based on their relation to mode of control distribution.
Table 3.1: Severity score definitions used in Functional Observational Battery.

<table>
<thead>
<tr>
<th>Severity Score</th>
<th>Continuous measures</th>
<th>Rank and descriptive measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Within 1 SD of control group mean</td>
<td>Typically seen in untreated rats.</td>
</tr>
<tr>
<td>2</td>
<td>Between 1 and 1.5 SD of control group mean</td>
<td>Slightly atypical; can be seen occasionally in untreated rats but could be a subtle sign of toxicity.</td>
</tr>
<tr>
<td>3</td>
<td>Between 1.5 and 2 SD of control group mean</td>
<td>Somewhat atypical; could rarely be seen in untreated rats; would usually reflect moderate intoxication.</td>
</tr>
<tr>
<td>4</td>
<td>Greater than 2 SD of control group mean</td>
<td>Completely atypical; reflect marked intoxication.</td>
</tr>
</tbody>
</table>

3.2.4. DNBS-induced Colitis

Crude extracts of *O. indicum* (OI$_{aq}$) and *A. heterophyllum* (AH$_{aq}$) were tested against DNBS induced colitis in rats. Methanolic extract of roots of *A. marmelos* was reported previously to be protective in animal model of experimental colitis by Dr. Gandhi and co-workers$^{[109]}$ and hence not studied here.

3.2.4.1. Groups and Drug Treatments

Animals were divided in nine groups of six animals each and treated as follows:

- **Group 1: Control** – No drug/extract treatment. Normal animals.
- **Group 3: Drug Control** – DNBS treated. 5-Aminosalicylic acid 100 mg/kg p.o.
- **Group 4: OI$_{aq}$ 100 mg/kg/day** – DNBS treated. OI$_{aq}$ was administered in dose 100, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.
Material and Methods

- **Group 5**: OI\textsubscript{aq} 200 mg/kg/day – DNBS treated. OI\textsubscript{aq} was administered in dose 200 mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.

- **Group 6**: OI\textsubscript{aq} 400 mg/kg/day – DNBS treated. OI\textsubscript{aq} was administered in dose 400, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.

- **Group 7**: AH\textsubscript{aq} 100 mg/kg/day – DNBS treated. AH\textsubscript{aq} was administered in dose 100, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.

- **Group 8**: AH\textsubscript{aq} 200 mg/kg/day – DNBS treated. AH\textsubscript{aq} was administered in dose 100, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.

- **Group 9**: AH\textsubscript{aq} 400 mg/kg/day – DNBS treated. AH\textsubscript{aq} was administered in dose 100, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after DNBS treatment.

3.2.4.2. Induction of colitis

Colitis was induced using technique of acid induced colon inflammation, as described by Cuzzocrea et al.\cite{110}. In fasted rats lightly anaesthetised with ether, a catheter was inserted into the colon via anus until approximately splenic flexure (8 cm from the anus). DNBS (25 mg/rat) was dissolved in 50% ethanol. Thereafter, animals were kept for 15 minutes in a Trendelenburg position to avoid reflux and observed for three days after DNBS administration. On day 4, animals were euthanatized and abdomen was opened by a midline incision. Colon was removed, freed from surrounding tissues, opened along antimesenteric border, rinsed, weighed and processed for histology. Body weight and food intake were measured on day 1 and 4. Similarly stool consistency were observed and scored as Normal (0), Pasty and partially formed pellets (Max 2), Liquid pellet (Max 4), Blood in hemoccult (Max 2) and Gross bleeding from the rectum (Max 4). Colonic lesion area was measured as described by Khan\cite{111}.
Colonic lesion area

*Image capturing for morphologic visualization*

Opened colonic samples were flattened and carefully sandwiched between two layers of a transparent plastic folder of A4 size. Specimens within plastic folder were scanned using a scanner and captured image was saved (TIFF format) in computer hard drive.

*Quantitation of colonic lesions using Scion Image menu commands*

The scanned images were subjected to analysis for quantification of gastric lesions using public domain image processing and analysis program developed at National Institute of Health, USA. PC version of this program (2.15 MB), known as Scion Image, is available on Internet for free download from Scion (http://www.scioncorp.com) (Scion Image for Windows, Release Beta 4.0.2). The step-by-step procedure for quantitation of gastric lesions is given in Table 3.2. Briefly, protocol was composed of six tasks that were sequentially performed as follows: opening of image file, image conversion to gray scale, subtraction of unlesioned area, thresholding, scale setting, and area measurement. The subtraction units of 150 was obtained by conducting few trials using a range of 100 to 200 with the increments of 5 units until best-filtered image was obtained showing all lesions without any background.

**Table 3.2: Sequential steps for quantitation of colonic lesions using Scion Image software**

<table>
<thead>
<tr>
<th>Step</th>
<th>Menu</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>File</td>
<td>Open</td>
</tr>
<tr>
<td>2</td>
<td>Process</td>
<td>Convert to gray scale</td>
</tr>
<tr>
<td>3</td>
<td>Process</td>
<td>Arithmetic, subtract</td>
</tr>
<tr>
<td>4</td>
<td>Options</td>
<td>Threshold</td>
</tr>
<tr>
<td>5</td>
<td>Analyze</td>
<td>Set scale, 12 pixels = 1 mm</td>
</tr>
<tr>
<td>6</td>
<td>Analyze</td>
<td>Measure</td>
</tr>
</tbody>
</table>
Material and Methods

Histopathology

Colon tissues were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Tissues were then cut in to 3 μm sections with uniform shape and size, mounted on silane-coated glass slides and stained with haematoxylin eosin, periodic acid Schiff reagent. Selected tissue sections were fixed on glass slide with the help of egg albumin.

Biochemical estimation

1. Myeloperoxidase (MPO) Activity

Myeloperoxidase (MPO) activity\cite{110}, an indicator of polymorphonuclear leucocyte (PMN) accumulation was determined as previously described\cite{19}. Four days after intracolonic injection of DNBS the colon was removed and weighed. The colon was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged (REMI C24) for 30 minutes at 20 000 g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide/minute at 37°C and was expressed in milliunits per 100 gram weight of wet tissue.

2. Malondialdehyde (MDA) Measurement

1 ml of supernatant 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 a water bath at 85°C for 1hr. The intensity of pink color developed was measured against a reagent blank at 532nm. Malondialdehyde was calculated using molar extinction coefficient 1.56x10^5 M⁻¹ cm⁻¹ or from standard curve and reported as millimoles per mg of wet tissue\cite{110}.

3. Nitrite analysis

Accumulated nitrite (NO₂⁻) in the homogenate will be spectrophotometrically determined based on the Griess reaction. The samples (100 μl) were incubated with 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min and then NO₂⁻
concentration will be determined by the absorbance at 540 nm. The standard curve was obtained using the known concentrations of sodium nitrite\[^{110}\].

4. Measurement of GSH level in the colonic tissue

Colonic tissues were homogenized in ice-cold 125 mmol/L sodium phosphate buffer with 6.3 mmol/L EDTA (pH 7.5, 3 μL/mg tissue) for 30 s. The crude homogenate was centrifuged at 30 000 g at 4°C for 30 min. Then, 200 μL of 40 g/L sulfosalicylic acid was added to 100 μL of supernatant and allowed to stand on ice for 5 min to precipitate protein. The mixture was centrifuged again at 5 000 r/min at 4°C for 10 min. Subsequently, 100 μL of the de-proteinized supernatant was mixed well with 300 μL of 125 mmol/L sodium phosphate buffer (pH 8) and 2 μL of 10 mmol/L 5,5’-dithiobis-(2-nitrobenzoic acid). The solution was allowed to stand at room temperature for 15 min to develop yellow color. The absorbance was read against the reagent blank at 412 nm in a spectrophotometer. A standard curve of reduced GSH was used for the calculation of the concentration of GSH in the colonic tissues. The final values were expressed as nanomole per milligram protein\[^{112}\].

3.2.5. Anti-diarrhoeal activity

Diarrhoea is one of major symptoms of IBD. Crude extract of *O. indicum* (OI\(_{aq}\)) was evaluated for its anti-diarrhoeal potential which will provide symptomatic relief against IBD. *A. heterophyllum*\[^{113}\] and *A. marmelos*\[^{13}\] had anti-diarrhoeal potential and reported in previous literature.

3.2.5.1. Antibacterial and antifungal activity

3.2.5.2. Microorganisms

Microorganisms used included a reference fungal strain, Candida albicans (ATCC-9002), and four clinical bacterial isolates—Staphylococcus aureus (ATCC 12598), Salmonella typhi (ATCC-6539), Shigella flexneri (ATCC-25931) and Escherichia coli (ATCC-15223). These microorganisms were obtained from the Biotechnology and Microbiology Department C.G Bhakta Institute of Biotechnology, Gopal Vidyanagar, Tarsadi, Gujarat (India). Cultures of these bacteria were done in Mueller-Hinton broth and the fungus on Sabouraud Dextrose Agar all at 37°C. They were maintained on slopes of nutrient agar and stored at 4°C in the laboratory.
Material and Methods

3.2.5.3. Disc diffusion method\textsuperscript{(114)}

The innocula of the microorganisms were prepared separately from 12 h broth cultures (Mueller-Hinton broth for bacteria and the Sabouraud dextrose broth for Candida albicans) and incubated at 37\(^{0}\)C. Culture media and distilled water were sterilized at 115\(^{0}\)C for 15 min in an autoclave. These innocula were diluted with sterilized distilled water to obtain a density corresponding approximately to 0.5 of McFarland standard turbidity scale (108 colony forming unit “CFU” per ml for the bacteria and 103 spores per ml for Candida albicans). The 0.1 ml of each innoculum was introduced in the corresponding fluid agar medium, homogenised and poured in glass Petri dishes (90mm in diameter). The Petri dishes were allowed on the flat slab top for the medium to solidify within 30 min. Sterile discs of 6mm in diameter were made from Fisher filter paper P5. The discs were impregnated with each plant extract (10 \(\mu\)l) prepared at the concentrations of 2.5 and 25 mg/ml. The reference drugs were gentamicin (10 and 100 \(\mu\)g/ml), and nystatine (50 and 500 \(\mu\)g/ml). After moistening the discs, they were immediately transferred to the inoculated solid media. The plates were incubated at 37 \(^{0}\)C for 24 and 48 h for the bacteria and fungus, respectively. The results were recorded as percentage potency of the drug.

3.2.5.4. Castor oil induced diarrhoea\textsuperscript{(115)}

Male albino mice were screened initially by giving 0.5 ml of castor oil and only those showing diarrhoea were selected for final experiment. Animals were divided in five groups of six animals each and treated as follows.

- **Group 1: Model Control** – Castor oil was administered in dose 0.5 ml/mouse p.o. No drug/extract treatment.
- **Group 2: Drug Control** – Castor oil treated. Loperamide was administered in dose 2 mg/kg p.o. one hour before castor oil treatment.
- **Group 3: OI\textsubscript{aq} 100 mg/kg/day** – Castor oil treated. OI\textsubscript{aq} was administered in dose 100 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.
- **Group 4: OI\textsubscript{aq} 200 mg/kg/day** – Castor oil treated. OI\textsubscript{aq} was administered in dose 200 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.
- **Group 5: OI\textsubscript{aq} 400 mg/kg/day** – Castor oil treated. OI\textsubscript{aq} was administered in dose 400 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.
Material and Methods

Each animal was placed in an individual cage, floor lined with blotting paper, which was changed every hour. 30 min after oral treatment of OI_{aq} on day 7, diarrhoea was induced by oral administration of castor oil (0.5 ml/mouse p.o.). Onset of diarrhoea, number of diarrhoeal episodes, stool mass and number of animals exhibiting diarrhoea was obtained over 5 h period of observation\(^{115}\).

3.2.5.5. Magnesium sulphate induced diarrhoea\(^{115}\)

Male albino mice were divided in five groups of six animals each and treated as follows.

- **Group 1: Model Control** – Magnesium sulphate was administered in dose 2 gm/kg p.o. No drug/extract treatment.
- **Group 2: Drug Control** – Magnesium sulphate treated. Loperamide was administered in dose 2 mg/kg p.o. one hour before Magnesium sulphate treatment.
- **Group 3: OI_{aq} 100 mg/kg/day** – Magnesium sulphate treated. OI_{aq} was administered in dose 100 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.
- **Group 4: OI_{aq} 200 mg/kg/day** – Magnesium sulphate treated. OI_{aq} was administered in dose 200 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.
- **Group 5: OI_{aq} 400 mg/kg/day** – Magnesium sulphate treated. OI_{aq} was administered in dose 400 mg/kg/day p.o. for 7 days prior to induction of diarrhoea.

Magnesium sulphate was administered in dose 2 gm/kg p.o. after 30 min. of oral treatment of OI_{aq} on day 7. The individual mouse cages were observed for 4 h after dosing with magnesium sulphate, by an observer unaware of particular treatment for presence of unformed water faecal pellets\(^{115}\).
3.2.5.6. *Gastrointestinal Transit*[^116]

Swiss albino mice of either sex were divided in five groups of six animals each and treated as follows.

- **Group 1: Model Control** – Two doses of croton oil (20 ml/mouse) in two consecutive days were administered orally. No drug/extract treatment.
- **Group 2: Drug Control** – Croton oil treated. Atropine was administered in dose 1 mg/kg p.o. 60 min before charcoal administration.
- **Group 3: OI<sub>aq</sub> 200 mg/kg/day** – Croton oil treated. OI<sub>aq</sub> was administered in dose 200 mg/kg/day p.o. for 7 days prior to induction of diarrhoea and continued for 5 days after first dose of croton oil.
- **Group 4: OI<sub>aq</sub> 400 mg/kg/day** – Croton oil treated. OI<sub>aq</sub> was administered in dose 400 mg/kg/day p.o. for 7 days prior to induction of diarrhoea and continued for 5 days after first dose of croton oil.

Inflammation was induced by administration of two doses of croton oil (20 ml/mouse orally) in two consecutive days on day 7 and 8 of OI<sub>aq</sub> administration. Four days after first administration of croton oil, upper gastrointestinal transit of mice was measured by oral administration of black marker (0.1 ml 10 g mouse<sub>-1</sub>; 10% charcoal suspension in 5% gum Arabic). After 20 min, mice were euthanatized and gastrointestinal tracts were removed. The distance travelled by marker was measured and expressed as percentage of total length of small intestine from pylorus to caecum[^116].

3.2.5.7. *Colonic propulsion*[^116]

Swiss albino mice of either sex were divided in four groups of six animals each and treated as follows.

- **Group 1: Control** – No drug/extract treatment.
- **Group 2: Drug Control** – Loperamide was administered in dose 2 mg/kg p.o.
- **Group 3: OI<sub>aq</sub> 400 mg/kg** – OI<sub>aq</sub> was administered in dose 400 mg/kg p.o.

60 min. after the treatment, a single 3-mm glass bead was inserted up to 2 cm into distal colon of each mouse with aid of a catheter and time to expulsion of glass bead was determined for each animal[^116].

[^116]: References should be included here or referenced in the text where needed.
3.2.5.8. Normal defecation\textsuperscript{[117]}

Three groups of 6 Swiss albino mice each, starved for 18 h, were treated as follows.

- **Group 1: Control** – No drug/extract treatment.
- **Group 2: Drug Control** – Atropine was administered in dose 1 mg/kg p.o. 60 min before glass bead insertion.
- **Group 3: OI\textsubscript{aq} 400 mg/kg** – OI\textsubscript{aq} was administered in dose 400 mg/kg p.o. 60 min before glass bead insertion.

Animals were placed individually in cages with filter paper at the bottom and observed for total number of faeces in each group up to 3 h. Percent reduction in number of faeces in treated group was obtained by comparison with control animals\textsuperscript{[117]}.

3.2.5.9. Gastric emptying\textsuperscript{[117]}

Wistar albino rat of either sex were divided in three groups of six animals each, fasted for 24 hrs and treated as follows.

- **Group 1: Model Control** – No drug/extract treatment. 3 ml of semi-solid test meal (10% charcoal suspension in 2% methylcellulose) was administered.
- **Group 2: OI\textsubscript{aq} 200 mg/kg** – OI\textsubscript{aq} was administered in dose 200 mg/kg p.o. 60 min before administration of 3 ml of semi-solid test meal (10% charcoal suspension in 2% methylcellulose).
- **Group 3: OI\textsubscript{aq} 400 mg/kg** – OI\textsubscript{aq} was administered in dose 400 mg/kg p.o. 60 min before administration of 3 ml of semi-solid test meal (10% charcoal suspension in 2% methylcellulose).

After 1 h of administration of 3 ml of semi-solid test meal, animals were sacrificed and stomachs removed. The full stomachs were weighed, opened and rinsed. Excess moisture was removed and empty stomach reweighed. Difference between full and empty stomach was subtracted from weight of 3 ml test meal to obtained quantity emptied from stomach during test period\textsuperscript{[117]}.
3.2.5.10. **Intestinal fluid accumulation**\(^{[116]}\)

Wistar albino rat of either sex were divided in three groups of six animals each and treated as follows.

- **Group 1: Control** – No drug/extract treatment.
- **Group 2: OI\(_aq\) 200 mg/kg** – OI\(_aq\) was administered in dose 200 mg/kg p.o. 60 min before administration of castor oil (2 ml/rat).
- **Group 3: OI\(_aq\) 400 mg/kg** – OI\(_aq\) was administered in dose 400 mg/kg p.o. 60 min before administration of castor oil (2 ml/rat).

After 30 min of castor oil administration, animals were euthanatized by cervical dislocation. Small intestine was clamped at pyloric valve and ilio–cecal junction and removed carefully from abdomen. Small intestine was weighed (W1), emptied of fluid, reweighed (W2) and length (L) measured. The difference in weight divided by length showed “enteropooling” in mg of fluid per centimeter of intestine.

\[
\text{Enteropooling} = \frac{(W1 - W2)}{L}
\]

3.2.6. **Efficacy of fractions isolated from O. indicum and A. marmelos**

Animals were divided in groups of three animals each, treated for seven days with extract fraction prior to induction of colitis and continued for next 4 days after DNBS treatment. Colitis was induced using technique of acid induced colon inflammation, as described by Cuzzocrea et.al\(^{[110]}\). In fasted rats lightly anaesthetised with ether, a catheter was inserted into the colon via anus until approximately splenic flexure (8 cm from the anus). DNBS (25 mg/rat) was dissolved in 50% ethanol. Thereafter, animals were kept for 15 minutes in a Trendelenburg position to avoid reflux and observed for three days after DNBS administration for signs of diarrhoea. On day 4, animals were sacrificed and abdomen was opened by a midline incision. Colon was removed, freed from surrounding tissues, opened along antimesenteric border, rinsed, weighed and observed for damage. Lesion area was measured as described by Khan\(^{[111]}\) (Similar procedure was followed as described in ‘Damage lesion area’ in DNBS induced colitis section).
3.2.7. Evaluation of mechanism of action of OI-F and AM-E fractions

3.2.7.1. Effect of OI-F and AM-E against Colitis associated Colon Cancer

3.2.7.1.1. Groups and Drug Treatments

Male Wistar rats were divided in four groups of ten animals each and treated as follows.

- **Group 1: Control** – No treatment
- **Group 2: Model control** – Azoxymethane (AOM) (20 mg/kg ip) followed by DSS.
- **Group 3: OI-F** was administered in dose 50 mg/kg/day p.o. for 7 days prior to AOM and DSS treatment and continued throughout experimental procedure.
- **Group 4: AM-E** was administered in dose 50 mg/kg/day p.o. for 7 days prior to AOM and DSS treatment and continued throughout experimental procedure.

After 7 days extract treatment, rats were injected i.p. with a single dose of azoxymethane (AOM, 20 mg/kg bodyweight in bicarbonate buffer) to induce colon cancer.[118] Inflammation was induced 1 week later (Day 14) by adding 2.0% DSS (molecular weight 36,000 to 44,000 g/L; Sigma-alderich) to drinking water for 7 consecutive days. During this time, rats were weighed on a daily basis and examined by blinded observers for clinical signs of disease associated with colitis (i.e., perianal soiling, rectal bleeding, diarrhea and piloerection). Rats were observed for signs of diarrhoea and accessed as stool consistency throughout DSS treatment. On Day 21 (Day 8 of DSS challenge), rats were switched to regular drinking water. Stool consistency and body weight determinations were continued weekly until the end of study. Mortality was checked daily. Dead rat colon were removed; checked for lesion area and incidence of tumours. Rats survived were euthanized after 80 days of carcinogen injection. Colons were removed, opened longitudinally and were screened for tumours. Tissue was homogenized and used for determination of Nuclear Factor kappa B and Interleukin – 6.

3.2.7.1.2. Determination of NF-κB translocation

Colon tissues were homogenized in cold PBS and then were centrifuged at 500×g for 5 min at 4 °C. The resulting supernatants were discarded. Precipitation was resuspended in 200 μL buffer A (10 mM HEPES, 10 mM KCl, 0.5mM EDTA, 0.5
mM dithiothreitol (DTT), and 0.05% NP-40, pH 7.9) and 5 μL PMSF, then allowed to
swell on ice for 10 min. After incubation for 10 min on ice, cells were centrifuged at
12,000×g for 5 min at 4 °C. The pellet was resuspended in 200 μL buffer B (5mM
HEPES, 1.5 Mm MgCl2, 0.2mM EDTA, 0.5mM DTT and 26% glycerol (v/v), pH
7.9) and 5 μL PMSF. Then tubes were incubated for 40 min on ice. Nuclear extracts
were then centrifuged at 12,000×g for 15 min at 4 °C and supernatant was frozen in
aliquots at −80 °C for measurements of NF-κB. Nuclear translocation levels of NF-κB
were determined by a commercially available enzyme immunoassay kits
(WUHAN EIAAB SCIENCE CO., LTD., Wuhan, China.). Measurement was
completed using an enzyme-linked immunosorbent assay with an absorbency
maximum at 450 nm. The results were expressed as nanograms per gram of wet tissue
(ng/g tissue)\textsuperscript{[118]}. 

3.2.7.1.3. Determination of IL-6 levels
Colon tissues were homogenized in cold PBS and then were centrifuged at
500×g for 5 min at 4 °C. Resulting supernatant were used for determination IL-6
levels. IL-6 levels were determined as per procedure supplemented with a
commercially available enzyme immunoassay kits (WUHAN EIAAB SCIENCE CO.,
LTD., Wuhan, China.). Measurement was done using an enzyme-linked
immunosorbent assay with an absorbency maximum at 450 nm. The results were
expressed as picogram per gram of wet tissue (pg/mg tissue)\textsuperscript{[118]}.

3.2.7.2. In-Vitro experiments
Efforts were made to find out mechanism of active fractions OI-F and AM-E
in prevention of colitis and colon cancer using simple in-vitro methods.

3.2.7.2.1. Effect of OI-F and AM-E on isolated rabbit ileum
A rabbit was fasted for 12 h before the experiment, and thereafter it was
sacrificed by a blow on head and exsanguinated. Segments of ileum, about 2 cm long
were cut. The ileum was suspended in 25ml organ bath containing Tyrode’s solution
[NaCl (136.8), KCl (2.7), CaCl2 (1.3), NaHCO3 (11.9), MgCl2 (0.5), Na2PO4 (0.45)
and glucose (5.5)] at a temperature of 37 (±1°C), and aerated with air. 1 h
equilibration time was allowed during which physiological solution was changed
every 15 min. Preparations were set up under a tension of 0.5 g and responses were
recorded on smoked kymograph paper through an isotonic frontal writing lever
(magnification × 10). Responses to acetylcholine, barium chloride and electrical field
stimulation (via a pair of platinum electrodes) were recorded in absence and in presence of increasing (noncumulative) concentrations of OI-F and AM-E (1–1000 µg/ml) added 15 min before (i.e. after washing the tissue) (Preliminary experiments showed that, a 15 min contact time was sufficient for maximal inhibitory effect). Each agonist was used on separate preparation\textsuperscript{119}.

In other experiments, effect of OI-F and AM-E on acetylcholine induced contractions was evaluated in presence of verapamil (10µM) (contact time: 15 min). The presence of such inhibitor/ antagonist does not affect reproducibility and stability of the contractions induced by acetylcholine.

3.2.7.2.2. Effect of OI-F and AM-E on store operated Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} channels

In other experiment with fresh tissue preparation, equipotent responses were obtained with carbachol, then tissues were treated with Cyclopiazonic acid (CPA) (10 µM) for 26 min. Carbachol was added in increasing concentrations to obtained maximum response (Ceiling effect). Ceiling dose (1.6 ml of 10 µl Carbachol solution) was selected for the further experiment\textsuperscript{120}.

After the treatment of CPA and carbachol (as above); increasing concentrations of Ca\textsuperscript{2+} were added in organ tube in presence and in absence of OI-F in separate experiments. The extracts were instilled in organ tube 4 min. before the treatment with CPA. All these experiments were carried out in presence of verapamil (10 µM) to eliminate the possible involvement of voltage-dependent Ca\textsuperscript{2+} channels.

3.2.7.2.3. Effect of OI-F and AM-E on Histamine H1 receptor

Effect of AM-E on histamine induced contractions on rat ileum was studied using Tyrode solution. The ileum was suspended in 25ml organ bath containing Tyrode’s solution [NaCl (136.8), KCl (2.7), CaCl\textsubscript{2} (1.3), NaHCO\textsubscript{3} (11.9), MgCl\textsubscript{2} (0.5), Na\textsubscript{2}PO\textsubscript{4} (0.45) and glucose (5.5)] at a temperature of 37 (±1°C), and aerated with air. Preparations were set up under a tension of 0.5 g and responses were recorded on smoked kymograph paper through an isotonic frontal writing lever (magnification × 10). Responses to Histamine were recorded in absence and in presence of increasing (noncumulative) concentrations of AM-E (1–1000 µg/ml) added 15 min before (i.e. after washing the tissue) (Preliminary experiments showed
that, a 15 min contact time was sufficient for maximal inhibitory effect) and reported as percentage inhibition of response\textsuperscript{[121]}.

3.2.8. Statistical analysis

All values in the figures and text are expressed as mean ± S.E.M. of \( n \) (number of animals) observations. The results were analysed by one-way analysis of variance followed by a Dunnet post hoc test for parametric data, whereas statistical significance was analyzed by Wilcoxon simple paired test for arthritic index and percentage body weight change of control. Kruskall–Wallis test followed by Dunn’s multiple comparison tests was used for scored data analysis. P value less than 0.01 were considered significant.