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1 INTRODUCTION

Inflammatory Bowel Disease (IBD) is a set of chronic inflammatory conditions resulting from inappropriate and persistent activation of the mucosal immune system, driven by presence of normal intraluminal flora. The two disorders known as IBD are Crohn’s disease (CD) and ulcerative colitis (UC). These disease share many common features but have distinctly different clinical manifestations\(^1\);\(^2\).

Ulcerative colitis is a relapsing non-transmural inflammatory disease that is restricted to the colon. Dependent on the anatomic extent of involvement, patients can be classified as having proctitis, left-sided colitis (involving the sigmoid colon with or without involvement of the descending colon), or pancolitis. A few patients also develop ileal inflammation (backwash ileitis), which occasionally complicates differentiation from Crohn’s ileocolitis. Patients typically present with bloody diarrhoea (often nocturnal and postprandial), passage of pus, mucus, or both, and abdominal cramping during bowel movements. Severe symptoms are less common in left-sided colitis and proctitis\(^3\).

Crohn’s disease is a relapsing, transmural inflammatory disease of the gastrointestinal mucosa that can affect entire gastrointestinal tract from mouth to anus. Typical presentations include discontinuous involvement of various portions of gastrointestinal tract and development of complications including strictures, abscesses, or fistulas\(^1\);\(^3\). The Vienna classification was developed to describe the distinct clinical phenotypes of Crohn’s disease with respect to disease location and occurrence of complications\(^4\);\(^5\).

IBD is observed most commonly in Northern Europe and North America with the incidence of 70-150 cases per 100,000 individuals alone in US. It is a disease of industrialized nations. Incidence among whites is approximately 4 times that of other races and higher in Ashkenazi Jews (i.e., those who have immigrated from Northern Europe) than in other groups. Females are slightly more prone to IBD than males. Incidence peaks in the second and third decades of life. A second smaller peak occurs in patients aged 55-65 years. CD and UC can occur in childhood, although the incidence is much lower in children younger than 15 years\(^3\).
In India, ulcerative colitis was first reported in 1964 and Crohn's disease was considered almost nonexistent till 1986. During the last 10 years, Crohn's disease is being reported more frequently from different parts of India, especially southern India\textsuperscript{[6]}.

Pathogenesis of IBD mainly involves environmental factors (smoking, psychological stress, improved hygiene, consumption of sterile and nonfermented foods, vaccination and age at first exposure to intestinal pathogens)\textsuperscript{[7-9]}, genetic predisposition (NOD2 gene, HLA DRB1 gene including chromosome 12 (IBD2), 6 (IBD3) and 14 (IBD4)\textsuperscript{[10; 11]}, intestinal microbial flora\textsuperscript{[10]}, and immunoinflammatory factors (arachidonic acid metabolites like prostaglandins, hromboxanes, and leukotrienes, reactive oxygen species, nitric oxide, nuclear factors, growth factors, various cytokines like tumor necrosis factor-α (TNF-α), interleukins (IL), and interferons\textsuperscript{[12-14]})

Increasing evidences showed correlation between IBD and Colorectal cancer (CRC) which is one of the most common fatal malignancies worldwide\textsuperscript{[15]}. In patients with inflammatory bowel disease (IBD), such as ulcerative colitis (UC), risk of CRC development is much higher than in general population\textsuperscript{[16]}. Long-standing UC predisposes to development of colitis-associated cancer (CAC), the major cause of death in UC patients\textsuperscript{[17]}. It has been proposed that noxious compounds released during chronic colonic inflammation damage DNA and/or alter cell proliferation or survival, thereby promoting oncogenesis\textsuperscript{[18]}. While chronic inflammation may contribute to oncogenic mutagenesis through production of reactive oxygen and nitrogen species\textsuperscript{[19]}, experimental evidence suggests that it mainly acts as a tumor promoter rather than as an initiator\textsuperscript{[20]}.

NF-κB and IL-6 was also found to promote colitis associated colon cancer. Cytokines or growth factors produced upon NF-κB activation in intestinal myeloid cells stimulate proliferation of premalignant IECs generated during early stages of CAC tumorigenesis. Inactivation of NF-κB in myeloid cells through ablation of IKKβ, protein kinase required for its activation, inhibits production of inflammatory mediators such as IL-6 and TNF-α, and prevents IEC proliferation during CAC induction. As a result, tumour load is reduced due to decrease in tumor frequency and size\textsuperscript{[21]}. 
Management of inflammatory bowel disease involves the use of 5-aminosalicylic acid and immunosuppressive agents such as corticosteroids and 6-mercaptopurine as well as its precursor azathioprine. Long term use of glucocorticoids is associated with high rates of relapse and unacceptable toxicity. On the other hand, 6-mercaptopurine and its prodrug azathioprine are effective in maintaining remission, however, a significant number of patients are resistant or intolerant to thiopurines. Novel agents such as monoclonal antibodies against TNF-α have been developed and demonstrate clinical efficacy. However, these agents are expensive and not without side effects. Consequently, there is a need for alternative agents that may be equally or more effective as well as being cheaper\cite{7}.

CD4\(^+\) T cells are thought to be major players in the processes leading to inflammatory bowel disease (IBD). Both Crohn’s disease (CD) and ulcerative colitis (UC) are characterized by an increased mucosal infiltration and activation of CD4\(^+\) T lymphocytes\cite{22}. Studies using models of experimental colitis have shown that blocking CD4\(^+\) T cell activation is useful for limiting ongoing mucosal inflammation. Recently, understanding of the molecular nature and regulation of the most important Ca\(^{2+}\) signalling pathway in T cells, the store operated Ca\(^{2+}\) (SOC) entry through Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels, has suggested that interference with Ca\(^{2+}\) signalling may also be a useful approach to control excessive T cell activation in vivo\cite{23, 24}. A rise in intracellular free Ca\(^{2+}\) concentration is one of the early critical steps in the activation of T lymphocytes and it then has an essential role in determining the strength and the type of the T cell response\cite{22}.

Alternatively histamine was found to involve in pathogenesis and promotion of IBD. It is a biogenic amine, mainly released from stimulated mast cells, plays important pathophysiological roles in central and peripheral tissues. The pathophysiological functions of histamine are mediated through four distinct G-protein coupled receptors that are classified as H1, H2, H3 and H4\cite{25-27}. Previously inhibition of histamine H1 and H4 receptors were reported to attenuate the progression of UC by inhibiting translocation of NF-kB to nucleus thereby inhibiting release of IL-6 and hence inflammation.
In recent years, there has been renewed interest in plant medicine for the treatment against different diseases, as herbal drugs are generally out of toxic effect reported from research work conducted on experimental animal models[28]. Herbal medicines are recognized by WHO as an essential building block for primary health care, especially in developing countries like India, but the herbal and other indigenous sources have not adequately been explored for the presence of safe and effective plants for the treatment of inflammatory bowel disease.

*Oroxylum indicum* (Syonakh) is a traditional herbal medicine in India, China and Japan, belonging to family Bignoniaceae. It is one of important ingredients in most commonly used Ayurvedic preparation, named as “Dasamula”. Root bark is also been used in other Ayurvedic formulation such as Amartarista, Dantyadyarista, Narayana Taila, Dhanawantara Ghrita, Brahma Rasayana, Chyavanaprasa Awalwha, etc. The plant is reported in Indian ancient text “Ayurveda” to possess diuretic and antibacterial activity[29]. It have been used as an analgesic, antitussive and anti-inflammatory agent for treatment of cough, bronchitis and other diseases[30]. These findings form a good basis for its use in Inflammatory bowel disease.

*Aconitum heterophyllum* Wall commonly known as “Atis” or “Patis” belongs to family Ranunculaceae and is reported for its medicinal and pharmaceutical values since long. The roots which have been used mostly as poison than as drug are now reported to possess significant antipyretic and analgesic properties with a high therapeutic index[31]. The plant was used for treatment of diseases of nervous system, digestive system, rheumatism and fever[31]. It also exhibits anti-fungal and antibacterial activities[31] which indicate its usefulness as a promising drug in treatment of IBD.

*Aegle marmelos* (L.) Correa commonly known as Bael/Bilva belonging to the family Rutaceae has been widely used in indigenous systems of Indian medicine due to its various medicinal properties. The decoction of root and root bark is useful in intermittent fever and unripe fruit is said to be an excellent remedy for diarrhoea, especially useful in chronic diarrhoeas. Additionally, *A. marmelos* has been shown to be effective in experimental models of inflammatory bowel syndrome and physiological diarrhoea[32]. Identification and isolation of active fraction useful against IBD was not reported in previous literature.
Aim of current study was to evaluate effect of some plants like *Oroxylum indicum*, *Aconitum heterophyllum* and *Aegle marmelos* against experimentally induced Inflammatory Bowel Disease. Efforts were made to fractionate the aqueous extract of *Oroxylum indicum* and methanolic extract of *A. marmelos* by using bioassay guided fractionation method and evaluate potential fractions against colitis associated colon cancer in rats.
2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND EXTRACT PREPARATION

2.1.1 Aconitum heterophyllum

2.1.1.1 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).

2.1.1.2 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\textsubscript{aq}) and administered in different doses.

2.1.2 Oroxylum indicum

2.1.2.1 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).

2.1.2.2 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\textsubscript{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.

2.1.3 Aegle marmelos

2.1.3.1 Plant material

Fruits of Aegle marmelos were collected from Bardoli region. The plant was authentified 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).

2.1.3.2 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.

2.2 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 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[33].

Powdered roots of *Aconitum heterophyllum*, root bark of *Oroxylum indicum* and fruits of *Aegle marmelos* were used to determine ash value (like total ash, water soluble and acid insoluble ash), extractive values, loss on drying and were characterized for various chemical constituents like Alkaloids, Glycosides, Phenolic compounds, amino acid, carbohydrate, flavonoids, proteins, steroids and triterpenoids[33].

2.3 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.

2.4 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.

2.5 PHARMACOLOGICAL EVALUATION

2.5.1 Oral toxicity study
Acute and chronic (repeated dose toxicity study for 28 days and 90 days) oral toxicity study of AH\textsubscript{aq} was performed as per OECD guidelines\textsuperscript{34}. In previous literature roots of \textit{A. heterophyllum} was reported to be toxic\textsuperscript{35} and hence AH\textsubscript{aq} was examined for its toxicity before administration. Acute oral toxicity of OI\textsubscript{aq} was not reported previously and evaluated in this study. Toxicity study of methanolic extract of \textit{A. marmelos} fruit was reported in previous literature\textsuperscript{36} and not done here.

2.5.2 DNBS-induced Colitis
Crude extracts of \textit{O. indicum} (OI\textsubscript{aq}) and \textit{A. heterophyllum} (AH\textsubscript{aq}) were tested against DNBS induced colitis in rats. Methanolic extract of roots of \textit{A. marmelos} was reported previously to be protective in animal model of experimental colitis by Dr. Gandhi and co-workers\textsuperscript{37} and hence not studied here.

2.5.2.1 Groups and Drug Treatments
Animals were divided in nine groups of six animals each and treated as follows:

\textbf{Group 1:} Control – No drug/extract treatment. Normal animals.


\textbf{Group 3:} Drug Control – DNBS treated. 5-Aminosalicylic acid 100 mg/kg p.o.
**Phytopharmacological evaluation of some plants useful in treatment of Inflammatory Bowel Disease**

**Group 4:** OI<sub>aq</sub> 100 mg/kg/day – DNBS treated. OI<sub>aq</sub> 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 5:** OI<sub>aq</sub> 200 mg/kg/day – DNBS treated. OI<sub>aq</sub> 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<sub>aq</sub> 400 mg/kg/day – DNBS treated. OI<sub>aq</sub> 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<sub>aq</sub> 100 mg/kg/day – DNBS treated. AH<sub>aq</sub> 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<sub>aq</sub> 200 mg/kg/day – DNBS treated. AH<sub>aq</sub> 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<sub>aq</sub> 400 mg/kg/day – DNBS treated. AH<sub>aq</sub> 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.

### 2.5.2.2 Induction of colitis

Colitis was induced using technique of acid induced colon inflammation, as described by Cuzzocrea et al.<sup>[38]</sup>. 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 sacrificed 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.

### 2.5.2.3 Parameters

Parameters like body weight, stool consistency, food intake, damage lesion area were measured. Colon were isolated and processed for histopathology. Levels of malondialdehyde, myloperoxydase, nitric oxide and GSH were measured in colonic tissue.<sup>[38]</sup>
2.5.3 Preliminary activity for effectiveness of fractions extracted

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\textsuperscript{[38]}. 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\textsuperscript{[39]} (Similar procedure was followed as described in ‘Damage lesion area’ in DNBS induced colitis section).

2.5.4 Colitis associated Colon Cancer

2.5.4.1 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\textsuperscript{[40]}. 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.

2.5.4.2 Determination of NF-κB translocation$^{[40]}$

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).

2.5.4.3 Determination of IL-6 levels$^{[40]}$

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).

2.5.5 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.
2.5.5.1 Effect on isolated rabbit ileum[41]

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.

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.

2.5.5.2 Inhibition of store operated Ca2+ release operated Ca2+ channels[42]

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.

After the treatment of CPA and carbachol (as above); increasing concentrations of Ca2+ 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 Ca2+ channels.
2.5.5.3 **Inhibition of Histamine H1 receptor**[^43]

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), 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. 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.

2.5.6 **Anti-diarrhoeal activity**

Diarrhoea is one of major symptoms of IBD. Crude extract of *O. indicum* (OI) was evaluated for its anti-diarrhoeal potential which will provide symptomatic relief against IBD. *A. heterophyllum*[^44] and *A. marmelos*[^32] had anti-diarrhoeal potential and reported in previous literature.

2.5.6.1 **Antibacterial and antifungal activity**

2.5.6.1.1 **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.

2.5.6.1.2 **Disc diffusion method**[^45]

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°C. Culture media and distilled water were sterilized at 115°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 Mc

[^43]: 2.5.5.3 Inhibition of Histamine H1 receptor
[^44]: 2.5.6 Anti-diarrhoeal activity
[^45]: 2.5.6.1.2 Disc diffusion method
Farland 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 µl) prepared at the concentrations of 2.5 and 25 mg/ml. The reference drugs were gentamicin (10 and 100 µg/ml), and nystatine (50 and 500 µg/ml). After moistening the discs, they were immediately transferred to the inoculated solid media. The plates were incubated at 37 °C for 24 and 48 h for the bacteria and fungus, respectively. The results were recorded as percentage potency of the drug.

2.5.6.2 Castor oil induced diarrhoea

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.

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\textsubscript{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.

2.5.6.3 Magnesium sulphate induced diarrhoea

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\textsubscript{aq} 100 mg/kg/day – Magnesium sulphate 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 – Magnesium sulphate 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 – Magnesium sulphate treated. OI\textsubscript{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\textsubscript{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\cite{46}.

2.5.6.4 Gastrointestinal Transit\cite{47}

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\textsubscript{aq} 200 mg/kg/day – Croton oil treated. OI\textsubscript{aq} 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\textsubscript{aq} 400 mg/kg/day – Croton oil treated. OI\textsubscript{aq} 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\textsubscript{aq} 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\textsuperscript{-1}; 10% charcoal suspension in 5% gum Arabic). After 20 min, mice were killed 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\cite{47}.

### 2.5.6.5 Colonic propulsion\cite{47}

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\textsubscript{aq} 400 mg/kg – OI\textsubscript{aq} 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\cite{47}.

### 2.5.6.6 Normal defecation\cite{48}

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\cite{48}.

### 2.5.6.7 Gastric emptying\cite{48}

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: $\text{OI}_{aq}$ 400 mg/kg – $\text{OI}_{aq}$ 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\[^{[48]}\].

2.5.6.8 Intestinal fluid accumulation\[^{[47]}\]

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: $\text{OI}_{aq}$ 200 mg/kg – $\text{OI}_{aq}$ was administered in dose 200 mg/kg p.o. 60 min before administration of castor oil (2 ml/rat).
Group 3: $\text{OI}_{aq}$ 400 mg/kg – $\text{OI}_{aq}$ 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 killed by cervical dislocation. Small intestine was clamped at pyloric valve and ilio–caecal junction and removed carefully from abdomen. Small intestine was weighed ($W_1$), emptied of fluid, reweighed ($W_2$) and length ($L$) measured. The difference in weight divided by length showed “enteropooling” in mg of fluid per centimeter of intestine.

$$\text{Enteropooling} = \frac{W_1 - W_2}{L}$$

2.6 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.
3 RESULTS AND DISCUSSION

3.1 STANDARDIZATION OF PLANT MATERIAL

It is of utmost important to standardize the plant material since it may vary in its phytochemical content according to diverse places of collection, with different times in a year for collection, with collection at the same time and places but in different years and with different environmental factors surrounding the cultivation of a particular medicinal plant. These phytochemical alterations may vary its therapeutic effect as well and hence standardization of plant material used in this study was done with various methods like ash value, extractive value, loss on drying and phytochemical screening for various constituents.

*O. indicum* was shown Total ash – 13.85 %w/w, Acid insoluble ash – 6.68%w/w and Water soluble ash – 11.03%w/w, while *A. heterophyllum* revealed total ash – 3.84%w/w, Acid insoluble ash – 1.06%w/w, Water soluble ash – 8.94%w/w and *A. marmelos* was found to have Total ash – 3.85%w/w, Acid insoluble ash – 2.05%w/w, Water soluble ash – 2.9%w/w.

Extractive values was found to be - *O. indicum* (Ethanol soluble – 7.6%w/w, Water soluble 19.59%w/w), *A. heterophyllum* (Ethanol soluble – 13.85%w/w, Water soluble 27.64%w/w), *A. marmelos* (Ethanol soluble – 24.76%w/w, Water soluble 13.65%w/w)

*O. indicum, A. heterophyllum* and *A. marmelos* were shown 5.6 %w/w, 3.8%w/w and 6.2%w/w of loss on drying.

Most of the drugs have definite specific chemical constituents to which their biological or pharmacological activity is attributed. And hence its qualitative characterization should be done before its pharmacological evaluation. The phytochemical screening of *O. indicum, A. marmelos* and *A. heterophyllum* were done and revealed presence of various constituents expressed in Table 3.1, 3.2 and 3.3 respectively.
**Table 3.1: Phytochemical screening of *Oroxyllum indicum* fractions**

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>OI-A</th>
<th>OI-B</th>
<th>OI-C</th>
<th>OI-D</th>
<th>OI-E</th>
<th>OI-F</th>
<th>OI-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds &amp; Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3.2: Phytochemical screening of *Aegle marmelos* fruit pulp powder**

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>AM-A</th>
<th>AM-B</th>
<th>AM-C</th>
<th>AM-D</th>
<th>AM-E</th>
<th>AM-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds &amp; Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Phytopharmacological evaluation of some plants useful in treatment of Inflammatory Bowel Disease

Table 3.3: Phytochemical screening of *A. heterophyllum*

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th><em>A. heterophyllum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td>+</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>-</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Phenolic compounds &amp; Tannins</td>
<td>+</td>
</tr>
</tbody>
</table>

Variations in quantity of extract in specific solvent can be attributed to number of contents present, polarity of contents, properties of solvent used and varying pH and temperature at the time of extraction. The quantity is expressed in % yield (w/w) of crude extract. Crude powder of *O. indicum, A. heterophyllum* and *A. marmelos* were extracted in various solvents by using bioassay guided fractionation method and results were summarised in Table 3.4, 3.5 and 3.6 respectively.

Table 3.4: Percentage yield of aqueous extract of root bark of *O. indicum* and its fractions

<table>
<thead>
<tr>
<th>Extract</th>
<th>Fractions</th>
<th>% yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract of dried root bark of <em>Oroxylum indicum</em> (OI-A)</td>
<td>OI-B</td>
<td>14.38</td>
</tr>
<tr>
<td>OI-A was dissolved in three solvents</td>
<td>OI-C</td>
<td>16.87</td>
</tr>
<tr>
<td></td>
<td>OI-D</td>
<td>38.34</td>
</tr>
<tr>
<td>OI-D was fractioned by column chromatography</td>
<td>OI-E</td>
<td>3.56</td>
</tr>
<tr>
<td></td>
<td>OI-F</td>
<td>6.37</td>
</tr>
<tr>
<td></td>
<td>OI-G</td>
<td>1.93</td>
</tr>
</tbody>
</table>
Table 3.5: Percentage yield of methanolic extract of dried fruits of *A. marmelos* and its fractions

<table>
<thead>
<tr>
<th>Extract</th>
<th>Fractions</th>
<th>% yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extract of dried fruits of <em>A. marmelos</em> (AM-A)</td>
<td>AM-B</td>
<td>21.17</td>
</tr>
<tr>
<td>AM-A was dissolved in three solvents</td>
<td>AM-C</td>
<td>11.65</td>
</tr>
<tr>
<td></td>
<td>AM-D</td>
<td>34.87</td>
</tr>
<tr>
<td>AM-C was fractioned by column chromatography</td>
<td>AM-E</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>AM-F</td>
<td>4.78</td>
</tr>
</tbody>
</table>

Table 3.6: Percentage yield of aqueous extract of roots of *A. heterophyllum*

<table>
<thead>
<tr>
<th>Extract</th>
<th>% yield (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract of dried roots of <em>A. heterophyllum</em></td>
<td>28.89</td>
</tr>
</tbody>
</table>

3.2 **Oral Toxicity Study**

Previous literature considered roots of *A. heterophyllum* as toxic (they contain an alkaloid aconitine) and following ingestion of roots, toxicity manifests in form of tingling numbness of mouth and throat, abdominal pain, loss of muscle power, visual and auditory disturbances and finally clonic convulsions\[35\]. And hence 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\[34\] before administration.

Acute toxicity study showed that AH\(_aq\) did not cause any mortality up to 5000 mg/kg. Animals were observed for signs of side effects or adverse effects after administration of AH\(_aq\) for 4 hrs, after 24 hrs and after 15 days. No adverse effect was seen and hence AH\(_aq\) was considered safe. Limit test for repeated dose oral toxicity assay was performed with dose 1000 mg/kg/day on both sexes of rats. No toxicity was observed in rats for 28 days and 90 days in two different treatment studies. Change in body weight was in accordance with body weight change in control group rats. There was no alteration in haematological, hepatic and kidney function parameters in rats treated with AH\(_aq\) for 28 as well as 90 days. No significant
difference was noted in weight of any organ from AH\textsubscript{aq} treated animals when compared with control.

Acute oral toxicity of OI\textsubscript{aq} was not reported previously and evaluated in this study that showed OI\textsubscript{aq} did not cause any mortality up to 5000 mg/kg. Animals were observed for signs of side effects or adverse effects after administration of OI\textsubscript{aq} for 4 hrs, after 24 hrs and after 15 days. No adverse effect was seen and hence OI\textsubscript{aq} was considered safe.

3.3 Effect of Oroxylum indicum against DNBS induced colitis

Gross macroscopic inspection of cecum, colon and rectum of DNBS treated rats revealed presence of mucosal congestion, erosion, hemorrhagic ulcerations and severe inflammation. Colon appeared flaccid and filled with liquid stool. All rats had diarrhoea and a significant reduction in body weight (compared with normal group of rats). A significant increase in weight of spleen (an indicator of inflammation), distal colon (an indicator of tissue oedema) along with decrease in food intake was noted in vehicle treated rats received DNBS. Histopathological features included trans-mural necrosis, along with extensive morphological disorientation, oedema and diffuse leukocyte cellular infiltrate as well as lymphocyte in submucosa of colon section. Inflammatory response on day 4 after induction of colitis by administration of DNBS was supported by significant rise in MDA, MPO and NO levels while decrease in GSH levels.

Rats pre-treated with OI\textsubscript{aq} significantly attenuated extent and severity of symptoms dose dependently. Body weight was improved significantly especially with dose 400 mg/kg (0.55±0.67 %) as compared with model control (-8.04±3.25). Food intake did not show any significant change but colon weight (3.44±0.36g in 400mg/kg OI\textsubscript{aq} and 4.39±0.35g in model control), spleen weight (0.66±0.16g in 400mg/kg OI\textsubscript{aq} and 1.49±0.13g in model control) and stool consistency (1.66±0.96 in 400mg/kg OI\textsubscript{aq} and 7.33±1.63in model control) showed significant alteration dose dependently (especially at dose 400 mg/kg). Colonic lesion area was found to be decreased dose dependently in OI\textsubscript{aq} treated rats with maximum reduction in 400mg/kg dose (3.83±2.4 sq. mm.) when compared with model control (76.76±6.33 sq. mm.). MDA, MPO and NO levels were found to be decreased significantly as compared with model control and the reduction was dose dependent. GSH levels were found to be increased significantly which is an indicator of protection against oxygen free radicals.
3.4 **EFFECT OF ACONITUM HETEROPHULLUM AGAINST DNBS-INDUCED COLITIS**

Rats pre-treated with AH<sub>aq</sub> attenuated extent and severities of symptoms dose dependently but overall results were not significant. Body weight was improved significantly especially with dose 400 mg/kg (-3.32±0.64%) as compared with model control (-8.04±3.25%). Food intake did not show any significant change but colon weight (2.89±0.48g in 400mg/kg AH<sub>aq</sub> and 4.39±0.35g in model control), spleen weight (1.01±0.21g in 400mg/kg AH<sub>aq</sub> and 1.49±0.13g in model control) and Stool consistency (4.00±2.19 in 400mg/kg AH<sub>aq</sub> and 7.33±1.63 in model control) showed significant alteration at dose 400 mg/kg only. Colonic lesion area was found to be decreased dose dependently in AH<sub>aq</sub> treated rats with maximum reduction in 400mg/kg dose (34.73±4.00 sq. mm.) when compared with model control (76.76±6.33 sq. mm.). MDA, MPO and NO levels were found to be decreased when compared with model control. The reduction was found to be dose dependent showing maximum reduction at 400mg/kg dose. GSH levels were found to be increased (not significantly).

3.5 **PRELIMINARY ACTIVITY FOR EFFECTIVENESS OF FRACTIONS EXTRACTED**

*O. indicum* was fractioned in OI-B, OI-C and OI-D which were administered in dose 100 mg/kg p.o. in animals treated with DNBS. Comparatively OI-D fraction was found to reduce colitis associated diarrhoea. OI-D also decreased colonic mucosal damage in DNBS treated rats. OI-D was fractioned by column chromatography and probably chrysin & biochanin-A rich fraction was obtained as OI-E, baicalein rich fraction was obtained as OI-F and ellagic acid rich fraction was obtained as OI-G, depending previous reports on isolation and TLC study performed at our place. Amongst these three fractions, OI-F showed better activity and selected for further study.

When administered in dose 100 mg/kg p.o. fraction AM-C of *A. marmelos* was found to be comparatively potent than Fraction AM-B and AM-D in DNBS animals treated. Fractions AM-E and AM-F were obtained from AM-C by column chromatography and administered in dose 50 mg/kg p.o. AM-E was found to decrease colonic mucosal damage in DNBS treated rats.

3.6 **EFFECT OF OI-F AND AM-E AGAINST COLITIS ASSOCIATED COLON CANCER IN RATS**

Treatment with AOM followed by DSS, induced colitis associated colon cancer, characterized by decrease in length of colon, tumour formation, bloody diarrhoea and colonic
damage in rats. OI-F and AM-E fractions were found to inhibit progression of inflammation associated colon cancer.

In model control, length of colon was decreased along with formation of tumour like mass in perianal portion. Survival was decreased (06 out of 10 survived), 100% incidence of tumour was found and number of tumours per colon was found to be increased (8.6±1.83). Body weight decreased and stool consistency score was found to be increased along with increase in colonic lesion area.

In OI-F and AM-E treated rats, length of colon was normalised, formation of tumour like mass in perianal portion was decreased significantly. Survival was increased, 09 out of 10 survived in OI-F treated while 10 out of 10 survived in AM-E treated rats. Incidence of tumour was decreased up to 60% in OI-F and 30% in AM-E treated animals. Number of tumours per colon was found to be decreased (4.5±0.92 in OI-F and 2.8±0.82 in AM-E treated rats). Body weight was found to be normalised and stool consistency score was decreased along with decrease in colonic lesion area. OI-F and AM-E showed to decrease NF-κB (92.73±8.36 ng/gm of tissue in OI-F treated and 75.36±11.45 ng/gm of tissue in AM-E treated rats) and IL-6 levels (226.74±9.27 ng/gm of tissue in OI-F treated and 162.64±11.37 ng/gm of tissue in AM-E treated rats) as compared with model control (NF-κB - 119.63±9.22 ng/gm of tissue; IL-6 - 234.62±9.35 ng/gm of tissue).

3.7 IN VITRO EXPERIMENTS

In-vitro experiments were done to get an idea about mechanism of action of fractions involved in attenuation of symptoms of UC as well as protection against inflammation associated colon cancer progression in rats.

OI-F (1–1000 mg/ml) in a concentration dependent manner inhibited contractions induced by acetylcholine, barium chloride or by EFS in isolated rabbit ileum. Though, inhibition was not 100% in any case. The plant extract was more active in inhibiting contractions induced by acetylcholine (or barium chloride) than contractions induced by EFS. Verapamil (blocker of L-type Ca\textsuperscript{2+} channels) potentiates inhibitory effect of OI-F on acetylcholine-induced contractions in isolated rabbit ileum.

Dysregulated Ca\textsuperscript{2+} responses have been associated with several autoimmune and inflammatory diseases, including IBD\textsuperscript{[49]}. Consequently, CRAC channels have been proposed
as potential target in the therapeutic management of autoimmune and inflammatory disorders [22].

In present study OI-F fraction obtained from *O. indicum* was found to inhibit CRAC channel, thereby inhibiting T cell activation and ultimately experimentally induced colitis. Chrysin, a major component of OI-F, was reported previously for decreasing intracellular calcium levels in mast cell and thereby inhibiting mast cell degranulation[50], which leads to inhibition of nuclear factor-κB and caspase-1 dependent release of pro-inflammatory cytokines like tumor necrosis factor-α, IL-1β, IL-4, and IL-6 in mast cells[50]. We propose OI-F act by inhibiting CRAC channels in colonic cells, thereby inhibiting nuclear factor-κB and IL-6, ultimately delaying the progression of inflammation and colon cancer in rats.

AM-E was found to have no effect on contractions induced by acetylcholine, barium chloride or by EFS and hence different approach was tried to found its mechanism against UC and CAC. Phytosterol rich AM-E fraction, obtained from *A. marmelos*, was tested against and found to inhibit response of histamine on H1 receptors dose dependently, thereby inhibiting the progression of UC. Sterol especially β-sitosterol and stigmasterol (probable contents of AM-E) were reported previously to inhibit acute and chronic inflammation by suppressing cytokine generation. These sterols were also reported to inhibit release of histamine in local lung tissues[51]. We propose, phytosterol rich AM-E fraction, inhibits histamine H1 receptor, thereby inhibits activation of various cytokines and inhibits the progression of colitis.

3.8 EFFECT OF OI<sub>aq</sub> AGAINST EXPERIMENTAL DIARRHOEA IN RATS AND MICE

Croton oil is well known irritant used experimentally to induce inflammation in mouse small intestine. This inflammation is characterized by disruption of mucosa and infiltration of lymphocytes into submucosa associated with increase of intestinal transit[47]. OI<sub>aq</sub> administration, in croton oil untreated animals, had no effect on upper GI motility, both in upper gastrointestinal tract (37.11±1.17% distance travelled by marker) and in large intestine (5.81±0.42 min to expulsion of glass bid inserted intrarectally). However gastrointestinal motility was inhibited in animals treated with croton oil (upper GI tract - 74.73±2.21% distance travelled by marker; Colonic propulsion - 10.42±0.33 min).

It is well known that drugs which inhibit intestinal transit in pathophysiological state may be effective in alleviating diarrhoea[47]. In addition, because diarrhoea is a major pathophysiological feature in patients with inflammatory bowel disease, we evaluated
potential antidiarrhoeal effect of OIaq against castor oil induced and magnesium sulphate induced diarrhoea.

OIaq demonstrated remarkable dose-dependent reduction in castor oil-induced and magnesium sulphate induced diarrhoea. It also significantly inhibited castor oil-induced intestinal fluid accumulation (enteropooling) and weight of intestinal content.

Besides other reasons, major causative agents of diarrhoea in human include hyperactivation of commensal bacteria: *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli*[^45^]. *Candida albicans* has also been known to cause diarrhoea in humans[^52^]. OIaq was evaluated for its antimicrobial potential against these microorganisms in disc diffusion assay. Extract was found to inhibit growth of microorganisms with maximum activity against *S. aureus* (70.79%). It also inhibited growth of *C. albicans* to sufficient extent (45.71%). Antibacterial and antifungal activity demonstrated by OIaq can be ascribed to presence of chrysin and baicalein as well as biochanin-A which was previously reported to have antimicrobial activity.

To sum up, OIaq was found to be protective against DNBS induced colitis by inhibiting macroscopic and microscopic damage to colon, weight loss, diarrhoea, MPO, MDA and NO levels with increase in natural antioxidant GSH levels in rats. The activity can be ascribed to combined effect of baicalein, chrysin and biochanin-A found in OIaq. It also reduced castor oil and magnesium induced diarrhoea, a relevant finding in light of fact that OIaq administration is not associated with constipating effects under physiological conditions. AHaq was found to be non toxic and have protective effect against DNBS induced colitis. The activity can be ascribed to flavonoids present in it. Isolated fractions OI-F and AM-E was found to inhibit the progression of Colitis associated colon cancer by inhibiting CRAC channels and Histamine H1 receptor respectively.
4 BIBLIOGRAPHY

2 Kamm MA. Inflammatory Bowel Disease, 2 edn.: Taylor & Francis, 1999.
Phytopharmacological evaluation of some plants useful in treatment of Inflammatory Bowel Disease