Original Article

PHYTOPHARMACOLOGICAL EVALUATION OF HERBAL PLANTS USED IN TREATMENT OF INFLAMMATORY BOWEL DISEASE

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Abstract

Ethanolic extract of *T. Portulacastrum* and pet. Ether extract of M. Laxiflora was evaluated in mice against trinitrobenzene sulfonic acid (TNBS) induced colitis. Mice were pre-treated orally for 14 days and continued for four days after the induction of colitis with (10, 50, and 100 mg/kg). Colitis was induced by intracolonic instillation of 20 mg of TNBS per mice dissolved in 50% alcohol and 4 days later, the colonic mucosal lesion was analyzed along with Diarrhoea, GI transit, defecation, gastric emptying, intestinal fluid accumulation. myeloperoxidase (MPO) activity, malondialdehyde (MDA) levels, reduced glutathione (GSH), and nitric oxide levels in colonic tissue homogenate. Significant reduction in gross damage area, weight loss and decrease in colonic lesion were evident in test substance-pretreated animals dose dependently as compared to samle treated control. These effects were confirmed biochemically by a reduction in colonic myeloperoxidase activity, malondialdehyde levels, nitric oxide levels, and increase in reduced glutathione (GSH) levels. Furthermore, microscopic examination revealed diminution of inflammatory cell infiltration and submucosal edema in colon segments of mice treated with MP and TP . The results demonstrate the protective effect of both sample in the animal model of acute colitis possibly through an antioxidant, anti-lipoperoxidative or due to reduction in nitric oxide generation.

Keywords: Colitis, TNBS ,Diarrhoea, Gastric Emptying, Edema.

Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder of the small intestine and colon with enhanced risk of colorectal cancer (CRC). IBD affects millions of people and mainly occurs in genetically predisposed individuals having dysregulated immune response to various environmental conditions [1–3]. The burden of IBD is quite high, both on patient's quality of life and on the health care system, with estimated hospitalization rates of 8.2–17 per 100,000 annually and annual treatment costs of $6.8 billion [4]. Genetic, immunological, and environmental factors play important roles in its etiology [5]. Pathogenesis of IBD is not fully understood but two broad hypotheses have arisen regarding its fundamental nature. The first contends that the primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microflora. The second suggests that changes in the composition of gut microflora and/or deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system. Currently, it is well accepted that IBD is indeed characterized by an abnormal mucosal immune response but that microbial factors and epithelial cell abnormalities can facilitate this response [13]. So, a complex interplay between genetic predisposition, environmental trigger, and an aberrant immune reaction contributes to disease initiation and its progression [14,15]. The most common symptoms include regular abdominal pain, fever, vomiting, diarrhea, blood in the stool, and weight loss with enhanced risk of colorectal cancer [6,7] The factors

involved in intestinal inflammation include altered synthesis and release of pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, and IL-12), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), transforming growth factor (TGF)-β, and increased reactive oxygen species (ROS) that results in excessive damage to the intestinal tissues [6,8,9]. In IBD, intestinal permeability is predominantly abnormal with increased appearance of immune cells and increased mucus production [10–12].

Several recent reports document use of natural phytochemicals in IBD that possess anti-inflammatory and antioxidant activity such as flavonoids and phenolic compounds. They modulate various inflammatory mediators, such as IL-1β, IL-6, IL-10, TNF-α, prostaglandin E2 (PGE-2), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [10,11]. Additionally, natural macromolecules are also tested in suppression of IBD-associated biochemical and molecular inflammatory pathways. Recently, antioxidant and anti-inflammatory properties of biophenols derived from fruits and vegetables have been considered beneficial for IBD through various in vitro and in vivo studies [10] In general, currently used medical therapy in IBD consists of salicylates, corticosteroids, immunomodulators, and biological therapy. These drug treatments have the aim to induce and maintain the patient in remission and ameliorate the disease's secondary effects, rather than modifying or reversing the underlying pathogenic mechanism [16]. Their use may result in severe side effects and complications, such as an increased rate of malignancies or infectious diseases [17]. Drug delivery to the appropriate site(s) along the gastrointestinal tract also has been a major challenge, and second-generation agents have been developed with improved drug delivery, increased efficacy and decreased side effects. [18]

In present study attempt has been made to cope up with the need of alternative herbal medicine for treatment of IBD, which is devoid of side effects, do not show resistance or intolerance and cheaper as compared to current medication available. Meyna laxiflora (Rubiaceae) and trianthema portulacastrum (Aizoaceae) is a traditional herbal medicine in India reported in Indian ancient text "Ayurveda" to possess anti-diarrhoeal and antibacterial activity.[20] It has been used as an analgesic and anti-inflammatory agent. by traditional medical practitioner This forms a good basis for its use as a curative drug against IBD. Different parts of *Trianthema portulacastrum Linn*.(Aizoaceae) are traditionally used as analgesic, stomachic, laxative, treatment of blood disease, anemia, inflammation, and night blindness. Laboratory investigations on extracts of the plant have demonstrated significant pharmacological activities, such as antioxidant, diuretic, analgesic, hepatoprotective, and anticarcinogenic.[19]

Materials and methods

- **Animals**

Albino rats (Sprague-Dawley) and aged between 8 - 10 weeks were chosen for the experiments. These mice were acclimatized for 14 days in the laboratory before the commencement of the experiment. The animals were maintained at a relative humidity of 50-55% and temperature 22-24°C with a 12 hr light/ dark cycle. Normal drinking water and the commercially available pellet were given as a diet. 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.[27] Animal handling and study procedures were followed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.(reference no. CPCSEA/IAEC/DIPS/06/22/35).

- **TNBS-induced Colitis**

Crude extracts of M. laxiflora and T. portulacastrum were tested against TNBS induced colitis in rats.

- ***Groups and Drug Treatments***

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

Group 1: control : No drug treatment/Water treated

Group 2: Model Control : TNBS treated. No drug/extract treatment.

Group 3: Standard : TNBS treated. 5-Aminosalicylic acid 100 mg/kg p.o.

Group 4: ML 10 mg/kg/day –TNBS treated. **ML** was administered in dose 10, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after TNBS treatment.

Group 5: ML 50 mg/kg/day –TNBS treated. **ML** was administered in dose 50 mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after TNBS treatment.

Group 6: ML 100 mg/kg/day –TNBS treated. **ML** 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 TNBS treatment.

Group 7: TP 10 mg/kg/day –TNBS treated. **TP** was administered in dose 10, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after TNBS treatment.

Group 8: TP 50 mg/kg/day –TNBS treated. **TP** was administered in dose 50, mg/kg/day p.o. for 7 days prior to induction of colitis and continued for next 4 days after TNBS treatment.

Group 9: TP 100 mg/kg/day –TNBS treated. **TP** 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 TNBS treatment.

- **Induction of colitis**

Trinitrobenzenesulfonic Acid (TNBS) was used as an agent to induce ulcerative colitis. Prior to induction, the mice were starved for a day with access to drinking water. A mixture of 100 µL of 5% TNBS and absolute ethanol (1:1 ratio) was intrarectally administered using a catheter. After administration, the mice were held vertically for 2-3 min for better distribution of TNBS. Animals were administered test samples at a dose of 100mg/kg for 7 days. At the end of the treatment period the animals were sacrificed by cervical dislocation and the colons were dissected out and cleaned with cold saline.[28]

- **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.

- **Biochemical estimation**

Myeloperoxidase (MPO) assay

The colonic tissues were weighed and homogenized in an ice-cold 50 mM Potassium

Phosphate buffer (pH 6.0) containing 0.5% Cetyltrimethyl ammonium bromide (CTAB). The tissue homogenate is then mixed with O-dianisidine, 50 mM Potassium Phosphate buffer, and diluted H2O2. A yellow compound is formed and it is spectrophotometrically measured at 450 nm.

Lipid Peroxidase assay (MDA)

Add 600 µL of TBA reagent into each well containing 200 µL sample. Incubate at 95°C for 60 minutes. Cool to room temperature in an ice bath for 10 minutes Take 200 µL of the reaction mix (containing MDA-TBA adduct) and add into a 96-well microplate for analysis. Measure absorbance immediately on a microplate reader at OD 532 nm for colorimetric assay.

Nitric oxide scavenging assay:

Nitric oxide scavenging activity can be estimated by the use of Griess IlIosvoy reaction.The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with extract of each plant were dissolved in methanol and incubated at 300C for 2 hours. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H3P04 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm.

GSH Assay

Glutathione is a key antioxidant that mostly exists in reduced form GSH. Reduced glutathione was determined Colon tissues were homogenized in a potassium phosphate buffer (10 mM). 1-chloro-2, 4-dinitrobenzene (CDNB) was added to the tissue homogenate. CDNB is a substrate that conjugates with GSH present in the sample to form a conjugate that has an absorption maxima at 340 nm. GST is measured in terms of free sulfhydryls (mM).

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 [xL/mg tissue) for 30 s. The crude homogenate was centrifuged at 30 000 g at 4°C for 30 min. Then, 200 iL of 40 g/L sulfosalicylic acid was added to 100 \iL of supernatant and allowed to stand on ice for 5 min to precipitate protein. The mixture was centrifuged again at 5000 r/min at 4°C for 10 min. Subsequently, 100 fiL of the de-proteinized supernatant was mixed well with 300 {xL of 125 mmol/L sodiimi phosphate buffer (pH 8) and 2 iL 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.

Anti-diarrhoeal activity

Diarrhoea is one of major symptoms of IBD. Crude extract of ML was evaluated for its anti-diarrhoeal potential which will provide symptomatic relief against IBD. ML had anti-diarrhoeal potential and reported in previous literature.

Gastrointestinal Transit

Swiss albino mice of either sex were divided in five groups of six animals each.

Normal defecation

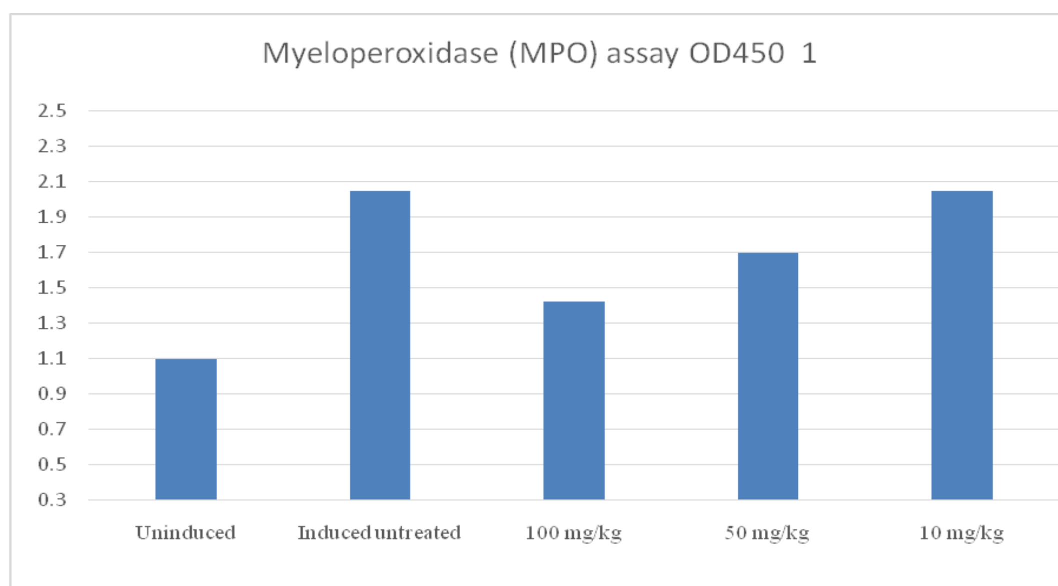
Swiss albino mice of either sex were divided in five groups.

Gastric emptying

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

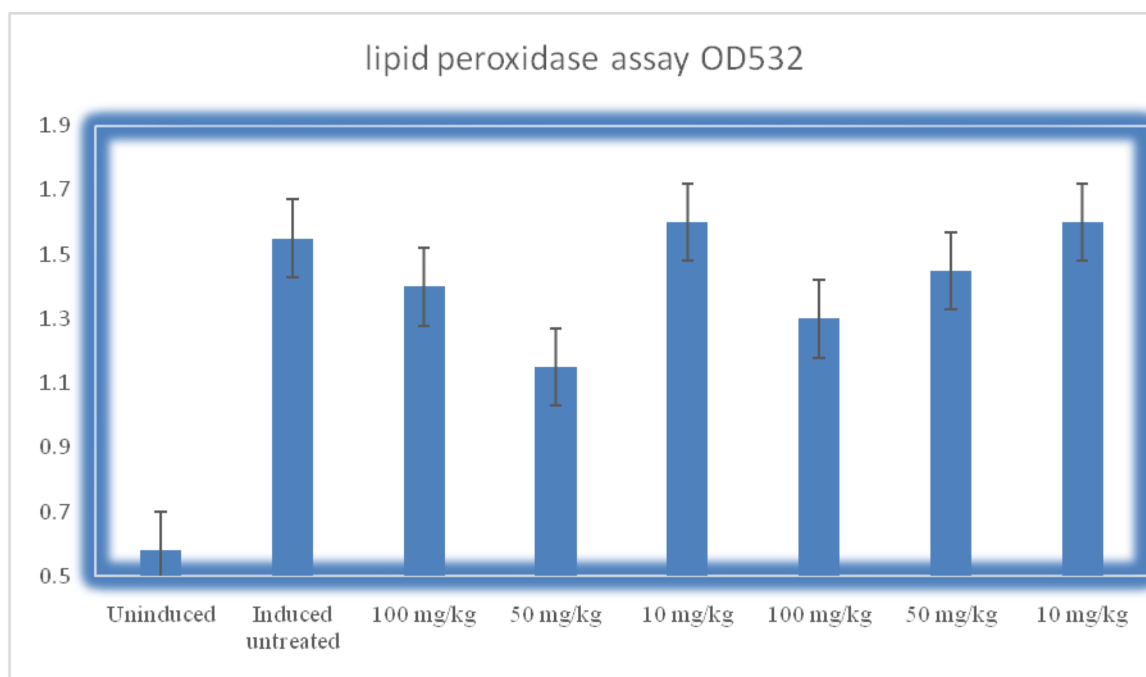
Intestinal fluid accumulation

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

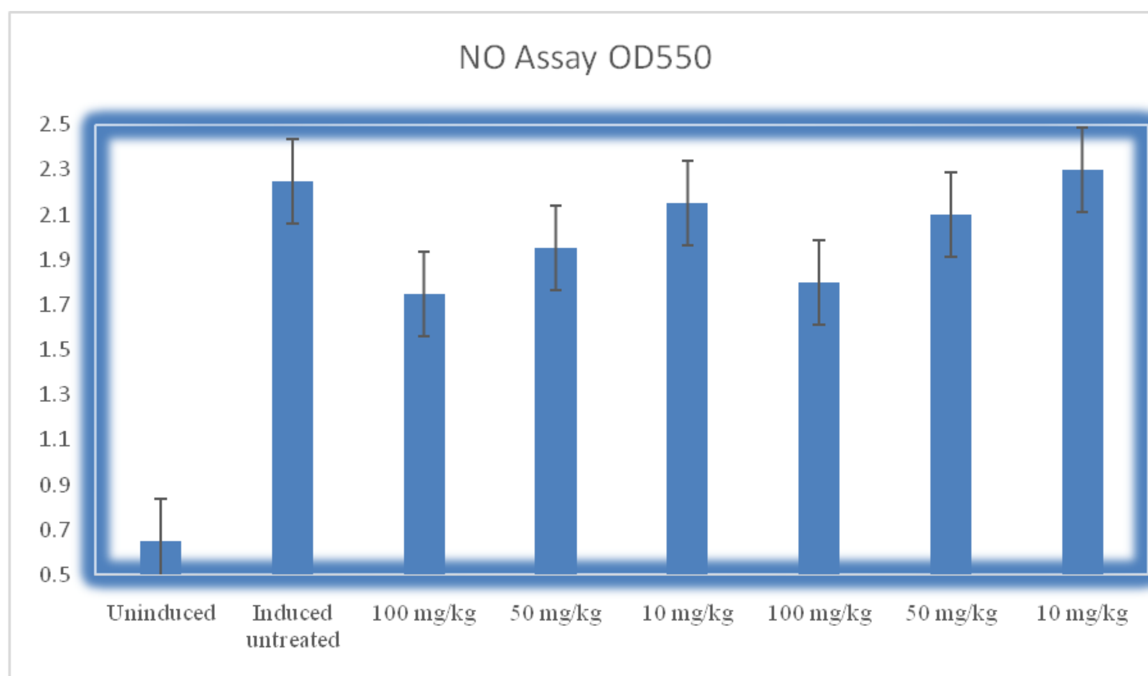


Myeloperoxidase (MPO) assay OD450 1									
Treatment		Animal						Average	STDEV
		1	2	3	4	5	6		
Uninduced		0.97	1.21	1.14	1.08	0.89	1.04	1.06	0.12
Induced untreated		2.14	2.16	2.24	2.09	2.28	2.31	2.20	0.09
Meyna laxiflora	100 mg/kg	1.62	1.65	1.58	1.12	1.14	1.53	1.44	0.24
	50 mg/kg	1.24	1.85	1.96	2.15	2.08	2.11	1.90	0.34
	10 mg/kg	1.82	2.06	2.24	2.16	2.14	2.35	2.13	2.13
Trianthema portulacastrum	100 mg/kg	1.57	1.48	1.67	1.52	1.37	1.38	1.50	1.11
	50 mg/kg	1.17	1.89	1.85	1.94	1.52	1.64	1.67	0.29

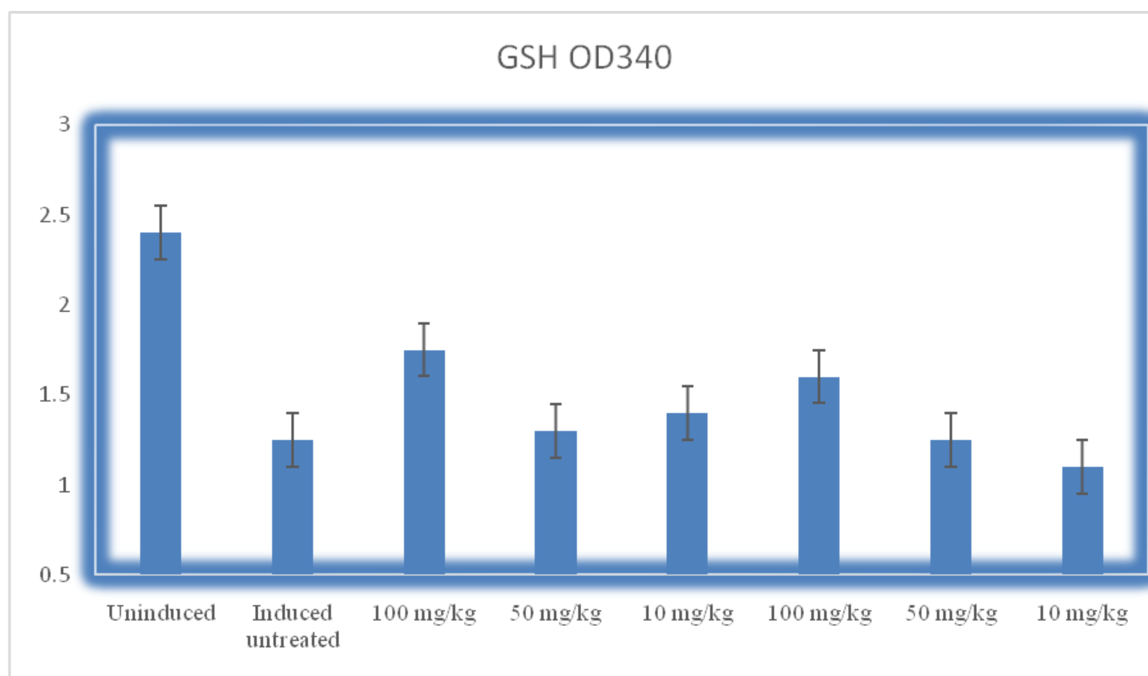
	10 mg/kg	2.31	2.14	2.16	2.24	2.18	2.21	2.21	0.06



lipid peroxidase assay OD532									
Treatment		Animal						Average	STDEV
		1	2	3	4	5	6		
Uninduced		0.58	0.48	0.62	0.59	0.52	0.66	0.58	0.07
Induced untreated		1.65	1.47	1.58	1.63	1.42	1.56	1.55	0.09
Meyna laxiflora	100 mg/kg	1.26	1.18	1.22	1.17	1.13	1.21	1.20	0.05
	50 mg/kg	1.42	1.38	1.36	1.44	1.46	1.39	1.41	0.04
	10 mg/kg	1.56	1.64	1.58	1.62	1.66	1.57	1.61	0.04
Trianthema portulacastrum	100 mg/kg	1.17	1.22	1.36	1.24	1.25	1.28	1.25	0.06
	50 mg/kg	1.47	1.48	1.32	1.43	1.52	1.35	1.43	0.08
	10 mg/kg	1.47	1.58	1.56	1.57	1.53	1.62	1.56	0.05



NO Assay OD550									
Treatment		Animal						Average	STDEV
		1	2	3	4	5	6		
Uninduced		0.75	0.64	0.71	0.58	0.55	0.76	0.67	0.09
Induced untreated		2.14	2.32	2.16	2.24	2.29	2.32	2.27	0.07
Meynalaxiflora	100 mg/kg	1.85	1.74	1.66	1.68	1.69	1.73	1.73	0.07
	50 mg/kg	2.12	2.18	1.95	1.62	1.66	2.31	1.97	0.28
	10 mg/kg	2.11	2.28	2.31	2.19	2.27	2.17	2.22	0.08
Trianthemaportulacastrum	100 mg/kg	1.75	1.62	1.66	1.82	1.77	1.84	1.74	0.09
	50 mg/kg	2.12	2.23	2.22	2.32	2.16	1.24	2.05	0.40
	10 mg/kg	2.11	2.36	2.41	2.32	2.16	2.22	2.2	0.12



GSH OD340									
Treatment		Animal						Average	STDEV
		1	2	3	4	5	6		
Uninduced		2.34	2.56	2.31	2.48	2.36	2.41	2.41	0.09
Induced untreated		1.35	1.28	1.17	1.32	1.28	1.21	1.27	0.07
Meynalaxiflora	100 mg/kg	1.84	1.81	1.76	1.78	1.64	1.69	1.75	0.08
	50 mg/kg	1.32	1.36	1.38	1.42	1.11	1.13	1.29	0.13
	10 mg/kg	1.42	1.5	1.22	1.31	1.36	1.38	1.34	0.07
Trianthemaportulacastrum	100 mg/kg	1.73	1.82	1.61	1.51	1.42	1.51	1.60	0.15
	50 mg/kg	1.21	1.23	1.25	1.45	1.06	1.12	1.22	0.13
	10 mg/kg	1.14	1.18	1.03	1.09	1.14	1.16	1.12	0.05

Result and discussion

Inflammatory bowel disease (IBD) results in a substantial burden to individuals and society, not only because of direct and indirect medical costs, but also by causing disability. The reduction in working capacity, especially in a young and active segment of the population, is the major economic and social burden of disease.[21]

In current study, aqueous extract of leaves M. laxiflora and trianthema portulacastrum were evaluated for their toxicity and found to be safe in acute and chronic toxicity protocols. This study provides scientific evidence for safety of these plant extracts and showed no mortality or morbidity in rats. These extracts were formed to be devoid of any adverse effects when evaluated by morphological, haematological and biochemical parameters.[22] TNBS treated mice showed decreased body weight and diarrhoea with pasty stools and presence of blood in stools. Treatment with ML and TP showed reduction in diarrhoea and improved body weight. Anorexia and malnutrition are the symptoms of chronic IBD due to nausea, vomiting, diarrhoea and abdominal pain. It is previously been reported that tissue Injury and infection trigger the release of proinflammatory cytokines that alter normal energy regulatory mechanisms resulting in anorexia, tissue catabolism, increased metabolic rate, and loss of body weight. Similar results were obtained in TNBS induced tissue injury and increased proinflammatory cytokines in rats.[23] In experimental colitis induced by TNBS, anorexia was reported in model control rats, while there was no significant alteration in food intake in ML and TP treated mice. This effect may be ascribed to protective and anti-inflammatory effects of these extracts. Animals treated with TNBS alone exhibited significant increase in colonic lesion area, compared with control. ML and TP treatment decreased colonic damage induced by TNBS. Histological sections from TNBS treated mice colon showed trans-mural necrosis, along with extensive morphological disorientation, oedema and diffuse leukocyte cellular infiltrate as well as lymphocyte in submucosa. While treatment with ML and TP reduced the disorientation of mucosa, prevented infiltration and oedema in higher dose levels (50 and 100 mg/kg doses). Weight of colon in TNBS treated mice was found to be increased, which is an indicator of oedema and increased fluid deposition in colonic tissue layers. Pretreatment with ML significantly decreased colon weight, compared with model control.

XO (xanthine oxidase) pathway seems to be discrete for UC. Reynolds and co-workers in one of clinical study illustrated that colonic XO activity was not elevated in UC patients.[24] It was suggested that XO may not be a major source of superoxide and ROS production in UC in present study MPO levels correlated well with severity of symptoms of TNBS induced colitis in mice[25]. Model control animals showed elevated activity of MPO in colon while pretreatment with ML and TP showed significantly reduction.

All these constituents phenolic compound, flavanoids (gallic acid) and lupeol affects synthesis of NO which justifies our observations in current study i.e. decreased NO production in mice colon due to pretreatment of MP and TP. Pathogenesis of IBD involves participation of Histamine and their receptors and was reported in previous literature . Previous studies showed that decrease in colonic GSH concentration following colitis induction is due to over-production of ROS that deplete GSH by inhibiting synthetic enzymes for GSH production in colonic tissue. Similar results were reported in current study showing significant decrease of GSH levels in TNBS treated mice. Levels of GSH were improved significantly with treatment of ML and TP treatment was unable to show significant effect. these natural antioxidants reduced colonic GSH level is crucial in inducing inflammatory changes.[26] Previous studies showed that decrease in colonic MDA concentration following colitis induction is due to over-production of ROS that deplete lipid peroxidase by inhibiting synthetic enzymes for production in colonic tissue. Similar results were reported in current study showing significant decrease of GSH levels in TNBS treated mice. Levels of GSH were improved significantly with treatment of ML and TP treatment was unable to show significant effect.

Conclusion

1. Ethanolic extract of TP and pet. Ether extract of ML was found to be protective against TNBS induced colitis by inhibiting macroscopic and microscopic defecation, gastric emptying , GI transit, intestinal fluid accumulation diarrhoea, MPO, MDA and NO levels with increase in natural antioxidant GSH levels in rats. The activity can be ascribed to combined effect of flavanoids ,lupeol quarcetin, gallic acid found in TP and ML.

2.Both ML and TP was found to be safe in acute toxicity study and in limit test performed at dose 1000 mg/kg/day in repeated dose toxicity in mice.

3 . TP showed inhibition of macroscopic and microscopic defecation, gastric emptying , GI transit, intestinal fluid accumulation diarrhoea MPO, MDA and NO levels with increase in natural antioxidant GSH levels in mice.

4. Potentialy active fraction obtained from TP and ML in dose 10, 50 and 100 mg/kg p,o., improved symptoms of TNBS treated mice. Body weight, stool consistency and colonic lesion area was found to be improved significantly.

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