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Protective effect of *Dillenia indica* L. on acetic acid induced colitis in mice

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The inflammatory bowel disease (IBD) is an idiopathic, immune mediated and chronic inflammation of the intestine. The study aimed to elucidate the ameliorative effect of methanolic extract of *Dillenia indica* (DIME), hexane fraction (HFDI) and chloroform fraction (CFDI) of *Dillenia indica* in acetic acid induced experimental colitis in mice. Macroscopic score, colon weight, colonic catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), myeloperoxidase (MPO), malondialdehyde (MDA), tumor necrosis factor (TNF-α), and histological changes were recorded after the treatment regimen of 7 days. Intra-rectal instillation of acetic acid caused enhanced macroscopic score, colon weight, colonic MPO, MDA, and TNF-α level. It caused significant decreased level of CAT, SOD and GSH. DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) treatment exhibited significant effect in lowering macroscopic score, colon weight, MPO, MDA, TNF-α levels and elevation of CAT, GSH and SOD levels. The results suggest that *D. indica* has ameliorating effects on experimental colitis by inhibiting the proinflammatory mediators like TNF-α production.

Keywords: Acetic acid, Anti-inflammatory, Antioxidant, *Dillenia indica*, Inflammatory bowel disease, Ulcerative colitis

Inflammatory bowel disease (IBD) is a chronic idiopathic inflammatory condition affecting the gastrointestinal tract that includes ulcerative colitis and Crohn's disease. Both the diseases are common in western and urban populations rather than rural areas[1]. The etiology of IBD is unknown, but the condition seems to be a result of the combination of environmental, genetic, and immunological factors in which an uncontrolled immune response within the intestinal lumen leads to intestinal inflammation[2]. This uncontrolled immune response results in excessive production of reactive oxygen and nitrogen species that may bring about the intestinal injury and dysfunction. Aminosalicylates, glucocorticoids and immunomodulators are generally used for the treatment of IBD, but these drugs are either sometimes ineffective or associated with unacceptable adverse effects. Therefore the use of complementary and alternative medicines, mainly herbal therapies, is increasing for the treatment of IBD[3]. Various herbal therapies have been screened against inflammatory colitis such as *Zataria multiflora*, *Curcuma longa*, *Camellia sinensis, Zingiber Officinale*[4]. Numerous animal models of experimental colitis have been established. Since the pathological changes of acetic acid induced colitis are similar to human inflammatory bowel disease, it is a commonly used model to evaluate the new IBD treatment drugs. Acetic acid causes massive intracellular acidification resulting in injury of epithelial cells and inflammatory response[5].

Dillenia indica L. (Family: Dilleniaceae) is commonly known as "Chulta" in Hindi and "Avartaki" in Sanskrit. *D. indica* is used in the indigenous system of medicine as anticancer, antidiarrheal, astringent and to regulate the heat of the body, tone up the nervous system, remove fatigue and stop abdominal pain[6]. Phytochemical studies have shown the presence of lupeol group of triterpenes like betulin, betulinic acid, flavanoids such as kaempferol, quercetin, dillenetin, isorhamnetin, myricetin, naringenin, phenolic materials, cycloartenone, n-hentriacontanol, stigmasterol and β-sitosterol in *D. indica*. Antioxidant, anti-diarrheal and anti-inflammatory[7], anti-leukemic[8], anti-diabetic and antihyperlipidemic activities[9] of *D. indica* have been reported.

However, so far *D. indica* has not been tested for its protective action against IBD. Hence, the present study has been aimed to assess the effect of *D. indica* L. in the mice model of acetic acid induced colitis.

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Material and Methods

Animals—Female Swiss albino mice (25-30 g) were procured from the animal house of Glenmark Pharmaceuticals Ltd, Mumbai, maintained under standard conditions (12-h light/dark cycle; 25±3 °C, 45–65% RH) and were fed with commercially available standard rodent pellet diet and water *ad libitum.* All animals were acclimatized to laboratory conditions for a week before commencement of the experiment. Study protocols were approved by Institutional Animal Ethics Committee (CPCSEA-BCP/2010/19) and experiments were performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Chemicals—Prednisolone (Wyeth Pvt Ltd, Mumbai, India), 5,5-dithiobis-2-nitrobenzoic acid, reduced glutathione,1,1,3,3-tetraethoxypropane, O-dianisidine hydrochloride (Sigma Aldrich, St. Louis, USA), hexadecyltrimethylammonoium bromide, thiobarbituric acid (Hi-Media Laboratories Pvt. Ltd, Mumbai, India) were purchased. All other chemicals used were of analytical grade.

Extraction and fractionation of leaves of D. indica—Fresh leaves of *Dillenia indica* L. (Dilleniaceae) were collected from Veer Mata Jijabai Bhosle Udyan, Mumbai during October-November. They were authenticated in the Department of Life Science, Ramnarain Ruia College, Matunga, Mumbai, India and voucher specimen was stored in the department. The leaves were air dried under shade to a constant weight and pulverized into a fluffy mass. Coarse powder (100 g) was extracted with 500 mL of methanol, using Soxhlet extractor. The resulting extract was evaporated to dryness under reduced pressure using rotavapour to provide crude methanolic extract with 17% yield (17 g). The methanolic extract of *D. indica* (10 g) was partitioned successively thrice with n-hexane and chloroform. Each fraction was evaporated under reduced pressure to yield the residues of n-hexane fraction (HFDI) 40% (4 g), and chloroform fraction (CFDI) 30% (3 g) respectively.

Administration of doses—All the doses were selected based on acute oral toxicity study (AOC 425) (unpublished data). Three doses of DIME (200, 400 and 800 mg/kg), two doses of HFDI (100 and 200 mg/kg) and CFDI (100 and 200 mg/kg) were selected for administration via oral route and were prepared as suspensions in distilled water using sodium carboxymethyl cellulose (sodium CMC, 0.5% w/v).

Induction of colitis—Animals were divided into following 10 groups of 6 each. Group I served as normal control (NS) and received only vehicle (0.5% CMC). Group II (AA) served as colitis control and received the vehicle (0.5% CMC). Group III was treated with prednisolone (PRED, 5 mg/kg body weight), Group IV, V and VI received DIME at 200, 400 and 800 mg/kg body weight Groups VII and VIII received HFDI at 100 and 200 mg/kg body weight. Groups IX and X received CFDI at 100 and 200 mg/kg body weight respectively. All these treatments were given for 7 days orally by using oral gavage. On the 4th day of the treatment, the animals were fasted overnight with free access to water. On the 5th day after 1 h of the aforementioned treatments, the animals in Groups II, III, IV, V, VI, VII, VIII, IX and X were anesthetized by ketamine-xylazine and a polypropylene tube with 2 mm diameter was inserted 4 cm into the colon. A solution of 0.1 mL of acetic acid (6%) in 0.9% saline was instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 sec to prevent the leakage of the intracolonic instillate. Animals of Group I were instilled saline, intracolonic. Four days pre treatment and three days post treatment was given in order to precondition the animals for antioxidant, anti-inflammatory, inhibition of neutrophil migration and to inhibit the progression of tissue damage at colon in acute model of acetic acid induced colitis. After 72 h i.e. 7^{th} day of single dose administration of acetic acid, the animals were sacrificed by cervical dislocation and colon was dissected out, flushed gently with saline, weighed and used for macroscopic scoring, histopathological and biochemical estimations.

Macroscopic damage score and colon weight—The severity of colitis was evaluated by an independent observer who was blind to the treatment. For each animal, the distal portion of the colon was cut longitudinally, cleaned to remove faecal residues and weighed. Colon (5 cm long) was scored for macroscopic features using the following scoring pattern. No percent area affected was counted as 0 point, 1–5% as 1 point, 5–10% as 2 points, 10–25% as 3 points, 25–50% as 4 points, 50–75% as 5 points and 75–100% as 6 points, respectively[5].

Biochemical analysis—Colonic tissue samples were homogenized in 10% (w/v) of ice-cold

potassium phosphate buffer (*p*H 7.4) and used for the measurement of TNF-alpha (TNF-α), myeloperoxidase (MPO) activity, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) levels.

TNF α was determined in tissue samples using TNF α sandwich ELISA kit (Bio-source International, CA, USA). The assay involved a four member sandwich reaction in which streptavidin peroxidase enzyme acted on a specific substrate, releasing a coloured product measured at 450 nm. Colour intensity was taken as directly proportional to the concentration of TNF α and the results were expressed as ng/g tissue. The colonic MPO assay was assessed as a marker of neutrophil infiltration as per Krawisz *et al*[10]. The MDA levels in the colon tissue were determined by the method of Ohkawa[11]. CAT activity was performed in tissue supernatant by method of Aebi[12]. SOD levels were estimated using the principle of inhibition of epinephrine auto-oxidation by the method of Misra[13]. A unit activity of SOD was defined as 50% inhibition of epinephrine auto oxidation per minute. GSH levels were determined in the tissue supernatant by the method of Sedlak[14].

Histopathological studies—Colon specimens were fixed in 10% formalin, cut into 5 µm thickness, stained using heamatoxylin–eosin and studied for histopathological changes i.e. any inflammatory change like infiltration of cells, submucosal edema, goblet cell loss etc.

Statistical analysis—The values were expressed as mean±SE. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparison test. P <0.05 was considered statistically significant.

Results

Macroscopic damage score and colon weight—Acetic acid caused severe macroscopic edematous inflammation in the colon. The macroscopic damage score and colon weight for colitis control group significantly increased as compared to normal control. DIME at 800 mg/kg and CFDI at 200 mg/kg showed a significant decrease in the damage score and colon weight (Table 1).

Biochemical studies—Intra-rectal administration of acetic acid showed a significant increase in the concentrations of TNF-α, MPO and MDA when compared with normal control, decreased GSH, SOD and CAT levels (Fig. 1). Pre-treatment with DIME at 800 mg/kg, HFDI at 200 mg/kg and CFDI at 200 mg/kg significantly reduced TNF-α, MPO activity and MDA levels, and increased GSH, SOD and CAT levels when compared to colitis control (Fig. 1). CFDI at 200 mg/kg was found to be more active and comparable to prednisolone at 5 mg/kg treatment.

Histopathological studies—The cellular architecture of the colonic tissue of the different groups of mice as studied by the histopathological analysis is presented in Fig. 2. The colonic tissue of normal animals showed intact mucosal and sub-mucosal architecture. Acetic acid induced colitis showed submucosal edema, loss of crypt and goblet cells, destruction of epithelial architecture, infiltration of neutrophils and lymphocytes into the mucosa and sub-mucosa. DIME (200 and 400 mg/kg), HFDI (100 mg/kg) and CFDI (100 mg/kg) exhibited a moderate improvement in the inflammatory response with slight goblet cell loss, sub-mucosal edema, and infiltration of neutrophils in the mucosa. DIME (800 mg/kg), HFDI (200 mg/kg), CFDI (200 mg/kg) and prednisolone (5 mg/kg) showed remarkable recovery of colonic mucosa from acetic acid induced colitis damage (Fig. 2).

Discussion

Reactive oxygen species, vasoactive amines and eicosanoids play a prominent role in the acetic acid induced colitis model of inflammatory bowel disease[15]. Acetic acid exerts its damaging effect by an

Table 1— Effects of methanolic extract of *D. indica* (DIME), hexane fraction (HFDI) and chloroform fraction (CFDI) of *Dillenia indica* on acetic acid (AA) induced mice macroscopic damage score and wet colon weight

[Values are mean ± SE of 6 mice in each group]

Treatment (mg/kg, po)	Macroscopic damage score	Wet colon weight (g)
NS (0.5% CMC)	0.00 ± 0.0	0.40 ± 0.012
AA (0.5% CMC)	3.80 ± 0.20 #	0.60 ± 0.043#
AA + PRED (5)	1.60 ± 0.24*	0.45 ± 0.017*
AA + DIME (200)	3.00 ± 0.31	0.58 ± 0.029
AA + DIME (400)	2.60 ± 0.24	0.50 ± 0.045
AA + DIME (800)	2.00 ± 0.31*	0.44 ± 0.02*
AA + HFDI (100)	3.00 ± 0.31	0.54 ± 0.034
AA + HFDI (200)	2.60 ± 0.24	0.52 ± 0.017
AA + CFDI (100)	2.50 ± 0.28	0.49 ± 0.011
AA + CFDI (200)	1.80 ± 0.20*	0.48 ± 0.033*

P<0.05; *compared to respective AA group; #compared to NS group (statistical analysis was done by one way ANOVA followed by Bonferroni's multiple comparison test).

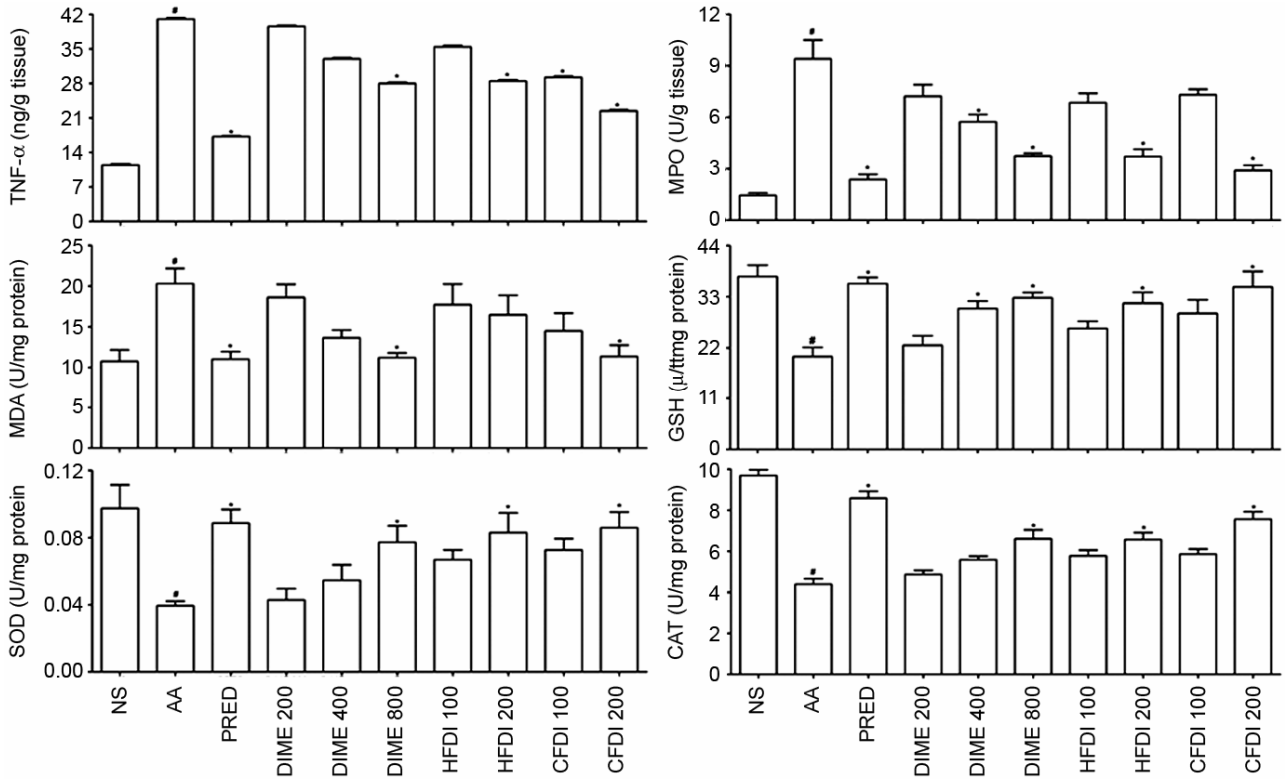

Fig. 1—Effect of DIME, HFDI and CFDI on biochemical profile in acetic acid induced colitis in mice. All values are expressed as mean ± SE for the groups of six animals each using Bonferroni multiple comparison test. $P< 0.05$; *between colitis control (AA) and treated groups, # between normal (NS) and colitis control (AA). Statistical analysis was done by one way ANOVA followed by Bonferroni's multiple comparison test.

acute inflammatory response following colonic injury, accompanied by widespread hemorrhage, release of mediators, and formation of lesions. The protonated form of the acid liberates protons within the intracellular space, causing a massive intracellular acidification resulting in an immense epithelial damage[16]. The weight of the inflamed colonic tissue is considered as a reliable and sensitive indicator for the severity and extent of intestinal inflammation[17]. In the present study, acetic acid administration showed a significant increase in the colon weight and macroscopic damage score, indicative of formation of ulcers, and edema, which is in agreement with previous reports[18]. Pretreatment with DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) significantly reduced colon weight suggesting an inhibition in edema formation, and macroscopic damage compared to colitis control group. TNF-α is a pro-inflammatory cytokine that plays an integral role in the pathogenesis of inflammatory bowel disease, which is evident by the fact that blockade of TNF-α with anti-TNF antibodies has already proven to be a highly effective treatment in Crohn's disease[19]. DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) significantly reduced TNF-α levels in colonic tissue, suggesting that the mechanisms by which *D. indica* attenuates acetic acid colitis involve inhibition of TNF-α production in the colon. Myeloperoxidase (MPO) is a green hemoprotein enzyme abundantly released from the azurophilic granules of neutrophils by inflammatory stimuli that catalyzes the formation of a number of reactive species[20]. The level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue. In the present study, acetic acid administration showed a significant increase in the MPO activity, indicative of the intestinal inflammation and neutrophil infiltration. Pre-treatment with DIME (800 mg/kg), HFDI (200 mg/kg), and CFDI (100 and 200 mg/kg) exhibited a significant decrease in the MPO activity when compared to colitis control.

Malondialdehyde (MDA) is a major lipid peroxidation end product. Increased MDA content may contribute to increased generation of free radicals and decreasing the activity of antioxidant defense systems. In the present study acetic acid resulted in

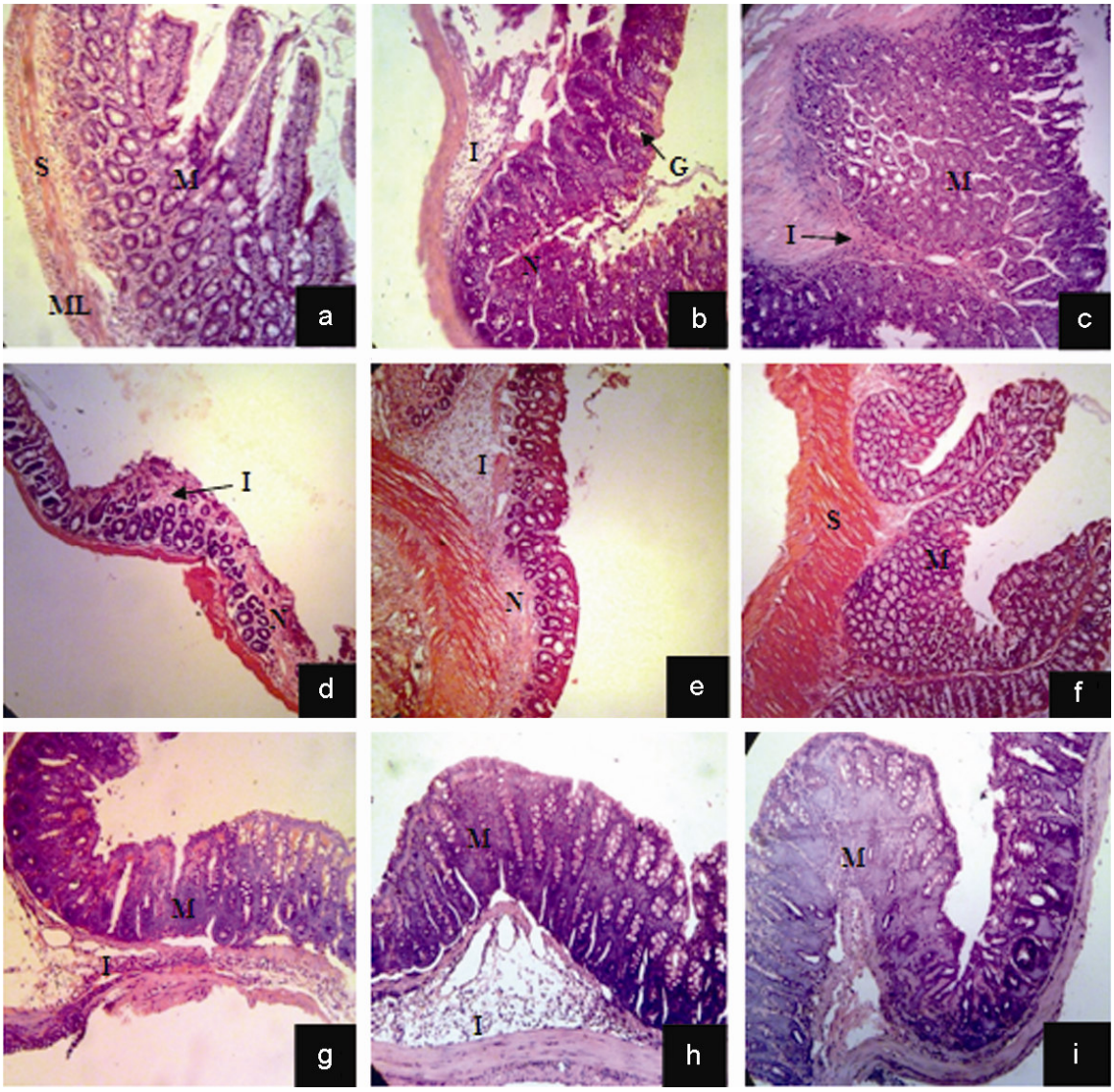

Fig. 2—Histopathological analysis of mice colon stained with H & E (100X). [(a) normal colon of untreated animals; (b) acetic acid (AA) control group; (c) AA+ prednisolone (5 mg/kg); (d) AA+DIME (200 mg/kg); (e) AA+DIME (400 mg/kg); (f) AA+DIME (800 mg/kg); (g) AA+HFDI (200 mg/kg); (h) AA+CFDI (100 mg/kg); (i) AA+CFDI (200 mg/kg) (M: mucosa, S: submucosa, ML: muscle layer, I: inflammatory infiltrate, G: goblet cell loss, N: necrosis)].

marked elevation of lipid peroxidation, expressed as MDA content, which is in line with the previous reports[21]. Pretreatment with DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) significantly reduced the MDA levels as compared to colitis control and this decrease can be attributed to the free radical scavenging potential of *D. indica*.

SOD is a metalloprotein that catalyses the dismutation of two superoxide radicals to form hydrogen peroxide and molecular oxygen. Decrease in the activity of SOD, catalase may be due to increased generation of reactive oxygen species, such as superoxide and hydrogen peroxide. Pre-treatment with DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) significantly attenuated the levels of SOD and CAT as compared to acetic acid colitis control. The histological observations of inflammation such as leukocyte infiltration, edema and tissue injury was found to be low following the pre-treatment with DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) respectively.

Glutathione (GSH) is an important intracellular nonenzymatic antioxidant that plays an important role in protecting cells from oxidative stress[22].

Pre-treatment with DIME (800 mg/kg), HFDI (200 mg/kg) and CFDI (200 mg/kg) significantly protected against colonic GSH depletion.

Based on the results, it could be concluded that prior administration of various fractions of *D. indica* ahead of acetic acid challenge alleviated the morphological and biochemical features of ulcerative colitis. The decrease in the release of inflammatory markers like TNF-α and MPO activity with subsequent modulation of oxidant/antioxidant balance in colonic tissue could possibly account for the protective role of *D. indica*. However, further investigations are necessary to evaluate whether a similar efficacy can be achieved in other models of experimental colitis that simulate human inflammatory bowel disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

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