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Anti-inflammatory effect of *Moringa oleifera* Lam. seeds on acetic acid-induced acute colitis in rats

Mohsen Minaiyan[1,4*], Gholamreza Asghari[2], Diana Taheri[3], Mozhgan Saeidi[4], Salar Nasr-Esfahani[5]

[1]*Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I. R. Iran*
[2]*Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I. R. Iran*
[3]*Department of Clinical Pathology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, I. R. Iran*
[4]*Isfahan Pharmaceutical Sciences Research Center, Isfahan, I. R. Iran*
[5]*School of Medicine, Isfahan University of Medical Sciences, Isfahan, I. R. Iran*

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**** Corresponding Author:***
Tel: +983117922623
Fax: +983116680011
minaiyan@pharm.mui.ac.ir

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Abstract

Objective: Anti-inflammatory, immuno-modulatory, and antioxidant properties of *Moringa oleifera* Lam. suggest that it might have beneficial effects on colitis. The present study was performed to investigate the anticolitis effect of *Moringa oleifera* seeds hydro-alcoholic extract (MSHE) and its chloroform fraction (MCF) on acetic acid-induced colitis in rats.

Materials and Methods: Both MSHE and MCF with three increasing doses (50, 100, and 200 mg/kg) were administered orally to separate groups of male Wistar rats, 2 h before ulcer induction (using acetic acid 4%) and continued for 5 days. Prednisolone (4 mg/kg) and normal saline (1 ml/kg) were used in reference and control groups, respectively. All rats were sacrificed 24 h after the last dose (at day 6) and tissue injuries were assessed macroscopically and pathologically.

Results: Extracts with three doses mentioned before were effective to reduce weight of distal colon (8 cm) as a marker for inflammation and tissue edema. Three doses of MSHE and two greater doses of MCF (100 and 200 mg/kg) were effective to reduce ulcer severity, area, and index as well as mucosal inflammation severity and extent, crypt damage, invasion involvement, total colitis index, and MPO activity compared with controls. MCF (50 mg/kg) was not significantly effective in reducing evaluated parameters of colitis compared with controls.

Conclusion: It is concluded that MSHE and MCF were both effective to treat experimental colitis and this might be attributed to their similar major components, biophenols and flavonoids. Since the efficacy was evident even in low doses of MSHE, presence of active constituents with high potency in seeds is persuasive.

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Introduction

Moringa is the sole genus in the flowering plant family Moringaceae. The genus Moringa in turn is made up of 13 species. The species that is more common and popular, called *Moringa oleifera* Lam. (*M. oleifera*), could be found even in the harshest and driest of soils (Foidi et al., 2001). *M. oleifera* (also known as the horseradish tree, drumstick tree) is a herb found in the tropics and sub-tropics commonly used for medicinal and nutritional purposes. That is native to the Sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan.

Fahey (2005) reported the nutritional and therapeutic qualities of *M. oleifera* which exist in both the scientific and the popular literature.

Genus Moringa contains more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and the protein quality of Moringa leaves is claimed to be similar to milk and eggs (Fahey, 2005). There are over 46 antioxidants (ascorbic acid, caretenoids, flavonoids, and phenolic compounds) and 36 anti-inflammatory (isothiocianate and phenolic derivatives) compounds all naturally occurring in the Moringa plant (Anwar et al., 2007).

Anti-inflammatory, antioxidant (Mahajan et al., 2007), and immuno-modulatory (Shaila et al., 2010) properties of biophenols are abundant in *Moringa oleifera* Lam. suggest that they may have beneficial effects on inflammatory bowel diseases (IBD). IBD is characterized by chronic intestinal inflammation and can be found in two forms: Crohn's disease and ulcerative colitis. Although its etiology is still unknown, immune dysfunction, inflammatory mediators, reactive oxygen species (ROS), and cytokines play crucial roles in its development (Sartor, 1997; Murata et al., 1995).

Sulfasalazine, mesalamine, and 5-aminosalicylic acid (5-ASA) derivatives, glucocorticoides and immuno-suppressives are among the current medications (McQuaid, 2007). Lack of specific and curative treatments with acceptable safety profile represents a growing need to develop new alternatives for IBD management (Sellin and Pasricha, 2006). The present examination was done to investigate anticolitis effect of oral administration of Moringa seeds hydroalcoholic extract (MSHE) and its chloroform fraction (MCF) on acetic acid-induced colitis in rats in comparison with related controls.

Material and Methods

Plant material and preparation of extract

Seeds of *M. oleifera* were purchased from Bangalore city, located in Karnataka states of India in October 2012 and its genus and variety were authenticated by Pharmacognosy Department of Isfahan School of Pharmacy and pharmaceutical Sciences, herbarium voucher No 2669.

For preparation of hydroalcoholic extract, dried and fine powdered seeds of *M. oleifera* (200 g) were macerated by 800 ml of EtOH-H_2O (7:3) and left for 24 h. To achieve most complete extract, percolation was consequently done using 800 ml of fresh EtOH-H_2O for 48 h. Then, the extract was filtered and divided into 2 equal parts. One part was evaporated in a rotary evaporator under reduced pressure until a semisolid extract yield 7.71% (w/w) was prepared. In fact, this is hydroalcoholic extract (MSHE) which was used during the study. Another part was decanted in triplicate with 100 ml of chloroform. The aliquot from each chloroform fraction was pooled and evaporated until a semisolid extract yield 67.96% (w/w) was obtained. In fact, this is the chloroform fraction of hydroalcoholic extract (MCF) which was used during the study (Ghassemi, 2002).

Chemicals

Prednisolone powder was procured from Merck (Darmstadt, Germany). Orto-

dianizidin dihydrocholoride and hexa-decyl trimethyl ammonium bromide were obtained from Sigma (St. Louis, USA). Chloroform and ethanol (96º) were analytical grade and purchased from Merck (Darmstadt, Germany) and Stalak (Tehran, Iran), respectively.

Animals

In the present study, male Wistar rats weighting 180-260 g were purchased from animal house of Isfahan School of Pharmacy (Isfahan University of Medical Sciences, Isfahan, Iran).

The rats were housed in wire bottomed cages under uniform and controlled conditions of temperature (20±4 °C), humidity (50-70%), and 12/12 h light/dark photoperiods. Rats were fed with chow pellets and tap water was freely accessible. All of the experiments were approved by the Ethics and Research Committee of Isfahan University of Medical Sciences, Isfahan, Iran.

Animal grouping

Animals were randomly divided into nine groups as normal (sham), negative control (control), positive control (reference), and test groups of 6 rats as following:

1: Sham group: received vehicle (normal saline) (1 ml/kg) orally (PO) without colitis induction.

2: Control group: received vehicle (1 ml/kg, PO) for 5 days. The first dose was administered 2 h before colitis induction.

3: Reference group: received prednisolone (4 mg/kg, PO) prior to colitis induction and continued for 5 days thereafter.

4, 5, and 6: MSHE groups; received 3 increasing doses of MSHE (50, 100, and 200 mg/kg, PO) 2 h prior to colitis induction and continued for 5 days thereafter.

7, 8, and 9: MCF groups; received similar increasing doses of MCF (50, 100, and 200 mg/kg, PO) 2 h prior to colitis induction and continued for 5 days thereafter.

Experimental protocol

All samples including suspensions, solutions of drugs, and plant extracts were freshly prepared. The extracts were used as a suspension in 0.5% v/v Tween 80 in normal saline (0.9%). For inducing acute colitis, first, rats were fasted for 24 h. Colitis was induced in rats using 2 ml acetic acid 4% via intra-colonic administration. Briefly, under light ether anesthesia, a soft and flexible catheter (2 mm inner diameter and 8 cm in length) was inserted to the anus for 8 cm and acetic acid was carefully injected. Before taking the catheter out, the rats were maintained in a head-down position for 30 seconds in order to prevent solution spreading out (Minaiyan et al., 2011).

Evaluation of colon macroscopic damage

Rats were sacrificed under over-dose ether anesthesia at day 6 (the day after receiving the last dose). Abdomen was opened and colon was exposed. Distal colon, 8 cm in length and 2 cm proximal to the anus, was excised and incised longitudinally. The tissue of colon was washed using normal saline and wet weight was measured. Then, tissue was fixed on a light and transparent sheet and a photo was taken using an appropriately adjusted Sony® camera. Tissue and mucosal injury were evaluated macroscopically through the grading scale according to Morris et al., 1989. The scores were 0=No macroscopic changes, 1=Mucosal erythema only, 2=mild mucosal edema, slight bleeding, or slight erosion, 3=moderate edema, bleeding ulcers, or erosions, and 4=severe ulceration, erosions, edema, and tissue necrosis. Ulcer area was assessed by Fiji-win 32 software (NIH Image for the Macintosh, 2004).

For each specimen, ulcer index was measured with summing the ulcer score and the ulcer area followed by this formula (UI =US+UA) (Minaiyan et al., 2006, 2008).

Evaluation of colon histological damage

Then, the colon specimen tissue was weighted and cut equally along its length.

One part was frozen in liquid nitrogen and kept at freezer (-20 ºC) in order to measure the myeloproxidase (MPO) activity. Another part was used for pathological examination and in this case the colon was fixed in 10% formalin, processed (dehydrated, cleared, impregnated with paraffin, blocked, and sectioned in 4 µm thick slices), and stained with hematoxylin and eosin (H&E).

Inflammation severity and extent as well as crypt damage were evaluated on H&E-stained and coded sections while a modification of a validated scoring system described by Cooper et al., 1993 and Dieleman et al., 1998 was used. Total colitis index was measured by summing three sub-scores (inflammation severity, inflammation extent, and crypt damage). Macroscopic and histological injuries were recorded by an independent pathologist unaware of the treatments using a Zeiss® microscope equipped with a Sony® color video camera for taking digital imaging.

Measuring the MPO activity

MPO activity was measured according to the method of Motavallian et al., 2012. Each tissue sample (0.1 g) was chopped in 5 ml of potassium phosphate buffer containing 0.5% w/v HTAB (Hexadecyl trimethyl ammonium bromide) and transferred to a homogenizing tube and homogenized for 3×45 seconds at 1 min interval. Next, the homogenate was sonicated in an ice bath for 10 sec and then subjected to a sequence of freezing and thawing and sonicated again for 10 more sec.

After that, the suspensions were centrifuged at 4000 rpm for 15 min and supernatant decanted for analysis. The MPO activity was assessed spectrophotometrically: 0.1 ml of the supernatant was added to 2.9 ml of potassium buffer (pH 6) containing orto-dianisidine dihydrochloride (0.167 mg/ml) and 0.005% hydrogen peroxide. The absorbance of mixture was measured at 450 nm by UV-Vis spectrophotometer. MPO activity was reported as units (U) per gram (g) weight of wet colon tissue.

Statistical analysis

Results are expressed as mean±SEM or median (range) and the minimal level of significance was considered at $p<0.05$. All statistical analyses were assessed using SPSS statistical version 17.0 software package. Differences among groups were tested by parametric one-way analysis of variance (ANOVA) with Tukey's HSD as post hoc test. Non-parametric data were analyzed using Kruskal–Wallis followed by Mann-Whitney U test.

Results

Yield value of the extract

The MSHE yielded 7.71 gram (7.71% w/w) and the MCF yielded 5.24 gram (67.96% w/w).

Macroscopic evaluation

With instillation of intra-colonic acetic acid 4%, macroscopic damage to rat colon was observed compared with Sham (normal) group. Observations revealed that no changes were observed in Sham group which suggests that handling and surgical procedure had no interference with experimental results. Treatment with prednisolone as corticosteroid reference drug reduced the ulcer score, ulcer area (cm^2), ulcer index, and the weight of 8 cm of wet colon (mg) ($p<0.001$) (Table 1).

Oral treatment with all doses of MSHE and MCF reduced the weight of 8 cm of colon (mg) compared with control group ($p<0.001$).

The severity of lesion scores, ulcer area, hemorrhage, and ulcer indices were significantly reduced with the medium and high doses (100 and 200 mg/kg) of both tested extracts while low dose of MCF (50 mg/kg) had no significant effect on assessed parameters (Table 1).

Pathological evaluation

In Sham group, no pathological and histological damage was seen while rats with acetic acid-induced colitis and vehicle treatment (control) expressed necrotic destruction of epithelium hemorrhage, edema, inflammatory cellular infiltration, crypt damage, and ulceration at mucus and sub-mucosal layers (Table 2, Figure 1). Treatment with prednisolone showed significant reduction in inflammation severity ($p<0.01$) and inflammation extent ($p<0.01$). The reference drug was also effective to decrease crypt damage ($p<0.001$) and total colitis index ($p<0.01$) after oral administration.

Administration of MSHE was invariably effective to reduce histopathology scores including inflammation severity, inflammation extent, crypt damage, and total colitis index ($p<0.01$) (Table2). Treatment with MCF was also effective to reduce histopathology scores ($p<0.01$). Lower dose (50 mg/kg) of MCF couldn't meaningfully reduce pathological factors similar to macroscopic parameters (50 mg/kg) (Table2).

MPO activity measurement

As it is shown in Table 3, MPO activity was diminished in groups receiving MSHE (100 and 200 mg/kg) and MCF (200 mg/kg) as well as prednisolone (4 mg/kg) ($p<0.001$). Moreover, in groups receiving MSHE (50 mg/kg) and MCF (100 mg/kg), the MPO activity was reduced (at least $p<0.05$) but no significant difference was observed in rats which were treated with 50 mg/kg dose of chloroform fractioned extract (Table 3).

Table 1. Effects of *Moringa oleifera* seeds hydroalcoholic extract (MSHE, 50, 100, and 200 mg/kg) and its chloroform fraction (MCF, 50, 100, and 200 mg/kg) on the macroscopic parameters of colitis induced by acetic acid in rats.

Groups	Score (0-4)	Ulcer Area (cm^2)	Ulcer Index	Weight of Distal Colon (mg)
Sham	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	786.2 ± 32.2
Control	4.0 ± 0.0	5.8 ± 0.8	9.9 ± 0.8	1920.0 ± 28.2
Pred.4	1.2 ± 0.2***	1.2 ± 0.2***	2.4 ± 0.2***	849.7±18.3***
MSHE50	2.3 ± 0.2**	2.6 ± 0.5**	3.5 ± 0.6***	952.2±69.6***
MSHE100	2.0 ± 0.4**	2.4 ± 0.4***	4.4 ± 0.8***	936.3±47.9***
MSHE200	1.8 ± 0.3**	1.7 ± 0.3***	3.5 ± 0.6***	862.3 ± 6.9***
MCF50	2.7 ± 0.4**	4.7 ± 1.2	7.4 ± 0.3	1304.0±102.3**
MCF100	2.1 ± 0.3**	2.8 ± 0.4**	5.0 ± 0.7***	960.7 ± 52.9***
MCF200	1.5 ± 0.2**	1.8 ± 0.1***	3.4 ± 1.5	868.7 ± 38.9***

Data are expressed as mean±SEM, n=6. **p<0.01, *** p < 0.001 indicate significant difference versus control (ANOVA).

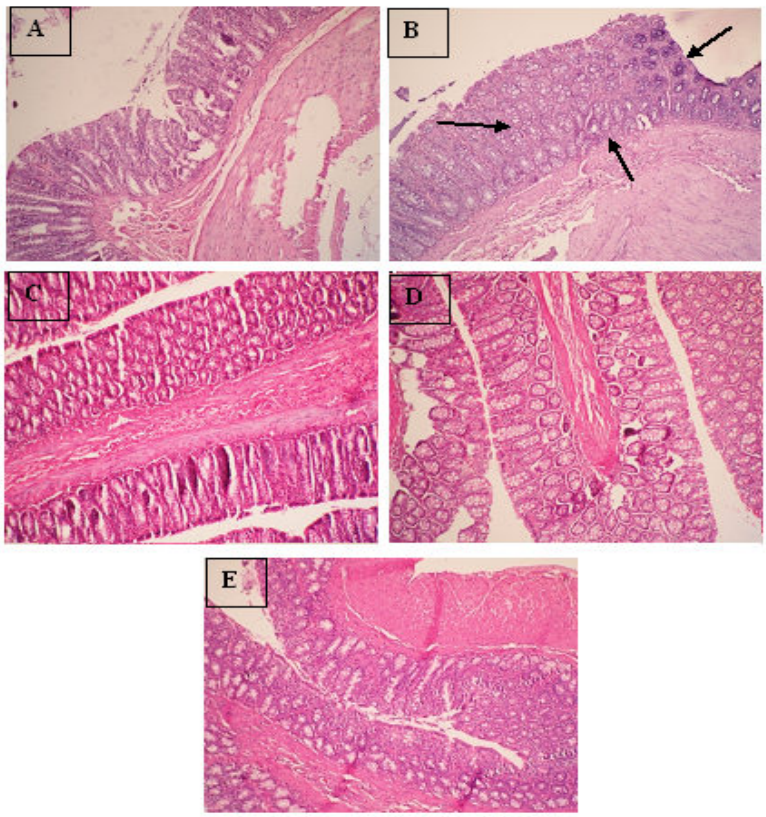

Figure 1. Microscopic illustration of colon tissue in rats. A) Normal tissue; crypts and mucosal layers are intact and leukocytes infiltration is absent. B) Colitis induced by acetic acid in control group; Crypt damage, mucosal layers destruction and leukocyte infiltration are evident. C, D, and E) Colitis tissue treated with MSHE (200 mg/kg), MCF (200 mg/kg), and prednisolone (4 mg/kg), respectively. H&E staining with 40× magnification.

Table 2. Effects of *Moringa oleifera* seeds hydroalcoholic extract (MSHE, 50, 100, and 200 mg/kg) and its chloroform fraction (MCF, 50, 100, and 200 mg/kg) on the pathologic parameters of colitis induced by acetic acid in rats.

Groups	Inflammation Severity (0-3)	Inflammation Extent (0-3)	Crypt Damage (0-4)	Total Colitis Index (0-10)
Sham	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Control	3 (3)	3 (3)	4 (4)	10 (10)
Pred.4	1 (0-1)**	2 (0-2)**	2 (0-2)***	4 (1-5)**
MSHE50	2 (0-3)*	2(0-3)*	3 (1-4)	8 (3-9)*
MSHE100	2 (0-3)**	2 (0-2)**	2 (0-3)**	6 (2-7)**
MSHE200	1 (0-3)**	2 (0-2)**	2 (0-3)***	5 (1-7)**
MCF50	3 (2-3)	3 (2-3)	3 (2-4)	9 (6-10)
MCF100	3 (2-3)	2 (0-3)*	3 (1-4)*	7 (3-9)**
MCF200	1 (0-2)**	2 (0-3)**	2 (0-4)**	5 (1-7)**

Data are expressed as median (range), n=6. *p<0.05, ** p<0.01, *** p<0.001 indicate significant difference versus control (Mann-Whitney U test).

Table 3. Effects of *Moringa oleifera* seeds hydroalcoholic extract (MSHE, 50, 100, and 200 mg/kg) and its chloroform fraction (MCF, 50, 100, and 200 mg/kg) on MPO activity (U/g weight of wet tissue) in colitis induced by acetic acid in rats.

Groups	Control	Pred 4	MSHE 50	MSHE 100	MSHE 200	MCF 50	MCF 100	MCF 200
MPO activity (U/g)	4.7±0.2	1.5±0.1***	3.4±0.3**	3.4±0.2**	1.8±0.1***	4.6±0.3	3.6±0.3*	2.3±0.1***

Data are expressed as mean±SEM, n=6. *p<0.05, **p<0.01, ***p<0.001 indicate significant difference versus control group (ANOVA).

Discussion

It has been described that acetic acid model of experimental colitis is both rapid and reproducible for the screening of drugs with anticolitis activity and has similarity to pathological and clinical features of the human ulcerative colitis (MacPherson and Pfeiffer, 1978). In the present study, results showed the efficiency of this method because an acute and invariably characteristic colitis was developed in experimental rats. Prednisolone was used as the reference drug to delineate the efficacy of two Moringa extracts and the results showed protection considering macroscopic and microscopic factors for applied drugs. Similar results were seen when MSHE (50,100, and 200 mg/kg) and MCF (100 and 200 mg/kg) were used in comparison with the control group. The exception was for the MCF (50 mg/kg) which was not effective in reducing both macroscopic and microscopic values.

Regarding to the macroscopic (ulcer index) and histological (total colitis index) results, it is evident that total extract of Moringa and its chloroform fraction possessed dose-independent anti-ulcerogenic effect. Gholap et al., 2012 examined a combination of *Moringa oleifera* root and *Citrus sinensis* (Rutaceae) fruit extracts in a similar colitis model induced by acetic acid in mice. They demonstrated that the examined mixture resulted in reduced hyperemia and ulceration as well as MPO activity in comparison with prednisolone as the reference drug. In the recent study, we used seeds of *Moringa oleifera* alone and the outcome was similar to those above-mentioned reports.

The period of five-day treatments with tested extracts produced an opportunity for delayed protective mechanisms such as scavenging of oxidoradicals and repairing mucosal layers to be involved. Polyphenolic compounds exist widely in Moringa and are common in leaves, flowering tissues, and seeds (Siddhuraju and Becker, 2003). Phenolic compounds are secondary plant metabolites and are essential in defense mechanisms of plants against pathogenic and free radicals (Maisuthisakul et al., 2007).

These compounds are used in humans to modulate lipid peroxidation because of their antioxidant and anti-inflammatory activity (Frankel and Meyer, 2000). The antioxidant compounds present in *M. oleifera* indicate the scavenging activity due to hydrogen proton donation (Verma et al., 2009). Flavonoids are related to a larger group, phenolic compounds. Most of the beneficial effects of flavonoids are because of their antioxidant properties (Heim et al., 2002).

Mechanism of antioxidant action is suppressing the formation of reactive oxygen species through inhibition of enzymes or chelating trace elements involved in free-radical production, scavenging reactive species, protecting antioxidant defenses, reducing alpha-tocopherol radicals, and activating

antioxidant enzymes (Heim et al., 2002; Montoro et al., 2005).

Isothiocyanates as another group of constituents of *M. oleifera* might play an important role in our research. According to Matsuda et al., 2007, powder of *M. oleifera* had significant protection against ethanol-induced gastric lesions and it was more potent than omeprazole, a potent and common anti-ulcerative drug. The researchers attributed these effects to its isothiocyanate constituents which have anti-inflammatory as well as immune-modulatory activities. According to Shaila et al., 2010 and Mahajan et al., 2007, extract of Moringa has a suppressive effect on macrophages and neutrophils and inhibits phagocytosis, so it can be considered as an immune-modulator and/or immune-suppressive which are suitable activities for IBD.

Activated macrophages and neutrophils are rich in inflamed intestine of IBD patients and these inflammatory cells reproduce excess amounts of ROS (Reactive Oxygen Species) with increases in oxidative stress (Shaheen and Annette, 2011). Moringa also suppresses prostaglandin biosynthesis through an inhibitory effect on COX-I and COX-II enzymes (Mehta and Agrawal, 2008). An extension of these results was the fact that Moringa also suppresses leukotriene biosynthesis; a mechanism which shares pharmacological property with glucocorticoids and may play a key role for their usefulness in human IBD (Koneni et al., 2009).

Increased tumor necrosis factor alfa (TNF-α) and interleukin-6 (IL-6) are evident in IBD disease (Podolsky, 1991; Chia et al., 2007). Mahajan et al., 2007, also found that Moringa was effective in blocking production of several cytokines including TNF-α, IL-4, and IL-6. It is probable that additional mechanisms such as mast cells stabilization and anti-spasmodic activities have a role in protective effects of Moringa (Caceres et al., 1992). Results of this animal experiment reveal that *M. oleifera* seeds extract possesses similar anti-inflammatory properties compared with those reported by glucocorticoides. These findings offer more pharmacological support to folkloric, ethno-pharmacological consumption of Moringa in IBD management.

In conclusion, our findings suggest a useful therapeutic activity for MSHE and MCF as an anti-inflammatory and antiulcerative medicinal plant for IBD conditions. Oral administration of MSHE, even with low doses, could be considered as an alternative remedy for IBD conditions and/or prevention of its recurrence. More studies are strongly recommended to distinguish the exact mechanism which is involved and study the active compounds which are really responsible for its beneficial pharmacologic actions.

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Conflict of interest

There is not any conflict of interest in this study.

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