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Evaluating the Effect of *Melillotus officinalis* L. Aqueous Extracts on Healing of Acetic Acid-Induced Ulcerative Colitis in Male Rats

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Abstract

Background: Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) that is characterized by acute and chronic inflammation. The etiology and pathophysiology of IBD is unidentified, and there are many obstacles on the definite treatment of this disease. Recently, the useful effects of some herbal medicine on improving UC have been studied. *Melillotus officinalis* L. (*M. officinalis*) is an herb with antioxidant and anti-inflammatory effects used as food, forage and medicine.
Objectives: This study evaluated the antioxidant effects of *M. officinalis* aqueous extracts in the acetic acid- induced ulcerative colitis in rats.
Methods: Fifty rats were randomly divided into five equal groups. Group I (Control healthy group) received 1 mL/kg of normal saline orally. Group II (control colitis group) received 1 mL/kg of normal saline orally. Group III (positive control) received 3 mg/kg prednisolone orally. Group IV received 1000 mg/kg *M. officinalis* aqueous extracts orally. Group V received 2000 mg/kg *M. officinalis* aqueous extracts orally. Ulcerative colitis was induced by intra-rectal acetic acid (3% v/v) administration. All treatments were done 24 hours after induction of colitis and continued for seven days. On the eighth day, the rats were sacrificed and colonic biopsies were taken for histopathological and biochemical studies. Data analysis was performed, using SPSS software and significance level was set at $P \leq 0.05$.
Results: Treatment with *M. officinalis* aqueous extract could enhance colonic antioxidant capacity and decrease inflammation and acute colonic injury induced by acetic acid, which is dose-dependent. In addition, administrating the extract significantly ($P \leq 0.05$) reduced the colonic level of malondialdehyde and myeloperoxidase, and significantly ($P \leq 0.05$) increased the level of reduced glutathione ($P \leq 0.05$). The extract had more effects at the dose of 2000 mg/kg than 1000 mg/kg dosage and prednisolone.
Conclusions: This study revealed that *M. officinalis* had apparent curative effects on treating UC because of its antioxidant and anti-inflammatory activities.

Keywords: Inflammatory Bowel Disease, Ulcerative Colitis, Antioxidant, *Melillotus officinalis* L

1. Background

Ulcerative colitis (UC) is a form of inflammatory bowel disease (IBD) that is characterized by acute and chronic inflammation of the mucosa, ulceration of the colon, bloody diarrhea, rectal bleeding, abdominal pain, cramping and weight loss (1, 2). UC is a non-transmittable inflammatory disease that is confined to the colon (3). The incidence of UC has been reported about 10 - 20 in 100,0000 per year, with a prevalence of 100 - 200 cases per 100,0000 in Western countries (4). The prevalence of UC is increasing in developing countries due to their lifestyle (5).

The etiology and pathophysiology of IBD is unidentified and depends on a number of factors such as environmental factors, genetic, reactive oxygen species and gastrointestinal infections (1, 6). Oxidative stress is an important factor in the pathogenesis of UC disease (7). A decrease in antioxidant defense against increasing oxidative materials in colonic mucosal of the patients with UC causes tissue damage and inflammation of the colon (8). Drugs such as amino salicylates, corticosteroids, and immune modulators are normally used to treat IBD, but these drugs have more adverse effects and are only for temporary relief, not definitive treatment (9).

Therefore, many IBD patients use complementary and

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alternative medicine (CAM) such as homeopathy, herbal medication and vitamin therapy for treatment (10). Experimental information acquired from animal models of colitis suggested the useful effects of the plant extracts such as *Teucrium polium* (11), *Calendula officinalis* (8), Agave *Americana Linn* (12), *Carum carvi* (13), *Naringenin* (14), and *Pterocarpus marsupium* (15) on improving UC.

Melilotus officinalis L. (sweet clover), belongs to the family of Leguminosae (Fabaceae). The plant is grown in Pakistan, Kashmir, India, Tibet, Russia, China, Turkey, Middle and Southern Europe (16). *M. officinalis* is an herb used as food, forage and medicine (17). The main phenolic compounds in *M. officinalis* are coumarin derivatives, rutin (flavonoid), and ferulic acid (phenol carboxylic acids) (18). It also contains vitamin C, allantoin, tannins, mineral salts, and flavonoids (19). *M. officinalis* grass has many effects like keratolytic, bio stimulant, regenerative, vasodilator, anticoagulant, expectorant, anti-inflammatory, softener and carminative (20, 21). In traditional medicine, it is used as softening, laxative, expectorant, sedative, diuretic, antibacterial, hypotensive, analgesic, and antispasmodic effects (22). As indicated in several studies, the excessive production of ROS in UC leads to oxidative damage in tissues (23). Hence, it is necessary to find antioxidant and anti-inflammatory compounds to reduce damages in UC disease. More in-depth research is suggested to find an efficient and useful treatment method.

2. Objectives

The aim of this study was to evaluate the antioxidant capacity of *M. officinalis* aqueous extracts at the doses of 1000 and 2000 mg/kg/d to reduce oxidative and tissue damages, histopathological changes, malondialdehyde (MDA), myeloperoxidase (MPO) and glutathione (GSH) level in experimental ulcerative colitis in rats.

3. Methods

3.1. Ethical Statement

This study was authorized by the animal care and apply committee of Shiraz University of Medical Sciences, Shiraz, Iran.

3.2. Preparation of the Extract

M. officinalis fresh plants were identified and gathered from north of Shiraz. The plants were dried in room temperature. All parts of the dried plant were powdered and cast in plenty of boiling water (a ratio of 1 to 15) and placed on the heater for 30 minutes at a temperature of 70 - 80°C. They were then wrapped in an aluminum foil container and kept in the dark room for 24 hours. First, the extract was flat with cotton and then by filter paper, and condensed in a rotary machine. The condensed extract was frozen at -20°C, and dried in a freeze dryer. Finally, the aqueous extract was prepared in different concentrations (24). The total phenols in the *M. officinalis* extract were determined by the Folin-Ciocalteua method. Results are given as gallic acid equivalent (GAE/g) of the extract (25).

3.3. Animals

Fifty male Sprague Dawley rats weighing 200 $\pm$ 20 g were purchased from laboratory animal center of Shiraz University of Medical Sciences. The rats were housed in standard condition as ambient temperature of 21 $\pm$ 2°C and 65 $\pm$ 5% relative humidity, with 12 lights and 12 dark cycles. Each animal received a balanced diet and had free access to water and chow.

3.3.1. Induction of the Experimental Colitis in Rats

All animals were fasted for 36 hours before the induction of colitis. A polyurethane cannula (diameter of 2 mm) was applied for rectal entrance of acetic acid 3%, and the tip was inserted up to 8 cm proximal to the anus. Two milliliters of acetic acid (AA) were administered transrectally into the colon by the cannula for 15 seconds, and to induce UC, the rats underwent anesthesia with ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) (26).

2.3.2. Experimental Design

The rats were randomly divided into five equal groups as follows:

- Group I (control healthy group) received 1 mL/kg of normal saline orally

- Group II (control colitis group) received 1 mL/kg of normal saline orally

- Group III (positive control) received 3 mg/kg prednisolone orally

- Group IV received 1000 mg/kg *M. officinalis* aqueous extracts orally

- Group V received 2000 mg/kg *M. officinalis* aqueous extracts orally

All treatments were provided 24 hours after induction of colitis and continued for seven days. On the eighth day, the rats were sacrificed, and colonic biopsies were taken for histopathological and biochemical studies. The doses were selected based on previous studies. The doses of 1000 and 2000 mg/kg/d *M. officinalis* aqueous extracts, and the dose of prednisolone were selected based on previous studies.

3.4. Histopathological Study

For histological examination, the colon tissues were sectioned with 5 μm thickness, and stained with haematoxylin and eosin (H & E). This was done by a pathologist who was unaware of the histological injury treatment records.

3.5. Biochemical Study

Colonic samples were stored immediately at -80°C till analysis. Tissue samples were homogenized in 1 mL of 10 mmol/L Tris-HCl buffer pH 7.1, and homogenation was used to measure malondialdehyde (MDA), myeloperoxidase (MPO), and glutathione (GSH) activities. In colonic tissue homogenate, MDA, MPO and GSH contents were expressed per gram wet tissue weight. MDA production was determined in the colonic tissue by thiobarbituric acid reaction (27). MPO activity was assessed in colonic tissue based on the reaction hydrogen peroxide and o-dianisidine dihydrochloride as substrates (28), and GSH was determined in colonic tissue, described by Owens and Belcher based on the reaction of 5,5-dithiobis-(2-nitrobenzoic acid) (29).

3.6. Statistical Analysis

For statistical analysis, the data were checked for normal distribution, using 1-sample K-S test. Differences between the groups, by normal distribution, were done using one-way ANOVA and Tukey HSD post hoc test, and the data not normally distributed were analyzed by Kruskall-Wallis test, followed by Dunn's test. All the statistical analyses were determined by SPSS software (Version 18, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

4. Results

4.1. Analysis of the Extract of M. officinalis

The aqueous extract yielded 18.21% w/w. The total phenols determination showed 26.51 mg GAE/g extract of the plant

4.2. Histopathological Study

In acetic acid control group, the colonic mucosa showed epithelial necrosis, granular atrophy mucousal tissue destruction with ulcer and migration of inflammatory cells (a). In prednisolone (3 mg/kg) treated rats, the relative improvement could be seen in the wound and mucosal tissue of the colon, but inflammatory cells were still present (b). The wound improved and intestinal mucosal tissue was repaired in *M. officinal* treated rats that received a dose of 1000 mg/kg orally, but inflammatory cell infiltration was still present (c). Administration of *M. officinal* at a dose of 2000 mg/kg orally, protected colonic mucosa, improved the wound and caused inflammatory cells to disappear (d) (Figure 1).

4.3. Biochemical Study

4.3.1. Effects of M. officinalis on MDA Activity

Figure 2 demonstrates the mucosal MDA concentrations in colonic mucosal biopsies of the rats. MDA increased in acetic acid control when compared to normal controls. Treatment with *M. officinalis* with 1000 and 2000 mg/kg orally and prednisolone for a period of seven days resulted in a significant decrease in MDA levels when compared to acetic acid induced control.

4.3.2. Effects of M. officinalis on MPO Activity

Figure 3 demonstrates the mucosal MPO concentrations in colonic mucosa of the rats. Tissue MPO levels significantly increased ($P < 0.05$), following the intrarectal administration of acetic acid. Treating rats with *M. officinalis* (1000 and 2000 mg/kg) such as prednisolone caused a significant reduction ($P < 0.05$) in the mean MPO activity when compared to acetic acid induced controls (Group 2).

4.3.3. Effects of M. officinalis on GSH Level

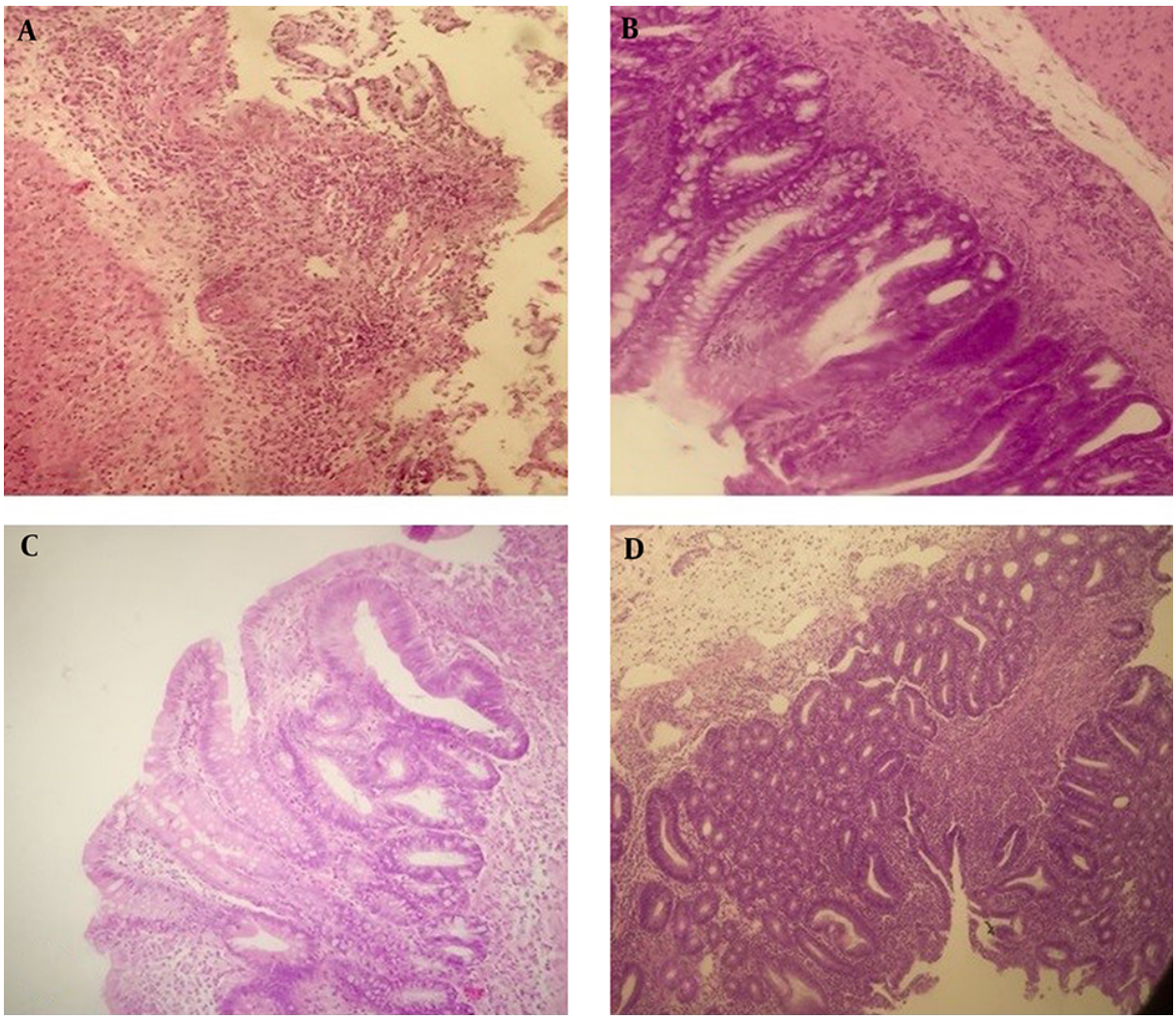
Reduced glutathione content significantly decreased in acetic acid control group. Treatment with *M. officinalis* in a dose of 1000 and 2000 mg/kg and prednisolone (3 mg/kg) orally resulted in a significant increase in GSH content (Figure 4).

5. Discussion

This study evaluated the healing effects of *M. officinalis* aqueous extract in 1000 and 2000 mg/kg dietaries against acetic acid-induced UC by measuring tissue histopathology and MDA, GSH and MPO level in rats. This study found that treatment with *M. officinalis* aqueous extract could lead to enhancement in colonic antioxidant capacity and a decrease in inflammation and acute colonic injury induced by acetic acid, which is dose-dependent. The results were confirmed by histopathological examinations. Moreover, the groups that received 2000 mg/kg dosage of oral *M. officinalis* extract administration for a period of seven consecutive days produced a better response when compared with the dose of 1000 mg/kg of *M. officinalis* extract. We used oral prednisolone as a reference drug, and found that the 2000 mg/kg dosage of the extract was more effective when compared with prednisolone.

Induced ulcerative colitis with AA causes neutrophils and macrophages infiltration in animals, which creates epithelial lesions and necrosis, colonic damage and inflammatory conditions (30).

Figure 1. Histopathological Changes Associated with Experimental Ulcerative Colitis Induction and Treatment with *M. officinalis* Extract

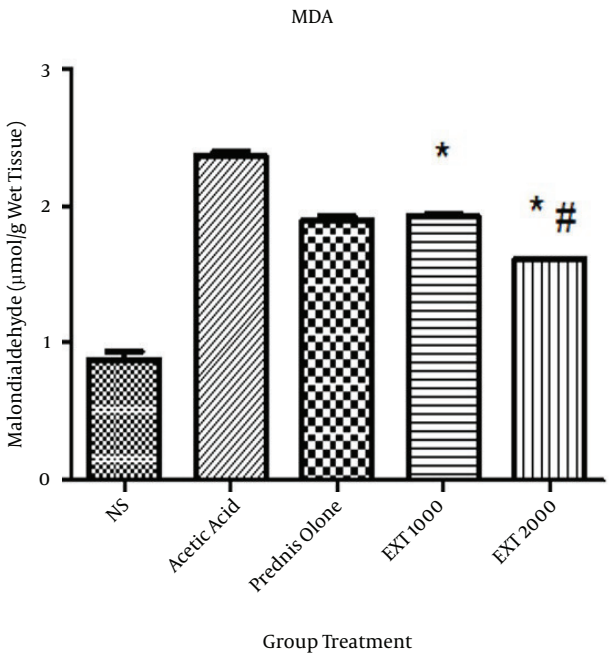


A, acute inflammation and granular atrophy in response to acetic acid 3%; B, 3 mg/kg prednisolone, orally; C, 1000 mg/kg of *M. officinalis* extract, orally; D, 2000 mg/kg of *M. officinalis* extract, orally (H and E, × 100 and 250).

It has been well demonstrated that oxidative stress has an important role in IBD initiation and continuance (31). Inconsistency between oxidant and antioxidant material in colitis caused oxidative damage, which is a characteristic feature of colitis (32).

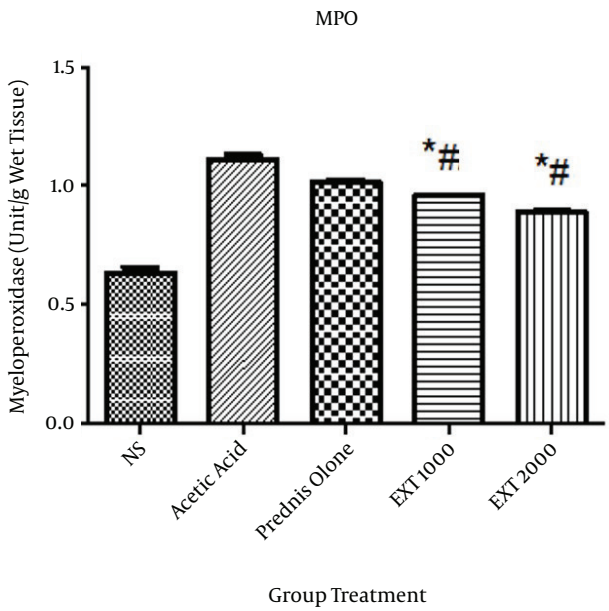
Antioxidant effects have been found in several compounds and can reduce the harmful effects of reactive oxygen species, created in biological systems by disabling these reactive. Natural flavonoids are important compounds as an effective source of exogenous antioxidants (33). *M. officinal* contains phenolic acid, flavonoids and coumarin and these components have notable antioxidant (34). Phenolic compounds of *M. officinalis* extracts have an inhibitory effect on arachidonic acid metabolism through the lipoxygenase pathway; also, *M. officinal* has anti-inflammatory effects by reducing circulating phagocytes and lowering citrulline production (35). Moreover, coumarin (the most effective component of *M. officinal*) has antioxidant properties that affect the organization and scavenging of ROS. In addition, it can suppress superoxide production in leukocytes via its antioxidant ability, which can influence phagocyte activity (36, 37). In this study, we found that *M. officinalis* has anti-inflammatory properties on acute inflammation in ulcerative colitis models. Similarly, Plesca-Manea found that *M. officinalis* had anti-inflammatory effects against acute inflammation induced

Figure 2. Effects of Treatments on Tissue Levels of MDA Experimental Rats



Results are presented as the mean ± SD of 10 rats in each group. *A significant difference in the level of P < 0.05 between treatments groups and the colitis control group (group receiving acetic acid); #a significant difference in the level of P < 0.001 between treatment groups and the colitis control group (group receiving acetic acid).

Figure 3. Effects of the Treatments on Tissue Levels of MPO Experimental Rats



Results are presented as the mean ± SD of 10 rats in each group. *A significant difference in the level of P < 0.05 between treatment groups and the colitis control group (group receiving acetic acid); #a significant difference in the level of P < 0.001 between treatments groups and the Colitis control group (group receiving acetic acid).

by turpentine oil in male rabbits (19).

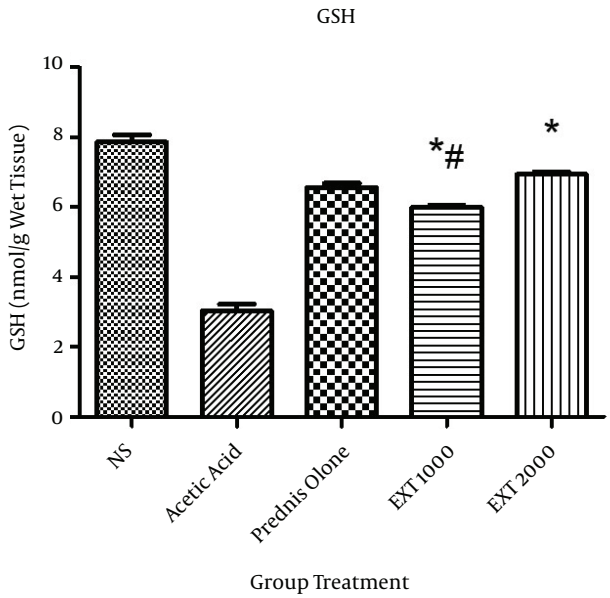
Previous studies reported the useful effects of the plant extracts such as *Calendula officinalis* (8), *Carum carvi* (13) and *Naringenin* (14) on improving UC. In addition, Safarpour et al. reported (38) some beneficial effects of *M. officinalis* in treating UC. In this study, we evaluated the healing effects of *M. officinalis* at the higher doses to investigate the probable side effects. We found that *M. officinalis* aqueous extract at the dose of 1000 and 2000 mg/kg did not have any toxic effects, but we noticed that UC treatment improved at the dose of 2000 mg/kg.

Several reports have shown that the contents of MDA increase in UC. MDA have acceptable relations with the degree of lipid peroxidation; hence, it is frequently used in measuring lipid peroxide levels (39, 40). Furthermore, studies have clearly demonstrated that depletion of GSH leads to the cellular damage as well as colonic injuries. GSH is one of the first lines of oxidative defense mechanisms against free radicals (41), and can prevent ROS oxidative injuries (42, 43).

In our study, the levels of GSH decreased in colonic tissue of the colitis in the control group, while MDA contents increased significantly. To date, no study has examined the effects of *M. officinalis* on the level of GSH in animal's model of UC. In this study, treatment with *M. officinalis* caused a remarkable increase in GSH and a decrease in MDA in colonic tissue, which was dose-dependent. *M. officinalis* provided protective effects in UC, perhaps through the scavenging radicals and its antioxidant properties. This can be one of the important and underlying mechanisms of M. *officinalis* protection against UC. Similar to findings of this study, Safarpour and et al. revealed that treatment by *M. officinal* in colitis rats decreased the level of MDA and improved UC symptoms (38).

MPO is an enzyme that exists in neutrophils. The level of MPO activity is directly related to the neutrophil concentration in the inflamed tissue Therefore, measuring MPO activity is considered as a quantitative and sensitive assay for acute intestinal inflammation (44). Many studies showed that herbs and their main components such as phenols and polyphenols decreased MPO level and improved inflammation in the colonic tissue of ulcerative colitis animals (45-47). In this study, a significant reduction was detected in neutrophil infiltration in colonic mucus and MPO activity of animal subjects with colitis treated with *M. officinal*. The beneficial effects of this plant on MPO and inflammatory condition may be due to its phenolic components like coumarin. Previously, no study examined the effects of *M. officinalis* on the level of MPO in animal's

Figure 4. Effects of Treatments on Tissue Levels of GSH Experimental Rats



Results are presented as the mean ± SD of 10 rats in each group. *A significant difference in the level of $P < 0.05$ between treatment groups and the colitis control group (group receiving acetic acid); #a significant difference in the level of $P < 0.001$ between treatment groups and the colitis control group (group receiving acetic acid)

model of UC.

In conclusion, this study demonstrated that daily oral administration of *M. officinal* extract could relieve the ulcerative colitis induced by acetic acid in the rats' colon. Results of tissue MDA, MPO and GSH and histopathological evaluations indicated a reduction in the inflammation. The healing, anti-inflammatory and antioxidant properties of *M. officinalis* can make it an appropriate choice of supplement for treating ulcerative colitis. However, further studies are required to confirm its clinical effect on humans.

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