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Original article

Biochemical and Histopathological Evidence for Beneficial Effects of *Pelargonium graveolens* Essential Oil on the Rat Model of Inflammatory Bowel Disease

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Abstract

Background and objectives: *Pelargonium graveolens* L'Hér. is an essential oil bearing plant used in traditional medicine of Iran for the treatment of inflammatory disease and pain, anxiety, sadness and also for the relief of gastrointestinal symptoms. Its pharmaceutical product is presently used in Iran for the treatment of depression disorders. Since colitis is a brain-gut interactive disorder, the authors were prompted to investigate the preventive effect of *P. graveolens* essential oil product (Deproherb®) to control the induced inflammatory bowel disease (IBD) in rats. **Methods:** Experimentally ulcerative colitis was induced by acetic acid in animals pretreated with three different doses of Deproherb® (100, 200 and 400 mg/kg, p.o.) for five consecutive days. Anti-inflammatory effects of Deproherb® were compared with orally administrated sulfasalazine (4 mg/kg). Biochemical [Myeloperoxidase (MPO) activity] and macroscopic and microscopic examinations of the colon was performed. Deproherb® was also analyzed by GC and GC/MS in order to identify the potentially responsible compounds for observed property. **Results:** The obtained results indicated that the activity of MPO increased in acetic acid-treated groups, while it was recovered by pretreatment with Deproherb® (100-400 mg/kg) and sulfasalazine. All doses of Deproherb® and sulfasalazine-treated groups showed significant lower score values of macroscopic and microscopic characters when compared to the acetic acid-treated group. It was concluded that Deproherb® inhibited the acetic acid toxic reactions in the rat bowel. **Conclusions:** The present study proved the anti-inflammatory potential of Deproherb® Oral Drop, in the experimentally induced colitis. Proper clinical investigations are suggested to confirm the observed activities in human.

Keywords: essential oil; myeloperoxidase; *Pelargonium graveolens*; ulcerative colitis

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Introduction

The causes of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are unknown. There are many factors contributing to IBD including disorders of immune system, abnormality and microbial contents of gastrointestinal system, oxidative stress, and abnormal activities of cyclooxygenase-2, nitric oxide and leukotriene B [1-3]. Common treatments for IBD are corticosteroids, amino salicylates, anti-tumor

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necrosis factors and immune-modulators which have a limited clinical usage due to their high cost as well as several side effects [4]. Due to a variety of active constituents, possibility of long-term use and rational prices, herbal medications and phytotherapeutics have been recently of great interest to clinicians [5]. Different pharmacological and animal studies have been conducted in the field of IBD. Some of these studies have focused on the treatment of ulcerative colitis using various herbal materials. *Berberis vulgaris, Copaifera langsdorffii,, Carum carvi, Moringa oleifera, Prunus armeniaca, Echium amoenum, Kelussia odoratissima Ziziphora clinopoides, Cydonia Oblonga, Zataria multiflora, Satureja khuzestanica* and *Zingiber officinale* were among medicinal plants with positive effects on induced ulcerative colitis in experimental animals [6-8].

Pelargonium species belong to the Geraniaceae family and have been traditionally used for intestinal problems, wounds and respiratory ailments in recent years. An oral drop containing *P. graveolens* essential oil has been formulated in Iran to relieve depressive disorders [8,9].

The essential oil from the fresh leaves using hydro distillation was found to have pharmacological activities [9,10]. Previous studies reported that the *Pelargonium* sp. oil suppressed the neutrophil accumulation in various inflammatory responses. It also suppressed the adherence response of neutrophils in vitro, and accumulation of neutrophils, induced by casein, in the peritoneal cavity [11]. Moreover, intraperitoneal and cutaneous functions of the oil suppressed the cellular inflammation and neutrophil accumulation in the inflammatory sites which were induced by a (1 → 3)- *β*-D-glucan, curdlan [10]. The anti-inflammatory activity of the methanol extract of *Pelargonium graveolens* has been reported in acetic acid-induced ulcerative colitis in rat [12]. In another study, the essential oil inhibited the carrageenan-induced edema [13] while in an animal study, the *Pelargonium* sp. oil showed significant antidepressant activity [14,15].

Since previous studies showed that *Pelargonium* had anti-inflammatory effect in some animal models, the present study was designed for assessing the anti-inflammatory activity of *P. graveolens* oil formulation (Deproherb®) in acetic acid-induced acute colitis in rat.

Material and Methods

Ethical considerations

Procedures involving animals and their care were conducted in accordance with the NIH guidelines for the care and use of laboratory animals [17] and the Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran (approval: IR.IAU.PS.REC.1396.3, 2017).

Herbal medicine

Depoherb (Barij Essence Co., Iran, 2017) was used in the present study. This product was standardized regarding β-citronellol (19-27 mg/mL); sulfasalazine powder was purchased from Sigma Aldrich (England). Acid acetic, organic solvents, formalin solution 35% w/w was obtained from Merck Company (Germany). Hexadecyl trimethyl-ammonium bromide (HTAB) and O-dianisidine dihy- drochloride were purchased from Sigma Chemical Co. (USA). All other solvents and chemicals were of analytical grade.

GC/MS analysis

The analysis was performed on a HP 6890 gas chromatograph equipped with FID and a DB-5 capillary column (30 m, 0.25 mm; 0.25 μm film thickness) and a temperature programmed as follows: from 60 to 240 °C at a rate of 4 °C/min. The carrier gas (nitrogen) was at a flow of 2.0 mL/min; the temperatures of the injector port and detector were 250 and 300 °C, respectively. Samples were injected by splitting at a ratio of 1:10. The analysis of GC/MS was performed on a Hewlett- Packard 6890/5972 system with a DB-5 capillary column (30 m, 0.25 mm; 0.25 μm film thickness). The operating conditions were the same as described above, except for the carrier gas that changed to helium. Mass spectra were taken at 70 eV, ranging from 40 to 400 m/z at a sampling rate of 1.0 scan/s. Electronic integration of FID peak areas resulted in the quantitative data. Oil components were identified by the time and indices of their retention, relative to C_9–C_{28} n-alkanes, computer matching with WILEY275.L library and also by comparison of their mass spectra with the data already available in the literature [17]. The composition percentage of identified compounds was obtained and relatively calculated from peak areas of GC with no correction factors.

Animals

Thirty-six male albino Wistar rats (Iran university, Tehran, Iran), weighing 200-250 g, were housed in an air-conditioned colony room in a 12 h light/dark cycle (21-23 °C and humidity of 30-40%) and supplied with standard diet and tap water ad libitum. The rats were fasted for 24 h before induction of colitis but were allowed free access to water**.** Six groups of rats with 6 animals in each were studied. The vehicle group orally (p.o.) received vehicle without ulcer induction. In the model group the rats with induced colitis received vehicle (1 mL, p.o.) in four consecutive days before induction of ulcer. In positive controls (sulfasalazine group) the rats with induced colitis received sulfasalazine (Iran Hormone Pharmaceutical Company, Iran) (4 mg/kg, p.o.) in four consecutive days before ulcer induction and in the test groups they were exposed to four consecutive days of pretreatment with Deproherb® (Barij Co., Iran) (100, 200 and 400 mg/kg, p.o.) and received 1 mL of 4% acetic acid solution. The rats were randomly assigned to different experimental groups. All test drugs and compounds were prepared freshly before administration.

For induction of colitis, overnight fasted rats were anesthetized using ether and then 1 mL of 4% acetic acid solution was instilled into their rectum on the 5th day. The animals were hung in the air by holding their tails for 1 to 2 min, which prevented the solution to spill from the rectum. After 24 h, the rats were sacrificed and dissected and their colon was removed. Ten cm of the colon was gently flushed with saline, cut open and scored for inflammation according to macroscopic features. Tissues were then fixed in formalin saline (10%) and were histopathologically examined. Biochemical assessment of colon inflammation was conducted by the assay of MPO activity.

Assessment of colon macroscopic damage

The animals were euthanized by overdose inhalation of ether 24 h after the colitis induction. The colon was excised from 2 cm proximal to the anus and 10 cm in length. Colon samples were cleaned up with normal saline. Photos were taken with camera. The pictures were analyzed subsequently by Fiji Image Processor Program to measure the ulcerated surface. Macroscopic mucosal damage was assessed according to the method proposed by Morris et al. as follows: no damage (0); localized hyperemia with no ulceration (1); linear ulcer with no significant inflammation (2); linear ulcer with significant inflammation at one site (3); two or more sites of ulceration and inflammation (4); and two or more sites of ulceration and inflammation or one major site of inflammation and ulcer extending >1 cm along the length of colon (5). The ulcer index was determined by summing the mean ulcer score and the mean ulcer area. Disease activity index (DAI) was measured as the ratio of colon weight to body weight, which was accordingly used for assessing the tissue edema, and to indicate the severity of colonic inflammation [18].

Assessment of colon histological damage

Tissue samples were divided into two parts with equal lengths and then weighed. One part was placed in 10% formalin for further pathological studies and the other was kept in the refrigerator at -80 °C in order to assess the myeloperoxidase activity.

For further microscopic studies, 5 µm thick paraffin sections were stained in hematoxylin and eosin. Stained colonic tissues were scored for histological damage using the criteria of Wallace and Keenan: 0 = intact tissue with no apparent damage; 1 = damage limited to surface epithelium; 2 = focal ulceration limited to mucosa; 3 = focal, transmural inflammation and ulceration; 4 = extensive transmural ulceration and inflammation bordered by normal mucosa; 5 = extensive transmural ulceration and inflammation involving the entire section [19].

Assay of colon MPO activity

MPO activity is used as a quantitative index of inflammation and a marker of neutrophil infiltration in the tissue. To measure the MPO activity, colonic samples were removed from the freezer. Then, 0.1 g of each sample was weighted and dissolved in 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethyl-ammonium bromide and finally, was homogenized. After sonication, the samples were centrifuged at 11000 rpm for 10 min at 4 °C. Then, 0.1 mL of the supernatant was combined with 2.9 mL of 50 mM phosphate buffer containing 0.167 mg/mL O-dianisidine hydrochloride and 0.005% H_2O_2. The change in absorbance was measured spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer) at 460 nm. One unit of MPO activity was defined as

the change in absorbance level per minute at room temperature in the final reaction. MPO activity (U/g) =X/weight of the piece of tissue taken, where X = 10 ×change in absorbance per minute/volume of supernatant taken in the final reaction [20].

Statistical analysis

One-way ANOVA test with Tukey post hoc test was used to analyze the parametric data. Kruskal-Wallis followed by Mann-Whitney U-test was also used for comparing the results of non-parametric data. Data was shown as Mean±SEM. p value <0.05 was considered as the significant level.

Results and Discussion

The present study was undertaken to evaluate the protective effects of essential oil of *P. graveolens* (Deproherb®) on acetic acid induced colitis. As shown by macroscopic, microscopic and biochemical evaluations of colon, the findings indicated that *P. graveolens* essential oil has the potential to alleviate colitis. The essential oil at three different doses of 100, 200 and 400 mg/kg b.w. had significant anti-inflammatory effects, compared to sulfasalazine.

Deproherb® Oral Drop was analyzed by GC and GC/MS and 28 components representing 95.3% of the oil were identified. According to table 1, β-citronellol (33.4%), geraniol (17.5%), γ-eudesmol (6.8%), linalool (6.5%) and menthone (6%) were characterized as the main components and included about 70% of the studied oral drop.

The antioxidative property of geranium essential oil could also be due to the presence of monoterpenes. The chemical composition of the essential oil of *P. graveolens*, produced in Iran, has been examined by GC and GC-MS. The β-citronellol, geraniol and linalool were the major monoterpenes detected in the chemical components. β-citronellol (33.4%) as the main constituent of *P. graveolens* essential oil has been found to have marked antioxidant and radical scavenging activity [22]. Geraniol could be considered as another active and main compound of *P. graveolens* essential oil (comprising 17.5% of the oil). Moreover, the antioxidative activities could be caused by the synergistic effects of two or more compounds present in the oils. In that regard, Lu et al. and Nivitabishekam et al. [23,24] reported that most of natural antioxidants often interact synergistically with each other in order to produce a wide spectrum of antioxidative properties which act as effective defense mechanism against free radicals. Therefore, the beneficial effects of *P. graveolens* essential oil on acetic acid induced colitis can be due to the strong antioxidative potential of the former.

Table 1. GC-MS analysis of Deproherb® oral Drop

Compound[a]	KI[b]	KI[c]	Percent
α-Pinene	938	939	0.4
cis-Linalool oxide	1072	1074	0.3
Linalool	1101	1097	6.5
cis-Rose oxide	1110	1108	1.0
trans-Rose oxide	1122	1126	0.4
Menthone	1151	1153	6.0
α-Terpineol	1192	1189	0.9
β-Citronellol	1225	1226	33.4
Z-Citral	1238	1240	0.3
Geraniol	1252	1253	17.5
Citronellylformate	1276	1274	3.9
Geranylformate	1301	1298	1.8
Citronellyl acetate	1356	1353	0.5
Geranyl acetate	1385	1381	0.6
β-Bourbonene	1390	1388	1.1
trans-Caryophyllene	1416	1419	2.0
Citronellyl propionate	1449	1446	1.3
α-Humulene	1459	1455	0.3
Geranyl propionate	1473	1475	1.0
Germacrene D	1488	1485	1.3
Ledene	1491	1493	0.3
γ-Cadinene	1511	1514	0.3
δ-Cadinene	1523	1523	1.2
cis-Calamene	1542	1540	1.1
Geranyl butyrate	1565	1562	1.9
γ-Eudesmol	1630	1632	6.8
Geranyltiglate	1701	1696	1.4
Total			93.5

[a]Compounds listed in order of elution; [b]KI: Kovats index measured relative to *n*-alkanes (C_9-C_{28}) on the non-polar DB-5 column under condition listed in the experimental section; [c]KI: Kovats index from literature.

Intra-rectal instillation of acetic acid caused significant inflammatory reactions as indicated by macroscopic and microscopic changes (figures 1 and 2). As it is shown in figure 1 and table 2, macroscopic observation in the model group showed maximum ulcer severity and disease activity index which are indicative of highest level of damage produced by acetic acid, compared to sham (normal) group that showed no change. Inflammation, hemorrhage, ulcer, necrosis, and thickened colon were evident in macroscopic presentation in model groups. However, there was no visible damage in the vehicle groups (figure 1). Data from the group pretreated with sulfasalazine (positive control) showed significant healing ($p<0.001$) in macroscopic parameters (table2). Pretreatment with Deproherb® (100, 200 and 400 mg/kg) was effective in attenuating all macroscopic parameters including ulcer severity, disease activity index and microscopic score in model group ($p<0.05$).

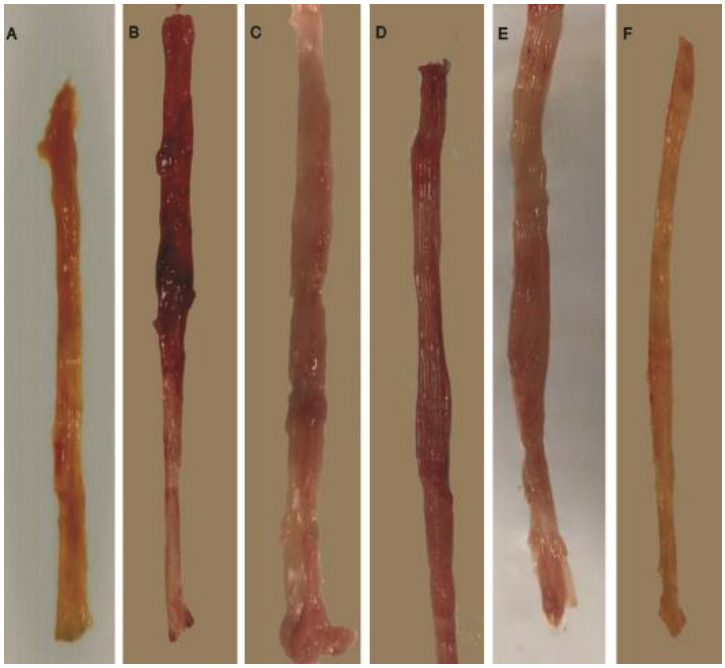

Figure 1. Macroscopic presentation of acetic–acid-induced colitis in rats; **A**: Vehicle; **B**: model group of colitis; **C**: Deproherb-treated colitis 100 mg/kg p.o.; **D**: 200 mg/kg; **E**: 400 mg/kg; **F**: Sulfasalazine pretreated colitis, 4 mg/kg; p.o

Table 2.Effects of Deproherb® drop on macroscopic and microscopic parameters of colitis induced by acetic acid in rats

Groups	W/L Ratio (mg/cm)	DAI (disease activity index: colon weight/total body weight)	Ulcer index	Macroscopic Score (0-5)	Microscopic Score (0-5)
Model group	99.93±20.70	$(7.16\pm2.70) \times 10^{-3}$	13.73±2.15	4.16 ± 1.32	4.3 ± 0.42
Vehicle	59.70± 13.03*	$(4.96\pm1.02)\times10^{-3}$*	2.50±3.13*	0.40±0.54*	0.0*
Sulfasalazine	76.66±15.86*	$(3.52\pm1.08)\times10^{-3}$*	5.75±1.50*	2.0±0.81*	3.0±0.36*
100mg/kg	62.33±15.88*	$(6.88\pm1.24)\times10^{-3}$	5.73±4.16*	1.25±1.5*	2.2±0.31*†
200mg/kg	61.52±17.51*	$(5.75\pm0.73)\times10^{-3}$*	2.42±2.50*†	1.33±1.15*	3.2±0.31*†
400mg/kg	41.24±23.75*†	$(5.52\pm1.26)\times10^{-3}$*	4.00±1.65*	0.66±0.57*†	2.5±0.56*†

Observations were scored as described in methods section. Each value represents mean ± SEM of six animals in each group.
* Significant ($p<0.05$) decrease in macroscopic score values, compared to model group;† The difference in macroscopic score values of Deproherb-treated and sulfasalazine-treated groups was significant.

Oral administration of sulfasalazine was effective in reducing the inflammation severity and extent as well as crypt damage and total colitis index in injurious colons (table 2).

The changes in MPO activity in colon homogenate of treated animals have been shown in figure 2. The colitis caused by acetic acid was associated with an increase in MPO activity, and the MPO activity of the model group showed significant increase, compared to vehicle group (63.14 versus 5.11 U/g colon). The MPO activity level significantly decreased in Deproherb®-treated (100, 200 and 400 mg/kg) and sulfasalazine-treated groups.

The Mean ± SEM of MPO activity in Deproherb® (100 mg/kg) and sulfasalazine groups were 32.89 ± 0.17 and 10.76 ± 0.15 (U/g colon), respectively.

The mechanisms by which acetic acid produces inflammation seem to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons within intracellular acidification that most likely account for the observed epithelial injury. The initiative inflammatory response induced by acetic acid activates the pathways of cyclooxygenase and lipoxygenase [25, 26]. Sulfasalazine is a standard treatment for acute inflammatory bowel disease as well as for maintaining remission. It is composed of 5-aminosalicylic acid and sulfapyridine joined by an azo bond. 5-Aminosalicylic acid is the active moiety responsible for the therapeutic efficacy of the drug in ulcerative colitis. The sulfapyridine component (acting only as the vehicle for 5-aminosalicylic acid) is evidently responsible for most adverse effects. The anti-inflammatory properties of 5-aminosalicylic acid are related to its topical effects on the inflamed colonic mucosa.

Moreover, 5-aminosalicylic acid inhibits the colonic formation of prostanoids, leukotriene B_4, leukotriene C_4, and platelet activating factor [27].

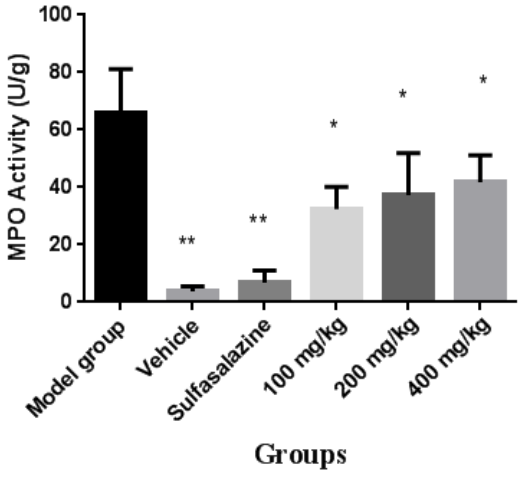

Figure 2. Effects of Deproherb® and sulfasalazine on MPO activity in acetic acid-induced colitis in rats. Each value represents Mean ± SEM of MPO activity in acetic acid-induced colitis in rats by treated compounds. * P<0.05, **P<0.01 compared to the model group

Thus, essential oil of *P. graveolens* may affect the release or synthesis of these inflammatory mediators. In support of finding, it has been shown that geranium oil has anti-inflammatory properties against the inflammation induced by carrageenan and collagen II in mice [13]. Amount of MPO activity has been found to be reliable biomarkers of oxidative stress [28-30]. In previous reports with rodent models of colitis, the utility of this assay has been established for detection of oxidative stress in vivo. Moreover, MPO activity has been found to be correlated with intestine providing further basis for selection of this biochemical assay [31-33]. Considering the macroscopic (ulcer index) and histological (total colitis index) results, it is evident that essential oil of *P. graveolens* possessed dose-independent antiulcerogenic effect. These results suggested that *P. graveolens* essential oil reduced the oxidative stress by preventing the generation of free radicals and, thus inhibiting the development of inflammation. In previous studies, reduction in the oxidative stress and lipid peroxidation of the geranium essential oils treated animals was reported which indicated the important role of essential oil as antioxidanst. Boukhris et al. in 2012 demonstrated that the treatment of alloxan-induced diabetic rats with the leaf essential oil of rose-scented geranium increased the superoxide dismutase (SOD), catalase (CAT) and glutathione (GPX) activities that might be due to the activation of enzymes by *P. graveolens* essential oil or reduction of reactive oxygen species level [34].

Figure 3 has shown the histological pictures of the colon sections of different treated groups. They are totally in agreement with macroscopic and microscopic scores (table 2).

No pathological and histological damages were observed in Sham group. The rats with acetic acid-induced colitis (model group) indicated necrotic destruction of epithelium hemorrhage, edema, inflammatory cellular infiltration, crypt damage, and ulceration at mucus and sub-mucosal layers (table 2 and figure 1). Treatment with sulfasalazine significantly reduced the severity of inflammation while pretreatment with Deproherb® was also effective in reducing the histopathology scores.

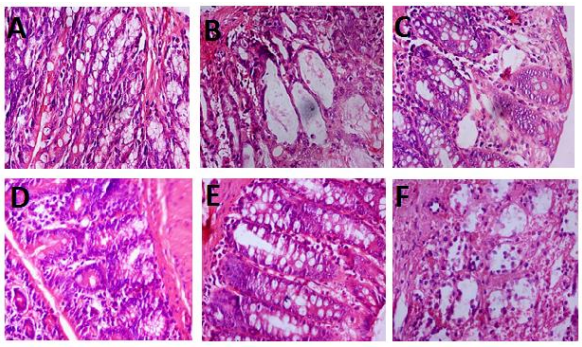

Figure 3. Microscopic presentation of acetic acid-induced colitis in rats (H&E staining with X40 magnification); A: vehicle, 2 mL/kg. Mucus layer and crypts are normal and leukocyte infiltration is absent; B: model group of colitis treated with normal saline, 2 mL/kg; mucosal and sub-mucosal inflammations as well as crypt damage and leukocyte infiltration are vastly evident; C: colitis pretreated with *Pelargonium graveolens* essential oil (Deproherb-treated colitis) 100 mg/kg .p.o.; D: 200mg/kg; E: 400 mg/kg administered p.o.; F: sulfasalazine treated colitis, 4 mg/kg

In Conclusion, it was demonstrated that *P. graveolens* essential oil was effective in protecting acetic acid-induced colitis in rat. It is possible that the known antioxidant, antimicrobial and anti-inflammatory potentials in the essential oil protected the animals against experimentally induced diseases. The present study proposed *P. graveolens* essential oil to be used as an anti-inflammatory and anti-ulcerative medicinal plant for colitis conditions. More studies are needed to distinguish the involved

mechanisms and also to study the active compounds responsible for the beneficial pharmacologic actions of this plant.

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Author contributions

Zahra Mousavi contributed in data collection and analysis, designing the study method and writing the manuscript; Marjan Bastani participated in designing the study method; Parvaneh Najafizadeh provided the data and contributed in drafting of the manuscript; Nassim Assar did the analysis and interpretation of data; Jinous Asgarpanah contributed in critical revision of the manuscript. All authors read and approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

MPO: myeloperoxidase; IBD: inflammatory bowel diseases; UC: ulcerative colitis; CD: Crohn's disease; PGE: *Pelargonium graveolens* essential oil