





Protective Effect of a Formulation Containing *Pistacia atlantica* Oleo-Gum-Resin and Honey on Experimental Model of Acetic Acid-Induced Colitis in Rats

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Abstract

Background and objectives: Inflammatory bowel disease (IBD) is a recurrent chronic inflammatory disease of the gastrointestinal tract. In Iranian traditional medicine, the oleo-gum-resin of the genus *Pistacia* is recommended for treatment of various diseases including gastrointestinal disorders. The present study investigated the therapeutic action of a combination of *Pistacia atlantica* subspecies *kurdica* oleo-gum-resin and honey in acetic acid-induced colitis in rats. **Methods:** *Pistacia atlantica* oleo-gum-resin was mixed with honey. The mixture was suspended in distilled water. Following induction of colitis with 4% acetic acid in all animals, except in sham group, the mixture was orally administered for two consecutive days at the concentrations of 100, 200, 400 mg/kg. Other groups included the control, sham and a standard group (dexamethasone). Microscopic and histopathologic examinations were conducted in inflamed colonic tissue. The inflammatory biomarkers of colitis including interleukin 6 (IL-6), tumor necrosis factor (TNF- α), and myeloperoxidase (MPO) and the gene expression level of toll like receptor-4 (TLR-4) were assessed. **Results:** *Pistacia atlantica* oleo-gum-resin+ honey induced significant progress in macroscopic and microscopic scores. Colonic levels of MPO, IL-6, and TNF- α significantly declined in rats treated with the mixture; while significant decrease in mucosal gene expression of TLR-4 and significant improvement of colitis were observed. *Pistacia atlantica* oleo-gum-resin (400 mg/kg) + honey (400 mg/kg) reduced inflammation of the bowel and colonic ulcer severity shown by downregulation of inflammation cytokines, reduction of neutrophil infiltration, and suppression of TLR-4 expression. **Conclusion:** The combination might be a promising supplement for treatment of inflammatory disorders.

Keywords: honey; inflammatory bowel disease, inflammatory mediators; *Pistacia atlantica*; toll like receptor

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Introduction

Inflammatory bowel disease (IBD) is a general term for preliminary addressing two medical conditions, ulcerative colitis (UC) and Crohn's disease (CD). Although these two conditions seem similar, their clinical manifestations are, to some extent, different [1,2]. The etiology of IBD is not fully known, although, it has been suggested that a genetic failure triggered by environmental factors can contribute to the disease development. Microbial infection, colon flora, and immunity impairments are also involved [3-5]. Concerning the inflammatory nature of the disease, a number of mediators have been nominated to play fundamental roles in IBD progression. Activation of the family of toll like receptors (TLRs) is known to induce several pro-inflammatory genes, the nuclear factor NF- κ B pathway, and many other inflammatory cytokines production, which altogether are responsible for activating the innate immune system. TLRs are widely present in immune cells and intestinal mucosa [6]. TLRs are transmembrane proteins that belong to the pattern recognition receptor (PRR) family, which are involved in recognition of the pathogen-associated molecular patterns (PAMPs). Up to date, more than 13 TLRs have been identified; of which, TLR-4 is the most important member and the most studied type. TLR-4 is activated in response to various stimuli such as viral proteins, bacterial lipopolysaccharide, and endogenous proteins i.e. heat shock protein. Upon inflammation, TLR-4 is stimulated by recognizing PAMPs in the intestinal tract, primarily through its adaptor, MYD-88. Following a series of structural conformations, several downstream effectors such as NF- κ B are activated, leading to the production of various pro-inflammatory, and inflammatory cytokines (i.e. tumor necrosis factor (TNF- α)) as well as chemokines. It is assumed that chronic inflammation may upregulate the expression of TLR-4 in colonic epithelial cells. TLR-4 function and activation is important to drive inflammatory events, particularly in IBD. Over expression of TLR-4 has been repeatedly stated in UC and CD patients [6–8].

It was also shown that activated monocytes and

macrophages are predominant source of interleukin 6 (IL-6) in patients with IBD. Moreover, IL-6 is associated with steroid resistance in severe pediatric UC [9,10]. Besides IL-6, TNF- α is also one of the most important inflammatory mediators that participate in IBD development and progression. Through re-induction of the NF- κ B pathway, TNF- α activates a complex cascade that leads to the simultaneous release of inflammatory and pro-inflammatory cytokines [11]. In addition to inflammatory mediators, immune cell infiltration including neutrophils, is a histopathological feature of IBD. Intrinsic immunity can improve intestinal damage. However, infiltration of neutrophils and monocytes into the lesion site, followed by secretion of inflammatory cytokines and mediated species such as reactive oxygen species (ROS), cause inflammation and contributes to the pathogenesis of the disease [12]. Myeloperoxidase (MPO) is mostly found in neutrophil granulocytes and to a lesser extent in macrophages. MPO produces a substance called hypochlorous acid (HOCL) that can cause biological and local damage in the intestinal tissues [13,14].

Up to now, there is no definite cure for IBD; however, the existing solutions such as corticosteroids, amino salicylates, anti-TNF- α , and immune-modulators are not efficient enough and usually cause adverse effects and an overall patient dissatisfaction [1,15].

Herbal remedies were always the center of public interests and have gained more popularity in recent decades. In a recent population study, it was shown that approximately 50% of the world population use complementary and alternative therapies [16]. Wild pistachio tree ("Baneh" in Persian), with scientific name of *Pistacia atlantica* Desf. subspecies *kurdica* (Zohary) Rech. f. belongs to the Anacardiaceae family. "Baneh" is a tree with a height of 2 up to 7 m and mostly grows in the mountains. The plant has five to seven inflorescences leaflets, which have different forms in different species. This plant is unisexual with five sepals and no petals. The male flowers have 4–5 flag folds and female flowers have a short style. There is a sap in the

inner tissues of the “Baneh” tree, which in summer is commonly collected from the pores of the bark [17].

It is frequently distributed in Western regions of Iran, including Kermanshah. The sap of this plant is called “Saqez”, *P. atlantica* gum or *P. atlantica* oleo-gum-resin [18–20]. In Iranian traditional medicine, wild pistachio is used entirely or only as oleo-gum-resin to treat gastrointestinal disorders such as peptic ulcer, dyspepsia, diarrhea, as well as skin wounds, kidney and liver diseases [20–24].

Wild pistachio has shown anti-inflammatory and antibacterial activities [25,26]. Due to the anti-inflammatory effects, this herb has been used to treat skin wounds [27] caused by burns, and has shown positive effects in diabetes [28], fungal diseases [29], and cutaneous leishmaniasis [30]. Due to the efficacy in reduction of free radicals, wild pistachio is beneficial for cardiovascular diseases and cancer [21]. Gas chromatography analysis showed that the plant's bark contains a variety of substances including alpha pinene, camphene, myrcene, and limonene. The amount of alpha pinene was shown to be higher than other substances [17,24,31]. It was reported that the extract of *P. atlantica* reduced the plasma levels of IL-6, c-reactive protein (CRP), TNF- α , thiobarbituric acid reactive substances (TBARS), super oxide dismutase (SOD), catalase (CAT), and MPO, reflecting the antioxidant and anti-inflammatory properties of this plant [17,31–34]. It is noteworthy that different parts or components of this plant such as leaves, oleo-resin-gum, and essential oils are reported to be show therapeutic effects. For instance, the essential oil of *P. atlantica* demonstrated antioxidant and antibacterial properties. In another study, the hydro-ethanolic extract of *P. atlantica* leaves showed nephroprotective and anti-inflammatory effects [31, 32].

Honey is widely used in traditional medicine for threatening a broad range of skin wounds and digestive problems by different cultures worldwide. Honey has also been mentioned in various traditional medicine databases as antitussive, throat lubricant, appetite suppressant, and for wound healing [35–38]. Generally, honey has a variety of ingredients, including vitamins and minerals, enzymes, flavonoids, amino acids, proteins, and sugars at different percentages [39–41]. Besides its anti-inflammatory and antioxidant properties [42,43], honey has

therapeutic effects like antibacterial, antifungal [44], antiviral [45], and anticancer [46] activities. Honey either directly or indirectly can modulate oxidative stress by reducing factors such as SOD, CAT, and TBARS. On the other hand, honey reduces inflammatory markers such as IL-1 β , TNF- α , IL-10, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and MPO [42]. Previously, it was shown that the combination of honey and sulfasalazine significantly reduced colonic inflammation [47]. In another study, combination of honey and *Spiroplina plantensis* exhibited protective effects against UC in rats [48].

Regarding the anti-inflammatory and antioxidant activities of *P. atlantica* gum and honey, and the inflammatory mechanisms involved in IBD, we aimed to investigate the curative effects of the combination formulation in an experimental model of acetic acid-induced colitis in rats.

Materials and Methods

Ethical considerations

The study was approved by Islamic Azad University of Medical Sciences guidelines for the care and use of laboratory animals with ethic number [IR.IAU.PS.REC.1398.156](#). Current animal study was performed according to the “Principles of Laboratory Animal Care” (NIH publication 82-23, revised in 1985 and 1996) and based on Guidelines and Protocols of the Research Ethics Committee of Islamic Azad Tehran Medical Sciences University-Pharmacy and Pharmaceutical Branch Faculty, confirmed by Ministry of Health and Medical Education. All efforts were made to minimize the number of animals and their suffering.

Chemicals

Chemicals were obtained from Sigma-Aldrich (GmbH, Munich, Germany). Rat-specific tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) enzyme-linked immunosorbent assay ELISA kits were prepared by Enzo Life Sciences, Lorrach/Germany.

Plant material

Pistacia atlantica Desf. *subspecies kurdica* (Zohary) Rech. f. gum was collected during May to July 2019 from Kermanshah province, Iran and was authenticated by Dr. Gh. Amin (Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical

Science). A voucher specimen (No. PMP-818) was deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.. Honey was collected from the slopes of the Alborz mountain in Lorestan.

Preparation

For preparation of honey-gum mixture, the gum and honey were suspended in distilled water (vehicle), sonicated and heated (up to 50 °C), just before use [49,50].

Animals

Forty-eight male Wistar rats (6-7 weeks old; 180–230 g) were provided by the animal laboratory of Medicinal Plants Research Center, Institute of Medicinal Plants, Alborz, Iran. The rats were housed under specific pyrogen-free condition and maintained on standard pellet chow and tap water ad libitum. They were kept in standard conditions (12-h light/dark cycle, 22 ± 0.1°C, and 50–55% humidity) (six rats in each cage).

Induction of colitis

Acetic acid-induced colitis is an animal model that mimics some of the acute inflammatory responses that happens in UC. Induction of colitis in rats using acetic acid is a classical method used to make an experimental model of human IBD [51]. Animals were fasted 24 h prior to any intracolonic studies and were just permitted to access water. Colitis was then induced according to the method of Fakhraei et al. [52]. Briefly, the rats were anaesthetized with an intramuscular injection of ketamine (4 mg/100 g) and xylazine (1 mg/100 g) mixture, and a medical-grade polyurethane canal for enteral feeding (external diameter 2 mm) was inserted into the anus, and the tip was advanced 8 cm proximal to the anus verge. Afterwards, 2 mL acetic acid 4% was injected into the colon of all animals, except for the sham group. There was no mortality in animals, either following colitis induction or after samples administration. Since the animal model of acute colitis was induced with acid solution, welfare of animals has been monitored carefully. Following colitis induction, some extent of stress, anorexia, and diarrhea was observed.

Experimental groups

The study period was four days for all groups. Twenty-four hours after induction of colitis,

experimental samples (drugs) were administered for two consecutive days (once a day). Animals were randomly chosen and divided into eight groups (n=6). The animals were grouped as follows;

1. Sham: healthy rats without any colitis were treated with 1 mL saline (gavage), for 2 days, once a day
2. Control group: colitis was induced by acetic acid (4%), the rats were treated with 1 mL saline (Oral gavage).
3. Dexamethasone: colitis was induced by acetic acid (4%). The rats were treated with 1 mL dexamethasone (1 mg/kg, intraperitoneally)
4. Comb100 group: colitis was induced by acetic acid (4%). The rats were treated with 1 mL honey (100 mg/kg) + *P. atlantica* oleo-gum-resin (PA gum) (100 mg/kg, oral gavage)
5. Comb200 group: colitis was induced by acetic acid (4%). The rats were treated with 1 mL honey (200 mg/kg) + PA gum (200 mg/kg, oral gavage)
6. Comb400 group: colitis was induced by acetic acid (4%). The rats were treated with 1 mL honey (400 mg/kg) + PA gum (400 mg/kg, oral gavage)
7. Honey 400 group: colitis was induced by acetic acid (4%). The rats were treated with 1 mL honey (400 mg/kg, oral gavage)
8. PA gum 400 group: colitis was induced by acetic acid (4%). The rats were treated with 1 mL PA gum (400 mg/kg, oral gavage) [17,53].

Assessment of colonic damage

Following 48 h of colitis induction, the animals were euthanized using carbon dioxide inhalation. In an ice bath, distal colons were cut open, cleansed gently with normal saline, and macroscopic scores were designated. Subsequently, colons were cut into two same pieces; one piece was used for histopathological index assessment (kept in 5 mL of 10% formalin) and the other part was used for analysis of biochemical markers (kept under -80 °C).

Determination of ulcer index

Isolated colonic segments were monitored by magnifying lens and macroscopic scoring was applied according to the Morris criteria: 0, intact epithelium with no damage; 1, localized hyperemia but no ulcer; 2, linear ulcer with no significant inflammation; 3, linear ulcer with inflammation at one site; 4, two or more sites of ulcer and inflammation; 5, two or more sites of ulcer and inflammation extending over 1 cm [54].

Microscopic evaluation of colonic damage

For microscopic (histologic) characterization, colonic tissues were fixed in phosphate-buffer formaldehyde, embedded in paraffin and 5 micrometer-thick sections were prepared. Colonic tissues were stained with Hematoxylin and Eosin and evaluated by light microscopy, being scored in a blinded manner by an expert pathologist [55]. Parameters including erosion, ulceration, mucosal necrosis, hemorrhage of mucosa, lamina propria and submucosal edema, and inflammatory cell infiltration were scored. These verities of changes were subjectively graded and compared with controls. Histological evaluation was carried out using a Zeiss® microscope equipped with an Olympus color video camera for digital imaging.

Biochemical assays

Determination of inflammatory mediators

Colonic levels of TNF- α and IL-6 were determined with an enzyme linked immunosorbent assay (ELISA kit) (Diaclone Rat TNF - α ELISA & ZellBio GmbH Rat IL-6 ELISA). To measure inflammatory cytokines, colon was cut up and homogenized in 50-mmol/L ice-cold potassium phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethylammonium bromide. Homogenates were centrifuged at 4000 rpm for 20 min at 4°C, and supernatants were separated subsequently and kept at -80°C until analysis. Wells pre-coated with a monoclonal antibody served to trap cytokine molecules in homogenate specimen. The results were expressed as picogram per milligram of wet tissue [56].

Determination of MPO activity

The test solutions and reagents were prepared and the MPO activity per g of tissue protein was determined and calculated by the following method. Colonic homogenate was prepared as described in previous section and the homogenate was sonicated in an ice bath for 10 s, then subjected to a sequence of freezing and thawing for 3 times, sonicated again for 10 s, and centrifuged for 15 min at 15,000 rpm at 4°C. Samples (0.1 g) were dissolved in 10 mM potassium phosphate buffer with pH 7, containing 0.5% hexadecyltrimethylammonium bromide and then were homogenized. Colonic samples were centrifuged with 20,000 rpm for 30 min at 4 °C. Supernatant fluid was mixed with

H₂O₂ (0.1 mM) and HCl (1.6 mM), then the absorbance was measured at 450 nm. Data expressed in units per gram (U/g) of protein [13].

Investigation of the TLR-4 gene expression with real time-reverse transcription polymerase chain reaction (RT-PCR)

Following preparation of colonic homogenate, RNA extraction was performed using the TRIzol reagent kit and kept in RNA later. Afterwards, cDNA was generated using the iScript cDNA Synthesis Kit. cDNA was synthesized using oligo dT specific primers;

Tlr-4-

F: 5'- GCAGGTCTGAATTGTATCGCC-3'

R: 5'- GGGTTTTAGGCGCAGAGTTT-3'

Gapdh-

F: 5'-ACTGAGCAAGAGAGGCCCTA-3'

R: 5'-TATGGGGGTCTGGGATGGAA-3'

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was considered as housekeeping gene. Quantitative RT-PCR was performed using LightCycler 96. Duplicate cycle threshold (CT) of each reaction was calculated from the amplification curve to determine the relative gene expression using the comparative cycle threshold method ($\Delta\Delta C_t$). Relative changes in gene expression were determined using the $2^{-\Delta\Delta C_t}$ method compared with endogenous reference GAPDH mRNA level [57].

Statistical analysis

All values are expressed as means \pm SEM SPSS (version 19.0, Chicago, USA). One-way analysis of variance was employed for analyzing the data, followed by Tukey's test for multiple comparisons. Significance ascribed when $p < 0.05$.

Results and Discussion

Sham group represented healthy intestinal condition, in which no wound was formed. In control group, induction of untreated wounds, displayed extensive necrotic lesions in most parts of the intestinal tissue, which was accompanied by swelling of mucosal layer and was significantly different from sham group ($p < 0.001$). Administration of dexamethasone in animals with colitis improved wound healing, adhesion, thickening of the intestinal wall, and inflammation. Combination of honey+PA gum (400 mg/kg) significantly reduced inflammation and improved wounds ($p < 0.01$), while in other groups [Comb 100, Comb200, honey (400 mg/kg)

and PA gum (400 mg/kg)], there was no significant decline in colonic inflammation as compared with control (Table 1).

Table 1. Macroscopic extent of colonic injury in acetic acid-treated rats. Data are presented as mean \pm SEM, (n=6)

Groups	Macroscopic score (Mean \pm SEM)
Sham	0
Control	4.4 \pm 0.24***
Dexamethasone (1 mg/kg)	2.2 \pm 0.37###
Comb100	4.6 \pm 0.40
Comb200	2.8 \pm 0.37
Comb400	1.8 \pm 0.20###
400 mg/kg honey	2.8 \pm 0.66
400 mg/kg PA gum	3.0 \pm 0.31

SEM: standard error of the mean; *Pistacia atlantica* oleo-gum-resin (PA gum); Comb100, 100 mg/kg honey +100 mg/kg PA gum; Comb200, 200 mg/kg honey +200 mg/kg PA gum; Comb400, 400 mg/kg honey +400 mg/kg PA gum ***: significantly different from Sham group ($p < 0.001$), ###: significantly different from control group ($p < 0.001$).

In microscopic studies, mucosal, submucosal, and muscular layers of colon in sham group were assessed as healthy with no significant pathologic changes.

In control group, severe inflammation, necrosis, edema and accumulation of polymorphonucleates were observed in mucosal and under mucosal tissues (Figure 1-A). In dexamethasone group, brief lesions and low mucosal inflammation were observed (Figure 1-B). Edema, congestion and surface erosion were

detected in submucosal area of the groups treated with Comb100, while in addition to edema, moderate inflammation and focal wounds were observed in mucosal layer, with significant difference to the control group (Figure 1-C).

In Comb200 treated animals, edema and moderate inflammation were noticed, in both mucosal and submucosal layers (Figure 1-D). The highest therapeutic effect was recorded in Comb400 group, where inflammation and edema were attenuated in mucosal and submucosal layers, with significance similar to dexamethasone and sham groups (Figure 1-E). In groups including PA gum (400 mg/kg) and honey (400 mg/kg) alone, moderate to severe inflammation, surface erosion, necrosis and focal ulceration were observed (Figure 1F/G). The amount of TNF- α in colonic tissue increased significantly in control group compared with sham group ($p < 0.001$). Administration of dexamethasone and combination of PA gum + honey in all concentrations, as well as separate usage of honey or PA gum significantly reduced the TNF- α levels ($p < 0.001$). However, groups treated with the mixture of resin and honey showed more inhibitory effect on TNF- α level compared with those that were treated with gum or honey alone. Comb400 suppressed the TNF- α activity more than other groups (Figure 2).

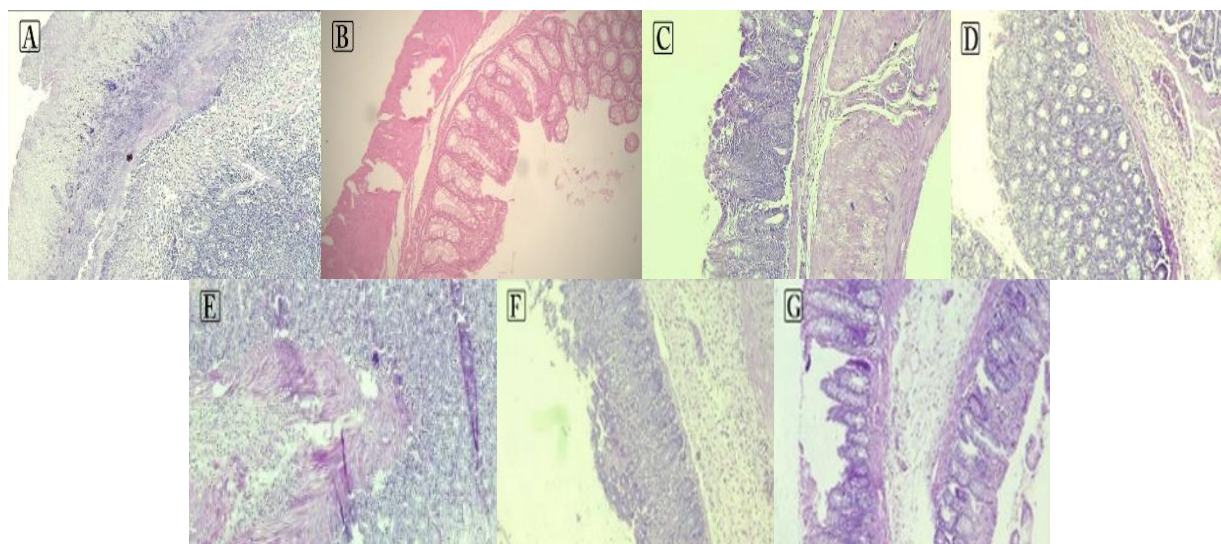


Figure 1. Microscopic evaluation of gastric tissue, stained with Hematoxylin and Eosin; A: control group: extensive ulceration and necrosis in most parts of tissue with inflamed mucosa; B: dexamethasone group, mild inflammation, no significant pathologic changes; C: Comb100, inflammation and edema in mucosa and submucosa with surface erosion; D: Comb200, moderate edema and inflammation in mucosa and sub mucosa; E: Comb400, mild edema and inflammation in mucosa and submucosa; F: PA gum (400 mg/kg), moderate to severe inflammation with surface erosion; G: Honey (400 mg/kg), moderate mucosal inflammation.

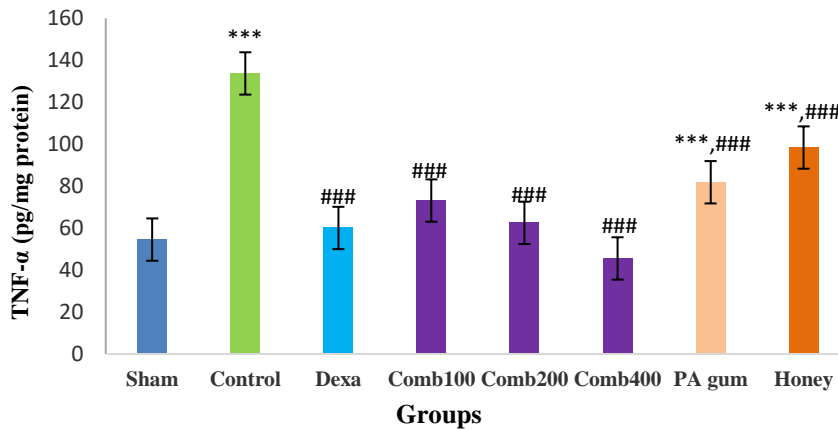


Figure 2. TNF- α levels in colon tissue (pg/mL protein) in rats with acetic acid-induced colitis. Results are expressed as means \pm SEM; (n = 6 rats/group). Dexa: dexamethasone; Comb100: 100 mg/kg PA gum +100 mg/kg honey; Comb200: 200 mg/kg PA gum +200 mg/kg honey; Comb 400: 400 mg/kg PA gum +400 mg/kg honey; PA gum: *Pistacia atlantica* oleo-gum-resin (400 mg/kg); Honey: Honey (400 mg/kg); *** significantly different from sham ($p < 0.001$); ### significantly different from control ($p < 0.001$)

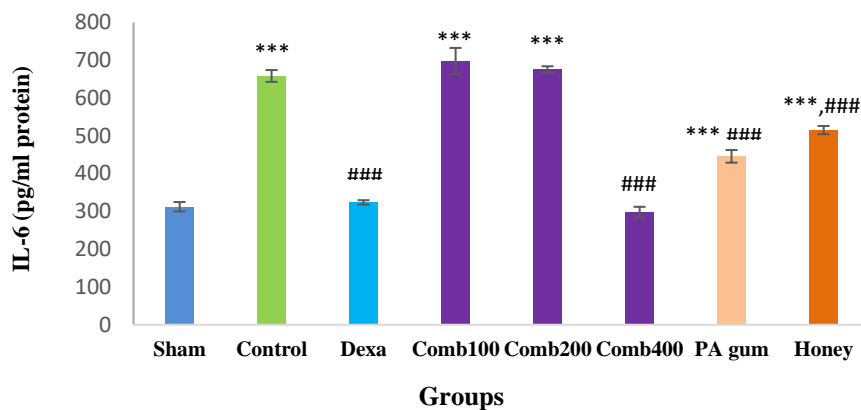


Figure 3. IL-6 levels in colon (pg/mL protein) in rats with acetic acid-induced colitis. Results are expressed as means \pm SEM; (n = 6 rats/group). Dexa: dexamethasone; Comb100: 100 mg/kg PA gum +100 mg/kg honey; Comb200: 200 mg/kg PA gum +200 mg/kg honey; Comb 400: 400 mg/kg PA gum +400 mg/kg honey; PA gum: *Pistacia atlantica* oleo-gum-resin (400 mg/kg); Honey: Honey (400 mg/kg); *** significantly different from sham ($p < 0.001$); ### significantly different from control ($p < 0.001$).

The inflammatory status of rats with IBD led to an increase in IL-6 level compared with sham group ($p < 0.001$). Administration of honey- PA gum combination with doses of 100 mg/kg and 200 mg/kg did not significantly alter the IL-6 level. However, administration of a higher dose of honey- PA gum (400 mg/kg) significantly reduced the IL-6 level in comparison with control animals ($p < 0.001$).

Treatment with PA gum (400 mg/kg), and honey (400 mg/kg) could also make a significant difference in IL-6 level compared with control group ($p < 0.01$) (Figure 3).

The activity of MPO enzyme reduced in all treatment groups compared with that of control;

however, this reduction was prominent in honey-gum treated rats (200 mg/kg and 400 mg/kg) ($p < 0.001$). Comb400 exhibited the highest inhibitory effect on MPO comparing with other treatment groups. The activity of Comb200 and Comb400 did not differ from dexamethasone significantly (Figure 4).

In $2^{-\Delta\Delta Ct}$ analysis, it was found that Comb200 and Comb400 suppressed the mRNA expression of TLR-4 substantially by nearly 13-fold in colitis colonic homogenates compared with control group, respectively. Although, this reduction was also significant in honey (400 mg/mL) treated rats by fold changes of 2 when compared with that of control rats. Comb400

showed the highest downregulation of TLR-4 gene in comparison with other test groups (Figure 5).

Overall, we found that the combination of honey and PA gum improved microscopic and macroscopic injuries. This combination, particularly at higher concentrations of *P. atlantica*, reduced inflammatory cytokines including the TNF- α , IL-6, and MPO. The formulation also suppressed the mRNA expression of TLR-4 gene in rat's colonic tissue, of which Comb400 mg/kg downregulated the mRNA expression of TLR-4 by nearly 13-fold compared with that of the control. In case of synergistic effect of honey and oleo-gum-resin formulation, our results confirmed the outcomes of previous studies that reported *P. atlantica* and honey (each one alone) have potent anti-inflammatory effects. For instance, Bahrami et al demonstrated that oral administration of *P. atlantica* gum essential oil significantly reduced macroscopic and microscopic scores of and suppressed the level of COX-2 in colon tissue of UC rats [58].

In another study, combination of *P. atlantica*, butyrate, and *Lactobacillus casei* reduced the severity of inflammation in 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis. Macroscopic and histopathological scores, as well as colonic MPO were also significantly attenuated. Those results revealed that combination therapy was more effective than treatment with each one of these agents alone [33]. Our study further confirmed the point that synergism of honey and oleo-gum-resin dramatically enhanced the healing process of UC

in the animal model. Even though the presence of neutrophil in intestinal inflammatory conditions is well known, recent findings showed a more significant role of neutrophils and their byproducts in intestinal inflammatory processes [59]. Honey-gum combination at the doses of 100, 200 and 400 mg/kg suppressed the MPO activity in colon tissues; however, this reduction was more prominent at 400 mg/kg dose of PA gum.

Despite lacking information about the definite etiology and pathology of IBD, it has been well established that unbalanced responses of both the innate and acquired immunity are of the major reasons behind the disease [60]. As a subsequent, the expression of pro-inflammatory cytokines such as IL-12, IL-6, IL-1 β , TNF- α , and IFN γ increases in this state [4,61]. Recent studies have shown that compounds that selectively inhibit the TNF- α activity, are also able to control UC and CD, and generally improve IBD [62,63].

T-cells and pre-inflammatory cytokines can also regulate inflammation and improve IBD. On the other hand, evidence suggests that high production of TNF- α , and IL-6 in inflammatory mucosa contributes to improved tissue injury of UC [64]. The outcomes of experimental, pre-clinical and clinical studies exhibited that the TLR-4 level enhances in the intestinal mucosa of patients with IBD and other immunodeficiency diseases. It was shown that the TLR 4 activity is initiated by an adapter called MYD and then activates the NF- κ B signaling pathway. Recent studies have examined a number of TLR inhibitors for treatment of UC, which could be a promising approach to treat or prevent IBD [6].

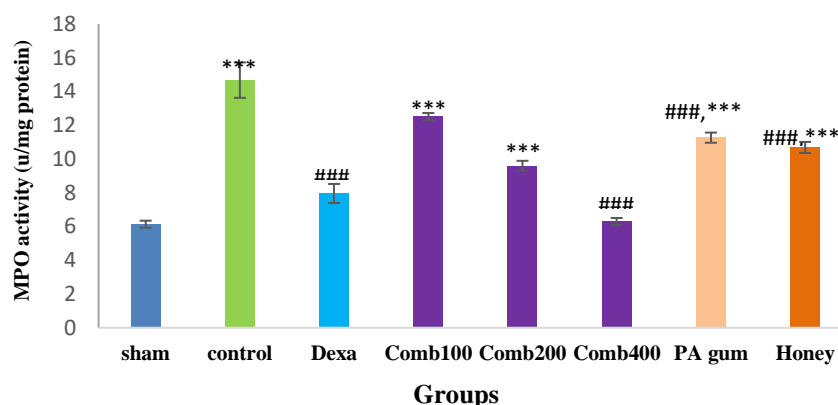


Figure 4. Colonic MPO activity (ng/mg protein) in rats with acetic acid-induced colitis. Results are expressed as means \pm SEM; (n = 6 rats/group). Dexa: Dexamethasone; Comb100: 100 mg/kg PA gum +100 mg/kg honey; Comb200: 200 mg/kg PA gum +200 mg/kg honey; Comb 400: 400 mg/kg PA gum+400 mg/kg honey; PA gum: *Pistacia atlantica* oleo-gum-resin (400 mg/kg); Honey: Honey (400 mg/kg); *** significantly different from sham (p<0.001); ### significantly different from control (p<0.001).

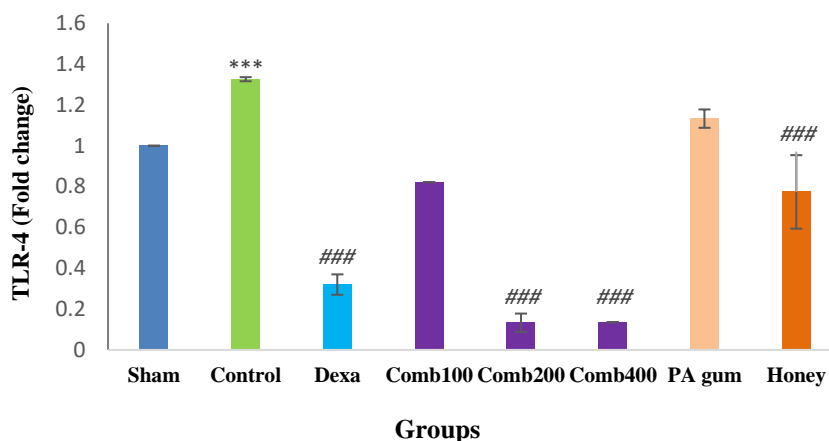


Figure 5. The mRNA expression of TLR-4 in colitis tissue of honey-PA gum, gum and honey treated rats. TLR-4: toll like receptor-4; Dexa: Dexamethasone; Comb100: 100 mg/kg PA gum +100 mg/kg honey; Comb200: 200 mg/kg PA gum +200 mg/kg honey; Comb 400: 400 mg/kg PA gum +400 mg/kg honey; PA gum: *Pistacia atlantica* oleo-gum-resin (400 mg/kg); Honey: Honey (400 mg/kg); *** significantly different from sham ($p < 0.001$); ### significantly different from control ($p < 0.001$).

Haghdoust et al. reported α -pinene as the main constituent in *P. atlantica* resin extract. α -Pinene dose-dependently improved healing of skin burn wounds in the animal model [27]. In 2014, therapeutic effect of the fruit extract of *P. atlantica* was investigated in acetic acid-induced colitis in rats. Animals were treated for 7 days, either orally (received 300 and 600 mg/kg *P. atlantica* fruit oil extract) or rectally (10% and 20% gel from *P. atlantica* fruit as enema). It was shown that both oral and rectal administration of the extract had acceptable preventive effects on the progression of microscopic, macroscopic, and pathological features of IBD [65]. Zho et al. demonstrated that α -pinene significantly inhibited the translocation of NF- κ B protein complex, which reduced intestinal inflammation [66]. In another study, the protective effect of the essential oil of *P. atlantica* (25, 50 and 100 mg/kg, orally) on ethanol-induced peptic ulcer was assessed in rat model. The *P. atlantica* essential oil improved peptic ulcer dose dependently, although, dose of 100 mg/kg considerably decreased the ulcer index [50]. In a study on wild pistachio, subsp. *kurdica*, the effectiveness of oleo-gum-resin was confirmed in acetic acid-induced UC model. This study assumed that α -pinene was the main ingredient of oleo-resin-gum. Oral administration of oleo-gum-resin significantly reduced the colitis indicators such as CRP, IL-6, and MPO activities [17]. In a recent study, the protective and therapeutic effects of PA gum (at doses of 100, 200, 400 mg/kg) were confirmed in UC model [67].

On the other hand, honey is shown to directly or indirectly act on oxidative stress and affect various bioactive molecules including IL-1 β , IL-10 TNF- α , P53, IFN γ , lipid peroxidase (LPO), and COX-2 [42]. High concentration of D-fructose in honey was shown to inhibit MPO exocytosis [68]. Medhi et al. reported that Manuka honey (5 g/kg) and sulfasalazine (360 mg/kg) improved inflammation in UC rat model, separately and in combination therapy with additive effect [47]. In rats with acute foot edema, honey reduced the plasma level of inflammatory mediators such as iNOS, COX-2, TNF- α , and IL-6 [43]. The protective effects of honey and *Spiroliina plantesis* against acetic acid-induced UC in rats was also confirmed in another study, which was implicated to the preventive effect of this mixture on oxidative stress and inflammatory parameters [48].

Conclusion

Overall, oral administration of honey and *P. atlantica* subsp. *kurdica* individually and in a combination could reduce inflammation and lesions and protected against acetic acid-induced colitis in rats owing to their anti-inflammatory and antioxidant effects. Honey and PA gum exerted their beneficial effects via down regulation of various inflammatory pathways, reduction of neutrophil infiltration, and suppression of the expression of a gene involved in IBD progression. We also proved that synergistic effect of honey and gum was more effective than single therapy in reducing

inflammatory biomarkers. Future studies should include clinical evaluation of honey-gum. Such studies should also investigate the role of other factors such as material cost and availability, and medicinal viability of honey-gum.

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None

Author contributions

Pardis Gharazi and Zahra Rezaei committed to the practical parts and drafted the manuscript; Amir Hossein Abdolghaffari, Saeideh Momtaz Mohammad Hossein Farzaei and Mohammad Abdollahi designed the study and all works have been done under their supervision; Kimia Zare, Maryam Baeeri, Saeideh Momtaz and Mahban Rahimifard contributed in biochemical assays and data analysis and participated in manuscript drafting; Saeideh Momtaz and Amir Hossein Abdolghaffari revised the manuscript.

Declaration of interest

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

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Abbreviations

TNF- α : tumor necrosis factor-alpha; MPO: myeloperoxidase; IL: interleukin; TLR: toll-like receptor; LPS: lipopolysaccharides; HTAB: hexadecyltrimethylammonium bromide; RT-PCR: real time-reverse transcription polymerase chain reaction; MAPK: mitogen-activated protein kinase; JNKs: c-Jun N-terminal kinases; STAT: signal transducer and activator of transcription; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B; IFN- γ : interferon gamma; (PI3K)/Akt: phosphatidylinositol-3-kinase; mTOR: mammalian target of rapamycin; iNOS: nitric oxide synthase; COX-2: cyclooxygenase-2; TBARS: Thiobarbituric acid reactive substances; SOD: superoxide dismutase; CAT: catalase; TNBS: 2,4,6-trinitrobenzene; PA gum: *Pistacia atlantica* oleo-gum-resin; IBD: inflammatory bowel disease; UC: ulcerative colitis; LPO: lipid peroxidase; INF- γ : interferon- γ