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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ORIGANUM ONITES L. ETHANOLIC EXTRACT ALLEVIATES ACETIC ACID-INDUCED ULCERATIVE COLITIS IN RATS BY REGULATING OXIDANT, INFLAMMATORY, AND APOPTOTIC MARKERS

ORİGANUM ONİTES L. ETANOLİK EKSTRAKTI OKSİDAN, İNFLAMATUAR VE APOPTOTİK BELİRTEÇLERİ DÜZENLEYEREK SIÇANLARDA ASETİK ASİT İLE İNDÜKLENEN ÜLSERATİF KOLİTİ HAFİFLETİR

Dilek OZBEYLI[1]*, Ali SEN[2], Leyla BITIS[2], Naziye OZKAN YENAL[1], Ayse Nur HAZAR YAVUZ[3], Deniz Mukaddes TURET[4], Meral YUKSEL[1], Ahmet DOGAN[5], Aslı AYKAC[6]

[1]Marmara University, Vocational School of Health Services, Department of Medical Services and Techniques, 34865, Istanbul, Turkiye
[2]Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, 34854, Istanbul, Turkiye
[3]Marmara University, Faculty of Pharmacy, Department of Pharmacology, 34854, Istanbul, Turkiye
[4]İstanbul University, Aziz Sancar Institute of Experimental Medicine, Department of Laboratory Animals Science, 34104, Istanbul, Turkiye
[5]Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 34854, Istanbul, Turkiye
[6]Near East University, Department of Biophysics, Mersin 10, Turkiye

ABSTRACT

Objective: *This study aims to evaluate the antioxidant and anti-inflammatory effects of Origanum onites ethanolic extract (O. onites EE) in vitro and its effects on inflammation, apoptosis, oxidative stress, and tissue damage-related parameters in the acetic acid (AA)-induced ulcerative colitis (UC) model in rats.*
Material and Method: *Dried parts of O. onites were extracted with ethanol using the maceration method. Then, the extract's anti-DPPH and anti-ABTS free radical scavenging, anti-5-lipoxygenase (LOX) activities, and total secondary metabolic quantities were evaluated in vitro. The rat groups were determined as control + saline (C), AA + saline (A), AA + O. onites EE (O), AA + Sulfasalazine (S). The treatments were applied once a day for 3 consecutive days.*
Result and Discussion: *O. onites EE showed high amount of total phenolic, total flavonoid, and total triterpene contents, high antioxidant activity against ABTS, DPPH radicals, and anti-inflammatory effects against 5-LOX enzyme. Due to AA-induction, increased luminol-lucigenin, TNF-α, IL-1β, IL-17, TLR-9, caspase-3, caspase-9, MMP-3, macroscopic and microscopic scores attenuated by O. onites EE and sulfasalazine treatments. In conclusion, O. onites EE has a significant therapeutic effect on rats' colitis due to its total flavonoid, phenolic, and triterpene content, and its Anti-5-LOX and free radical scavenging activity.*
Keywords: *Anti-5-LOX, apoptosis, IL-17, IL-10, Origanum onites, TLR-9, ulcerative colitis*

* **Corresponding Author / Sorumlu Yazar:** Dilek Ozbeyli
e-mail / e-posta: dilekozbeyli@marmara.edu.tr, **Phone / Tel.:** +905326619524

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ÖZ

Amaç: *Bu çalışmada Origanum onites etanolik özütünün (O. onites EE) antioksidan ve anti-inflamatuar etkileri in vitro olarak ve inflamasyon, apoptoz, oksidatif stres ve doku hasarıyla ilişkili parametreler üzerindeki etkileri asetik asit (AA) ile indüklenen sıçan ülseratif kolit (UC) modelinde değerlendirilmiştir.*

Gereç ve Yöntem: *O. onites'in kurutulmuş kısımları maserasyon yöntemi kullanılarak etanol ile ekstre edildi. Daha sonra, ekstrenin anti-DPPH ve anti-ABTS serbest radikal temizleme, anti-5-lipoksijenaz aktiviteleri ve toplam sekonder metabolik miktarları ölçüldü. Sıçan grupları kontrol + salin (C), UC + salin (A), UC + O. onites EE (O), UC + Sülfasalazin (S) olarak belirlendi. Tüm tedaviler 3 ardışık gün boyunca günde bir kez uygulandı.*

Sonuç ve Tartışma: *O. onites EE, toplam fenolik, flavonoid ve triterpen içeriklerine iyi miktarda, sahipti ve ABTS, DPPH radikallerine karşı yüksek bir antioksidan aktivite ve 5-LOX enzimine karşı yüksek bir anti-inflamatuar aktivite gösterdi. AA ile uygulanması ile artan luminol-lusigenin, TNF-α, IL-1, IL-17, TLR-9, kaspaz-3, kaspaz-9, MMP-3, makroskobik ve mikroskobik skorlar, O. onites EE ve sülfasalazin tedavileriyle azaldı. Sonuç olarak, O. onites EE'nin sıçanlarda kolit üzerinde iyi bir terapötik etkisi vardır; bu da esas olarak total flavonoid, fenolik ve triterpen içeriği ve Anti-5-LOX ve serbest radikal temizleyici aktivitesinden kaynaklanmaktadır.*

Anahtar Kelimeler: *Anti-5-LOX, apoptoz, IL-17, IL-10, Origanum onites, TRL-9, ülseratif kolit*

INTRODUCTION

Ulcerative colitis (UC) is classified as a treatment-resistant disease by World Health Organization [1]. Since UC is characterized by inflammation and ulcers in the mucosal membrane of the colon, it causes symptoms such as weight loss, diarrhea, and even bloody stools in patients. UC patients (including those whose symptoms are under control) are under significant psychosocial pressure due to the disease-related impact on their quality of life [2]. The incidence of UC, which can develop in individuals from all walks of life, regardless of gender and age (dominant age range of 10-30), due to genetic, environmental, and immunological factors, is increasing worldwide [3,4].

Oxidative stress is a condition that arises when there is an imbalance between the production of free radicals and the biological system's ability to detoxify these reactive substances or repair the resulting damage [5]. Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive molecules that possess one or more unpaired electrons, making them unstable and capable of initiating chain reactions. Free radicals are naturally produced during cellular metabolic processes. In addition, external factors, such as toxins and certain chemicals, can also amplify free radical production leading to damage to cellular molecules and playing an essential role in developing inflammatory diseases [6]. Oxidative stress stimulates various transcription factors, resulting in the altered expression of genes that upregulate inflammatory pathways. The inflammation initiated by oxidative stress plays a pivotal role in the development of numerous chronic diseases including ulcerative colitis [7,8]. Inflammation is a physiological response that serves as the organism's defensive system against injury. Inflammation happens after cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are released by activated inflammatory cells. The etiology of UC is multifaceted, with mucosal inflammation resulting from a complex interaction of several variables. Colonocytes, mucosal, and epithelial barriers are somehow disrupted, which is important in UC pathogenesis [9]. With all of this, the severity of UC can be directly linked to oxidative stress-induced tissue damage. Reactive oxygen species (ROS) are essential signaling molecules that significantly influence inflammation, highlighting their critical role in our body's response to injury and disease [10,11]. Controlling inflammation is crucial to therapy success in many chronic diseases. IL-10 deficiency or aberrant expression may boost the inflammatory response but also contribute to the development of certain autoimmune disorders [12]. IL-17 triggers a potent inflammatory response, activating granulopoiesis factors, neutrophil-specific chemokines, IL-6, pro-inflammatory cytokines (TNF-α and IL-1β), and matrix metalloproteinases (MMP) [13]. Toll-like receptor (TLR)-9 has been known to play a part in combating pathogens and inflammatory diseases [14]. IL-17 enhances recruitment of neutrophils and also epithelial barrier disruption and causes worsening mucosal inflammation in UC [15]. IL-1β also promotes neutrophil recruitment, enhances the production of other

inflammatory mediators, and disrupts epithelial integrity. Its overexpression in UC is linked to the persistence of mucosal inflammation and tissue damage [16]. MMPs are enzymes involved in extracellular matrix remodeling. In UC, overexpression of MMPs, contributes to tissue destruction by degrading the extracellular matrix and basement membrane [17]. Dysregulated TLR-9 signaling in UC contributes to excessive inflammation by activating downstream pathways caused by pro-inflammatory cytokines. Overactivation of TLR-9 in UC disrupts epithelial barrier integrity, increasing intestinal permeability. This causes luminal antigens and microbial substances to cross the mucosa, spreading chronic inflammation [18].

The fact that the medications employed in the treatment of UC do not give adequate success (treatment success rate is 33%) increases disease resistance and complicates treatment [19]. Natural medicine has existed almost since humankind's inception, and its impact on the advancement of clinical medicine is indisputable. The valuable ingredients found in medicinal plants have guided countless studies in quest of the best and most successful therapies, laying the groundwork for today's pharmaceutical industry [20]. Due to the limitations of current treatment, the search for a therapeutic regimen that can benefit from UC regulatory and preventive medications is ongoing. In this context, plants with medicinal uses known or tested to have anti-inflammatory, antioxidant, antiapoptotic, and neuroprotective properties have become the pivot. Many medicinal plant extracts are helpful in UC models [21,22]. When therapeutic plants with antioxidant, anticancer, antimicrobial, antiapoptotic, and anti-inflammatory activities are evaluated in terms of content, it is noted that the extracts of these plants contain high contents of flavonoids, phenolics, and polyphenols [23].

The genus Origanum (Lamiaceae) has around 43 species in the eastern Mediterranean region. The leaves of the *Origanum onites* (O. *onites*) plant is widely used in traditional medicine as they have a wide range of pharmacological properties [24-26]. *O. onites* contains fatty acids, hydroquinones, flavonoids, phenolic acids, sterols, pigments, terpenoids, and tocopherols. In compliance with the pharmacological studies, *O. onites* has shown the following activities: antimicrobial, antioxidant, anticancer, antidiabetic, antiviral, anti-inflammatory, hepatoprotective, and analgesic, effects, as well as effects on colitis and gastrointestinal tract [24]. *O. onites* L. essential oil, applied in various doses in experimental colitis, has been shown to have a considerable anti-inflammatory impact, especially when compared to traditional treatment approaches, particularly 5-aminosalicylic acid (5-ASA); it was shown to be more prominent than either. As a result of that study, the protective effect of oil applied rectally or intraperitoneally against colitis was shown [27]. Another study found that *O. onites* L. essential oil has antioxidant, anti-inflammatory, and antiapoptotic features [28].

The diagnosis rate in UC is low, and one of the main reasons is the scarcity of biomarkers for UC detection. In the long-term process of UC, before the ulcer or tissue deterioration becomes evident, different biochemical pathways, such as an elevation in epithelial permeability, activation of mucosal invasion of inflammatory cells, and mucosal cell apoptosis can be observed [29]. The underlying mechanisms of mucosal epithelial cell damage include TNF-α, which induces intestinal cell apoptosis [30], reactive oxidants such as superoxide and nitric oxide [31], and increased matrix metalloproteinases [32]. Studies evaluating the potential of biomarkers to increase mucosal healing and remission rates in patients and thus facilitate early intervention are of great importance. Depending on the data, biomarkers that indicate effective immune cell infiltration, and anti-inflammatory, and anti-apoptotic actions in UC should be further explored [33].

In our study, firstly, we targeted to investigate the in vitro anti-2,2-Diphenyl-1-picrylhydrazyl radical scavenging (DPPH) and anti-2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging activities and anti-lipoxygenase type V (LOX-5) activity of ethanolic extract (EE) of O. onites in addition to the amounts of total phenolic (TPC), total flavonoid (TFC) and total triterpene content (TTC). Secondly, we aimed to determine the effects of *O. onites* EE in terms of inflammation markers (TNF-α, IL-1β, IL-10, IL-17, TLR-9), apoptotic caspases (casp) (casp-3 and casp-9), oxidative stress markers (Superoxide dismutase (SOD), luminol, and lucigenin chemiluminescence (CL)) and tissue damage parameters (MMP-3, and histological evaluation with H&E) in AA-induced UC model in rats.

MATERIAL AND METHOD

Chemicals and Drugs

Ketamine and sulfasalazine were purchased from Pfizer, Türkiye (Ketalar and Salazopyrin, respectively). AA (100%), folin-ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin–Ciocalteu's phenol reagent, luminol, lucigenin and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (≥ 99.8%) and neutral buffered formalin were purchased from Merck (Darmstadt, Germany). All ELISA kits [(TNF-α, Cat. No. E0764Ra), (IL-1β, Cat. No. E0119Ra), (IL-10, Cat. No. E0108Ra), (IL-17, Cat. No. E0115Ra), (TLR-9, Cat. No. E0082Ra), (SOD, Cat. No. E1376Ra), (casp-3, Cat. No. E1648Ra), (casp-9, Cat. No. E1898Ra), and (MMP-3, Cat. No. E031Ra)] used in the experiments were obtained from Bioassay Technology Laboratory (BT LAB, China).

Plant Collection and Extraction

The aerial parts of *O. onites* were collected from Akpınar village of Bayramiç district of Çanakkale province (Türkiye) in July 2023 by Dr. Leyla Bitiş and identified by Dr. Ahmet Doğan. The plant sample was numbered (23479), recorded, and stored in the herbarium (Marmara University (MU), Faculty of Pharmacy, MARE). Dried and ground aerial parts of *O. onites* L. (21.83 g) were extracted with 90% ethanol (3 x 100 ml) using the maceration method. The solvent of the extract was evaporated to dryness at a temperature not exceeding 45 °C using a rotary evaporator to obtain EE with 11.3% yield (g/g) [34].

Assessment of the Antioxidant Activity of EE of *O. onites*

DPPH and ABTS, which demonstrate the radical scavenging activity of *O. onites* EE, were determined briefly as follows, as implemented by Zou et al. (2011): 190 µl of ABTS or DPPH radical solution was added to each well containing 10 µl of the extract (Stock solutions in the range 5000-9.77 µg/ml) in DMSO and mixed [35]. After incubation in the dark for half an hour, the absorbance by a spectrophotometer (Agilent, BioTek, Epoch) of all samples (triplicated for each sample), including Trolox or Ascorbic acid as a reference standard, was measured at 517 or 734 nm, respectively.

Assessment of the Anti-Inflammatory Activity of EE of *O. onites*

O. onites EE (10 µl), purified water (20 µl), ethanol (20 µl), 0.1 M sodium borate buffer (25 µl), and finally 25 µl type V soybean lipoxygenase enzyme solution (20,000 U/ml) dissolved in sodium borate buffer were added to each well. After incubating the cocktail for 5 min., linoleic acid solution (100 µl, 0.6 mM) was added and mixed. Using indomethacin as a reference standard, the absorbance values of the samples in the wells were measured at the wavelength of 234 nm. The IC_{50} (µg/ml) was calculated as the amount of *O. onites* EE and indomethacin needed to inhibit LOX activity by 50% [36,37].

Assessment of the Total Secondary Metabolic Amount of EE of *O. onites*

TPC of the *O. onites* EE was analyzed using the Folin-Ciocalteu colorimetric assay as described previously [38,39]. After mixing 20 µl of Folin-Ciocalteu reagent with 10 µl of *O. onites* extract (Stock solution at 5000 µg/ml concentration), 200 µl of H_2O, and 100 µl of 15% Na_2CO_3, the absorbance value was measured at 765 nm after leaving at room temperature for 2 h. The TPC value (mg GAE/g extract) was expressed using gallic acid (GA) (Stock solutions in the range 500 - 15.63 µg/ml) as the reference standard.

TFC analysis using the method of Zhang et al. (2013) and Yildirim et al. (2019) was performed briefly as follows: 125 µl of water (distilled) was added to the well containing 25 µl of *O. onites* EE, then 7.5 µl of sodium nitrite (5% w/v) was added and waited for 6 min, and finally 15 µl of $AlCl_3$ (10% w/v) was added [37,38]. After waiting for 5 min NaOH (50 µl) and distilled water (27.5 µl) were added to the mixture. The absorbance value was read with a spectrophotometer (510 nm). TFC was calculated as mg quercetin equivalent (mg QUE/g extract).

The TTC method of Chang et al. (2012) was adapted to the microplate [40]. After preparation of 10 µl of each of the stock solutions at a concentration of 5000 µg/ml these solutions were mixed with 15 µl of vanillin-glacial acetic acid solution (5% w/v) and 50 µl of perchloric acid solution. The plate was heated at 60°C for 45 min and then cooled to room temperature in an ice-water bath. After adding

225 µl of gallic acetic acid, the absorbance of the solutions was measured at 548 nm by a spectrophotometer. Absorbances corresponding to each concentration were measured using oleanolic acid (1200-37.5 µg/ml) for the standard curve plot. The TTC of the extract was calculated from this graph, and the results were expressed as mg oleanolic acid equivalent per g extract (mg OLE /g plant extract). The extract measurements were repeated three times, and the standard curve measurements were repeated five times.

Experimental Animals

All experimental protocols were carried out after obtaining ethical approval from Marmara University Animal Experiments Local Ethics Committee (50.2023mar. Date: 2023). Sprague-Dawley strain rats (3-4 months, male and female, 200-250 g, each group consisting of 6 rats) obtained from MU (Experimental Animal Application and Research Center) were housed under the following environmental conditions one week before the experiments: in a ventilated room with 12:12 h of day/night lighting, a temperature of 22 ± 2 °C, and a constant relative humidity of 60-65%. All rats were fed *ad libitum*.

Experimental Design

In the current study, in vitro, anti-LOX activity, ABTS, and DPPH free radical scavenging activity, and the total secondary metabolic amount of the EE of *O. onites* measurements were first executed. *In vivo* experiments were conducted, and after euthanasia, *in vitro* experiments were carried out (Figure 1).

In this study, 24 rats were divided into four experimental groups by randomly allocating six rats to each group (no casualties occurred). The four experimental groups and the treatments that the rats received based on the experimental group are as follows:

i) Control group (C), and ii) AA-induced colitis model group (A): The rats under mild ether anesthesia were through 8 cm proximal to the anus, 1 ml of saline (0.9% NaCl) or 1 ml of AA (5%; v/v in distilled water) (AA) intrarectally (i.r.) using a soft polyethylene catheter for 30 s. One ml/day saline was given orally to the C and A group rats once daily for 3 days [41,42].

iii-iv) Treatment groups: The rats with AA-induced colitis were given *O. onites EE* (O) (250 mg/kg/orogastric/day) [43] or sulfasalazine (S) (100 mg/kg/orogastric/day) [44] once daily for three days following UC. All rats in the control and model groups were fasted for 12 hours before creating an UC model.

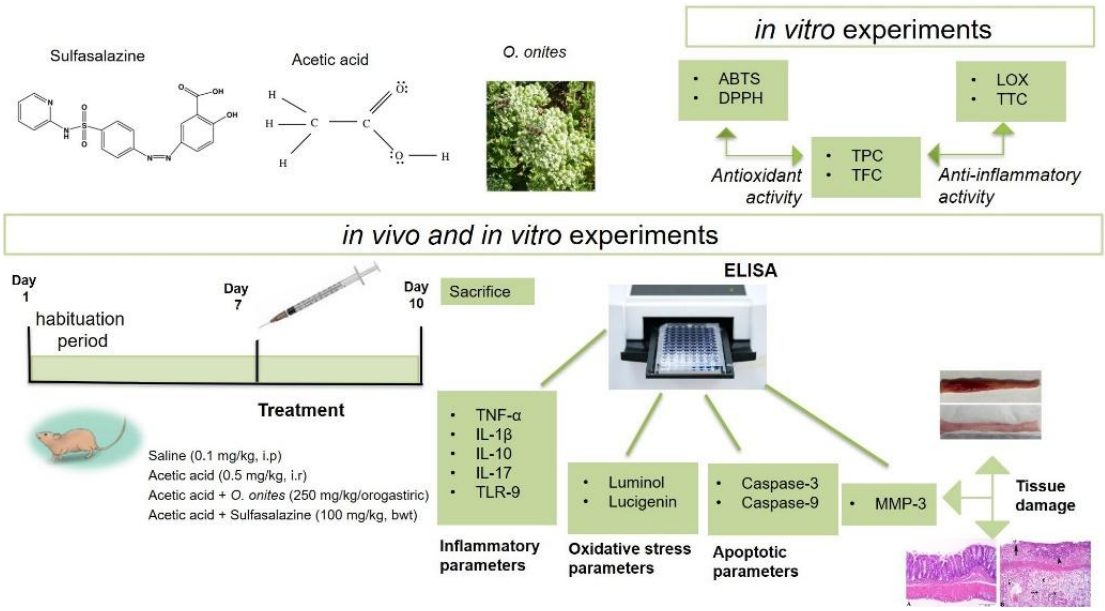

Figure 1. Experimental design

Appraisal of Colitis Degree: Macroscopic Measurement

At the end of 72 h, all rats were euthanized under ketamine and xylazine anesthesia (100 mg/kg and 10 mg/kg, respectively) and 8 cm of distal colon tissue was resected. After the luminal contents of the resected tissues were removed, they were washed with saline and allowed to dry with a blotting paper for a few sec. Macroscopic evaluation of colon lesions was carried out as instructed by Iseri et al. (2009) [45] (Table 1).

Table 1. Criteria for the macroscopic grading of lesions in the colon

Grade	Appearance
0	No injury
1	Local hyperemia
2	Hyperemia or gut wall thickening
3	Ulceration or inflammation in one area
4	Two or more areas of ulceration or inflammation
5	Major area of injury extending more than 1 cm along the length of the colon
6-10	If the damage extends more than 2 cm, increase by one for each + 1 cm

Evaluation of Colitis Severity

The Release of Cytokine and Reactive Oxygen Species of the Colon Tissues

Changes in inflammatory cytokine levels (TNF-α, IL-1β, IL-10, IL-17, and TLR-9), mitochondria-mediated apoptosis markers (casp-3 and casp-9), and tissue damage indicators (MMP-3) in colon tissue were assessed using ELISA tests carried out using a Bio Tek Epoch Microplate spectrophotometer (450 nm) (Agilent Technologies) following the manufacturer's guidelines.

The release of reactive oxygen species in the colon was determined using luminol and lucigenin probes CL levels were taken at 1-minute intervals for 5 min. The data is reported as relative light units per mg of tissue (rlu/mg) [46].

Histological Evaluation of the Colon Tissues

The rat colon tissues were fixed in formalin (10% neutral buffer), dehydrated in a graded alcohol series, and embedded in paraffin. To evaluate the overall histologic structure regarding damage/necrosis, submucosal edema, inflammatory cell infiltration, vasculitis, and perforation, 4 μm sections were cut from the tissues and stained with hematoxylin and eosin. The sections were examined under a BX51 photomicroscope and graded on a scale of 0 to 3 (n= 6) [47] (Table 2).

Table 2. Histopathologic assessment of the colonic injury was performed using the previously described criteria [39]

	None	Localised	Moderate	Severe
Damage/necrosis	0	1	2	3
Submucosal edema	0	1	2	3
Inflammatory cell infiltration	0	1	2	3
Vasculitis	0	1	2	3
	Absent		**Present**	
Perforation	0		1	
With a maximum score of 13				

Statistical Analysis

Statistical analysis of biological activity and total secondary metabolite content of *O. onites* EE was carried out using with Student's t-test. Each value was represented as mean ± SD. A p-value of less than 0.05 indicates a significant difference (GraphPad Prism 6.0, San Diego, CA, USA).

After verifying the normal distribution of other data of analyses by the Kolmogorov-Smirnov test, for group comparisons, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's testing for multiple comparisons. The data were provided as mean ± SEM. (GraphPad Prism 6.0, San Diego, CA, USA). A p-value of less than 0.05 indicates a significant difference.

RESULT AND DISCUSSION

Results of the Antioxidant and Anti-inflammatory Activity of *O. onites* EE

The IC_{50} value determined by DPPH radical was 82.51 ± 0.08 for *O. onites* EE and 40.23 ± 2.08 µg/ml for ascorbic acid, which was used as a reference. According to the ABTS test result, the IC_{50} value for Trolox was used as a reference standard, and *O. onites* EE was determined as 4.54 ± 0.08 and 88.38 ± 0.06, respectively. The extract showed low antioxidant activity compared to standards ($p<0.05$, Table 3). However, in an article by Indarti et al., antioxidant activity was classified as very strong when IC_{50} was <50 µg/ml, strong when IC_{50} values were 50-100 µg/ml, moderate when IC_{50} was 101-250 µg/ml, weak when IC_{50} was 250-500 µg/ml, and inactive when IC_{50} was >500 µg/ml [48]. According to this classification, the extract exhibited strong antioxidant activity against both DPPH and ABTS radicals.

TPC was determined using the equation [y = 0.093x + 0.062 (R2: 0.9987)] obtained from the calibration curve as GAE (mg GAE/g extract). The TPC of *O. onites* EE was determined at 125.20 ± 1.50 mg GAE per g of dried extract. The TFC of *O. onites* was determined using the equation [y= 0.003x + 0.015 (R2: 0.9644)] obtained from the calibration curve as QUE (mg/g extract). The TFC of *O. onites* EE was determined at 362.10 ± 0.92 mg QUE per g of dried extract.

The TTC of *O. onites* EE was determined using the equation [y = 0.027x + 0.016 (R^2: 0.9981)] obtained from the calibration curve as OLE (mg/g extract). The total triterpene of *O. onites* EE was determined at 194.50 ± 1.35 mg OLE per g of dried extract.

O. onites EE showed substantial anti-inflammatory action against a LOX enzyme, with an IC_{50} value of 19.08 ± 0.11 µg/ml (21.42 ± 0.48 µg/ml for standard indomethacin) ($p<0.05$, Table 3).

Table 3. Biological activity and total secondary metabolite content of *O. onites* EE

Tests		*O. onites* EE	Ascorbic acid	Trolox	Indomethacin
Antioxidant activity	DPPH radical scavenging activity ($IC_{50,}$ µg ml^{-1})	82.51±0.08[b]	40.23±2.08[a]		
	ABTS radical scavenging activity ($IC_{50,}$ µg ml^{-1})	88.38±0.06[b]		4.54±0.08[a]	
Anti-inflammatory activity	Anti-lipoxygenase activity ($IC_{50,}$ µg ml^{-1})	19.08±0.11[a]			21.42±0.48[b]
Total secondary metabolite contents	Total Phenolic (mg GAE/g extract)	125.20±1.50			
	Total Flavonoid (mg QE/g extract)	362.10±0.92			
	Total Triterpene (mg OAE/g extract)	194.50±1.35			

* OOE: Aqueous ethanol extract of *Origanum onites* aerial parts; GAE: Gallic acid equivalent; QE: Quercetin equivalent; OAE: Oleanolic acid equivalent.

** Each value in the table is represented as mean ± SD (*n*=3). The values with different letter superscripts in the same row indicate statistically significant differences between the means ($p<0.05$).

Changes in Inflammatory, Oxidative Stress, Apoptotic, and Damage Parameters

The current study determined that the AA-induced colitis group (A) dramatically elevated levels of pro-inflammatory cytokines such as TNF- α ($p < 0.001$) and IL-1β ($p < 0.001$) compared to the C group, which explains the increase in inflammatory response (Figures 2A, B). It was determined that TNF-α and IL-1β levels of the O and S groups after AA induction were significantly decreased compared to the A group ($p < 0.01$-0.001). IL-10 level was significantly reduced in the A group compared to the C group (Figure 3C). IL-10 level was significantly increased in the O group ($p < 0.05$) and S groups ($p < 0.05$) compared to the A group. IL-17 ($p < 0.001$) and TLR-9 ($p < 0.01$) levels were significantly increased in the A group compared to the C group (Figures 2D and E). IL-17 and TLR-9 levels were reduced considerably in the O group ($p < 0.01$-0.001) and S groups ($p < 0.001$) compared to the A group.

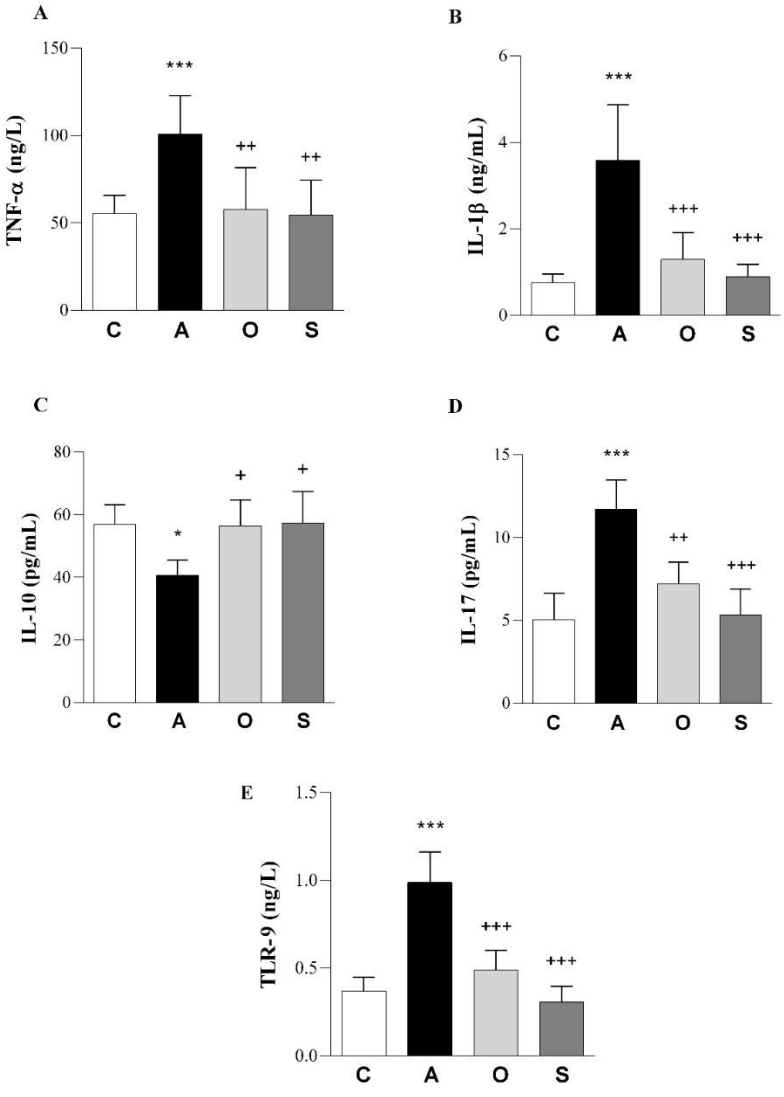

Figure 2. Evaluation of the levels of TNF-α (A), IL-1β (B), IL-10 (C), IL-17 (D), and TLR-9 (E) in colon tissue after *O. onites* EE or sulfasalazine treatment in the acetic acid-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

The activity of SOD, an antioxidative enzyme, was not found to be considerably different between the groups (Figure 3A). In the current study, it was discovered that the rats in the A group had higher levels of lucigenin and luminol than the values in the C group ($p < 0.001$, for both markers). The A group's increased lucigenin level dropped dramatically in both treatment groups and eventually recovered to control levels ($p < 0.001$, for both treatment groups) (Figure 3B). When the amount of luminol-enhanced CL in each treatment group was compared to that in the A group, it was found that the amount had decreased in both treatment groups ($p < 0.01$ for the O group and $p < 0.05$ for the S group) (Figure 3C).

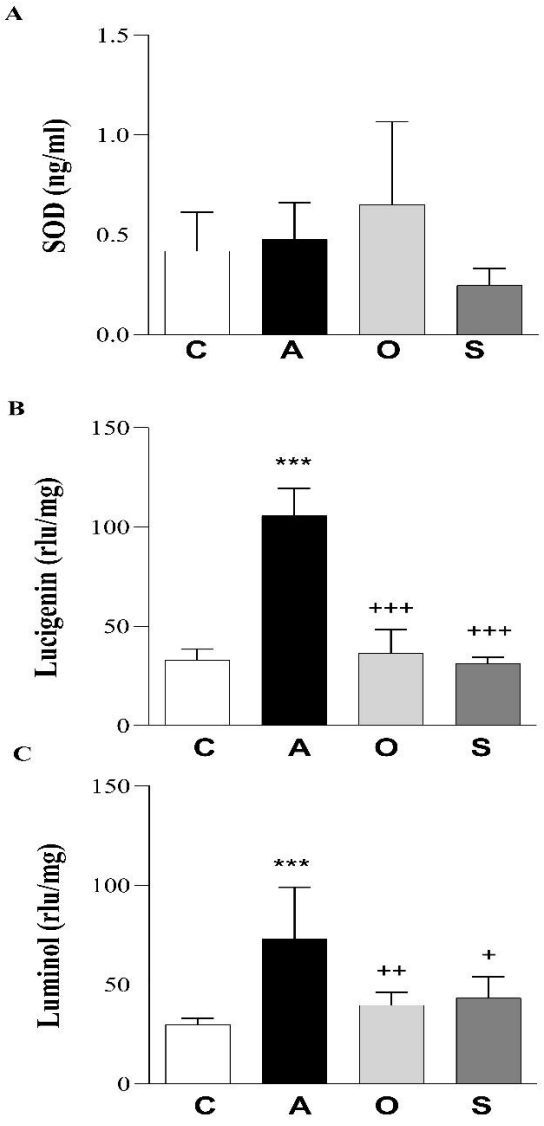

Figure 3. Evaluation of the levels of SOD (A), lucigenin (B), and luminol (C) in colon tissue after *O. onites* EE or sulfasalazine treatment in the AA-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

The activity of apoptotic markers, casp-3 and casp-9, were increased significantly in the A-group compared with the C group ($p < 0.001$, for both markers). *O. onites* EE and sulfasalazine treatment ameliorated the activity values of casp-3 ($p < 0.05$ for O group, $p < 0.01$ for S group) and casp-9 ($p < 0.01$ and $p < 0.001$, respectively) almost to the C group level (Figure 4A, B).

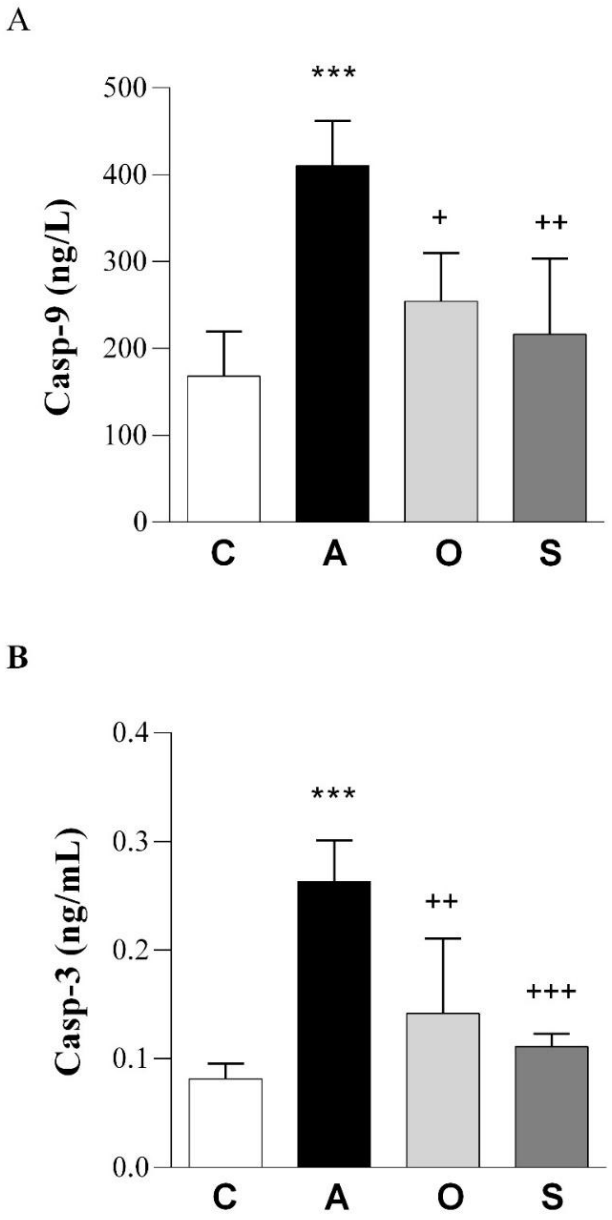

Figure 4. Evaluation of the levels of caspase-9 (A), and caspase-3 (B) in colon tissue after *O. onites* EE or sulfasalazine treatment in acetic acid-induced ulcerative colitis rat model, using ELISA method. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). The results were presented as mean ± SEM. ***$p < 0.001$ control vs. AA; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

Sample colon sections belonging to the experimental groups were photographed (Figure 5).

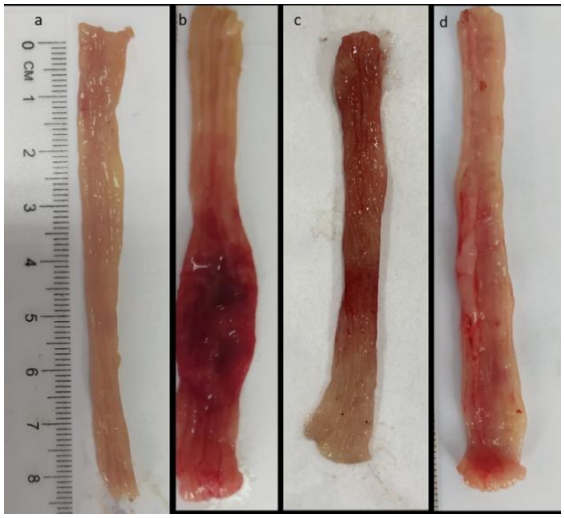

Figure 5. The effect of *O. onites* EE on macroscopic images of rat colons with AA-induced ulcerative colitis. (a) Saline-treated control group (C), (b) saline-treated AA-induction group (A), (c) AA + *O. onites* treated group (O), (d) AA + sulfasalazine treated (S) group

The A group had higher MMP-3 activity (an indicator of tissue damage) than the C group ($p < 0.01$). Both *O. onites* EE and sulfasalazine treatment groups showed decreased levels compared to the A group ($p < 0.05$, Figure 6A).

The total macroscopic damage score data obtained by evaluating the pathologic changes in the colon tissues of the experimental groups are given in Figure 6B. It was determined that macroscopic damage in the A group treated with AA was significantly higher than in the control group ($p < 0.001$). Compared to the A group, a decrease in the macroscopic damage was observed in the groups treated with *O. onites* EE or sulfasalazine ($p < 0.001$, in both treatments). In addition, while there was a significant decrease in macroscopic damage in the O and S groups compared to the A group, it was observed that this decrease in damage was almost at the level of the C group.

The total microscopic damage data obtained by evaluating the histopathologic changes in the colon tissues of the experimental groups are given in Figure 6C. It was determined that microscopic damage in the A group treated with AA was significantly higher than in the control group ($p < 0.01$). Compared to the A group, a decrease in the microscopic damage was observed in the groups treated with *O. onites* or sulfasalazine (O and S group, $p < 0.05$ and $p < 0.01$, respectively).

Figure **7**A-D illustrates how the EE of *O. onites* affected histopathological changes in the colon tissue of the experimental groups in terms of the following parameters: submucosal edema, inflammatory infiltration, and vasculitis. As a result of the microscopic examination of the colon tissue of the control (C) group, the images obtained were found to have a regular, that is, normal colonic mucosa, submucosa, and muscularis muscularis histologic structure (Figure 7A). Microscopic analysis of the AA-induced colitis group revealed markedly reduced and distorted colonic crypts, extensive inflammatory cell infiltration, vasculitis, and submucosal edema (Figure 7B). In the O group, mucosal, glandular degeneration, submucosal edema, and mild inflammatory cell infiltration were lower than in the A group (Figure 7C). In the S group, epithelial surfaces appeared more regular, and glandular structures were preserved (Figure 7D).

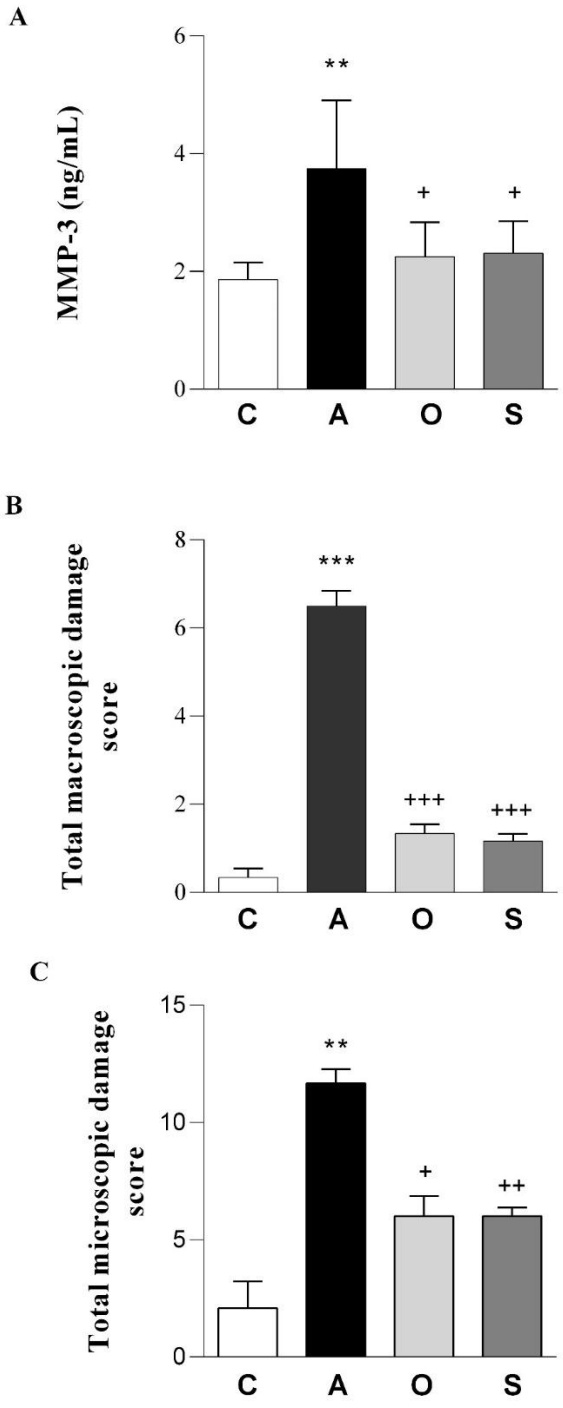

Figure 6. Evaluation of (A) MMP-3 level by ELISA method and evaluation of colon tissue macroscopically (B) and microscopically (C) after *O. onites* EE or sulfasalazine treatment in AA-induced ulcerative colitis rat model. Saline-treated control group (C), saline-treated AA-induction group (A), AA + *O. onites* treated group (O), AA + sulfasalazine treated (S) group (n=6). Results were presented as mean ± SEM. ** $p < 0.01$, ***$p < 0.001$ control vs. acetic acid; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ AA vs. treatment group

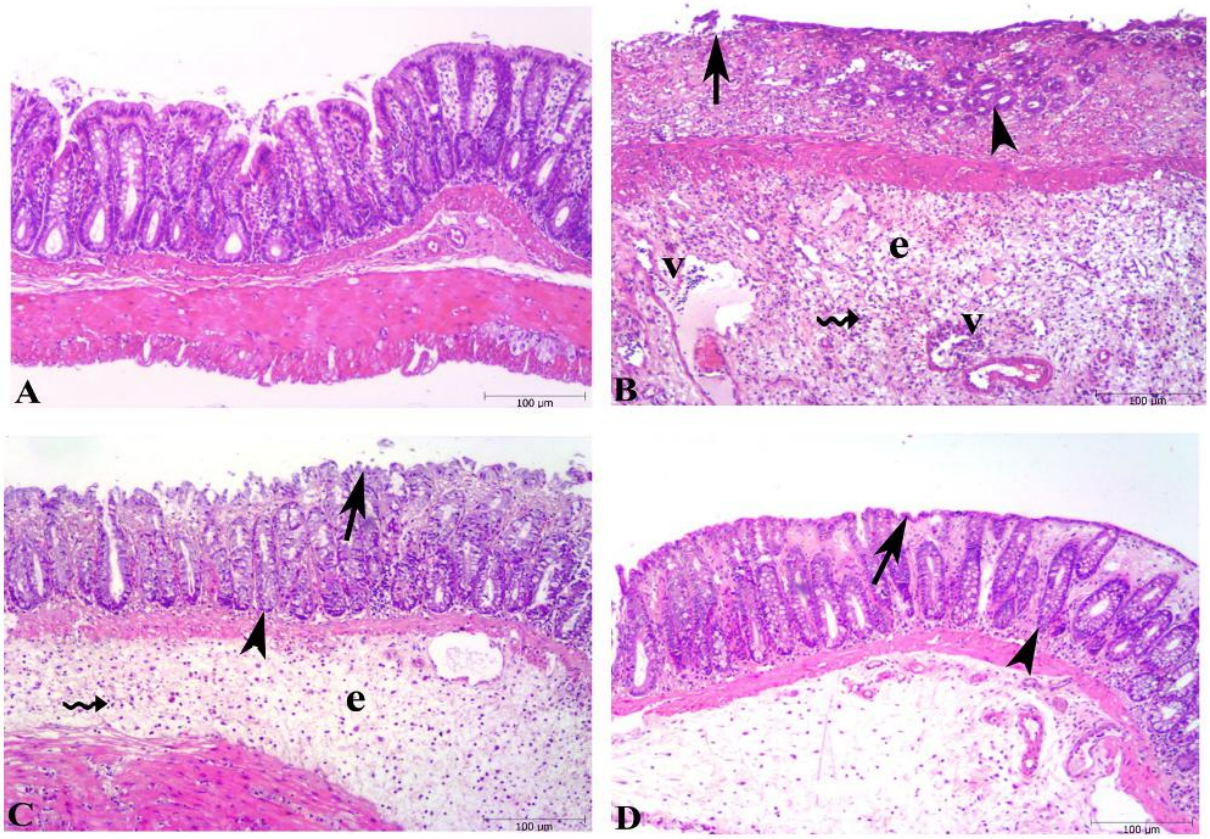

Figure 7. Histopathologically evaluated the effect of *O. onites* EE on the colon tissue of rats with acetic acid-induced UC. Representative photomicrographs shown were colons of (A) control (C group) rats [exhibited regular intestinal mucosa, submucosa, and muscularis], (B) AA (A group) rats [showed significant loss of colonic crypts (arrow) and glands (arrowhead), along with submucosal edema (e), vasculitis (v), and substantial inflammatory cell infiltration (wavy arrow)], (C) *O. onites*-treated (O group) rats [had less mucosal and glandular degradation (arrowhead), edema (e), and inflammatory cell infiltration (wavy arrow)], and (D) sulfasalazine treated (S group) rats [exhibited more homogeneous epithelial surfaces (arrow) and intact glandular structures (arrowhead)]-scale bar: 100 µm

In this study, total phenol, flavonoid, and triterpene compounds were found in very high amounts in *O. onites* EE. The extract also showed a high anti-LOX activity. In addition, it was demonstrated that *O. onites* EE while decreased colonic TNF-α, IL-1β, IL-17, casp-3, casp-9, MMP-3, TLR-9, CL (luminol and lucigenin) levels, macroscopic and microscopic damage score, and elevated IL-10 levels but had no effect on SOD levels.

Rodent models are widely used in studies evaluating the physiopathology of UC and the anti-inflammatory, antiapoptotic, antioxidative, and antiulcerative activities of new compounds. The intrarectal AA induction model is one of the most effective at mimicking the histological and biochemical characteristics of the human UC. AA induction results in acute and non-transmural inflammation of the colon tissue with overproduction of inflammatory mediators, edema, vascular dilatation, and necrosis [49]. In the present study, the model successfully mimicked human UC, which aligns with previous reports.

Multifaceted pro-inflammatory cytokines are involved in the development of UC, the worsening of the inflammatory response, and the maintenance of the inflammatory response. Downregulation of inflammatory responses is a critical component of homeostasis and is required to recover and maintain tissue and organ function in the presence of inflammation. In mice with induced colitis, elevated IL-1β, a critical biomarker associated with pyroptosis in the colon, was associated with worsening of colitis

symptoms in UC and targeting IL-1β significantly attenuated dextran sulfate sodium (DSS)-induced colitis [50,51]. TNF-α is one of the central cytokines involved in the pathogenesis of UC and promotes the production of interleukins such as IL-1β and IL-6. It also increases in AA-induced UC models [42, 50,52]. Pro-inflammatory cytokine IL-17 has an active role in mediating immune responses and inflammation [53]. Studies have revealed the importance of the IL-23/IL-17 immune axis in the pathogenesis of UC and the importance of IL-17 in the progression of UC [54]. In a study report assessment of the influence of a plant extract on the UC model, it was reported that the extract alleviated colitis by reducing inflammatory cell infiltration, TNF-α and IL-1β concentrations, and IL-17 expressions [55]. Other research results also mention the curative effect of inhibiting IL-17 on the disease in the rat UC model [56,57]. IL-17 expression levels in the intestinal mucosa and serum increased in humans with inflammatory bowel disease (IBD) [58]. In addition, a previous study in active IBD patients reported that the disease activity index, histological, and endoscopic score correlated with increased IL-17A expression [59]. Our findings are similar to those of previous studies; the IL-17 level increased in colitis, and we suggest that O. onites treatment might have anti-inflammatory properties that can regulate immunological responses by reducing IL-17 in UC. Our in vitro results supported that *O. onites* EE was rich in triterpenoid compounds known for their anti-inflammatory activities. Previously reported that oleanolic acid, a terpenoid, ameliorates UC in mice by repairing the equilibrium of Th17/Treg cells, downregulating IL-17, TNF-α, IL-1β, and increasing IL-10 levels [60]. Our data support the previous data, suggesting that the IL-17, IL-1β, and TNF-α lowering effect of *O. onites* EE may be due to its triterpene content.

Elevated cytokines and the destroying the intestinal barrier through increased apoptosis of intestinal epithelial cells contribute to UC [61]. Previously, it has been shown that Casp-3 and Casp-9 increased in UC, and inhibition of apoptosis alleviated UC by protecting the physical and immune barrier [62,63]. A limited number of colitis and toxicity studies have reported the anti-apoptotic activity of *Origanum* species L. extract in the literature [64-66]. The observed healing effect of *O. onites* EE could be the direct interruption of mitochondrial apoptotic signaling pathways.

Oxidative stress elevates lipid peroxidation, resulting in structural cellular damage, apoptosis, and necrosis [67]. The resultant necrotic/apoptotic tissues/cells exacerbate the inflammatory response, triggering the vicious cycle of UC pathogenesis [68,69]. In addition, oxidative stress, which results from an unregulated imbalance between the creation and elimination of ROS, is thought to contribute to the advancement of colon inflammation in UC [70]. The increase in luminol and lucigenin levels, which are oxidative stress factors increased by AA induction, was effectively suppressed by *O. onites* treatment. The present study revealed that *O. onites* EE has good DPPH/ABTS radical scavenging activity, which may be a reason for the decrease in the amount of luminol and lucigenin-enhanced CL.

Previously, it has been reported that pattern-recognition receptor TLR-9 expression increased in the colon with UC rats [71]. Furthermore, a positive correlation was found between the brutality of inflammation and TLR-9 expression in UC patients [72]. Our results show that *O. onites* EE and sulfasalazine treatment inhibit the AA-induced TLR-9 expression. This can lead to alleviating colonic inflammation.

SOD, an essential antioxidant enzyme, protects cells against ulcerative damage by facilitating the dismutation of superoxide anion and reducing lipid peroxidation [73]. SOD also inhibits leukocyte adherence in colon tissues. In UC, SOD enzyme levels are decreased due to oxidative damage mediated by free radicals in colon tissues [74]. However, in this study, neither the reducing effect of UC on SOD nor the extract on SOD activity was detected. It is observed that neutrophil-mediated reactive oxygen species increase more in tissues with inflammatory damage, such as colitis. On the other hand, although it was not analyzed in this study, it can be said that the nitric oxide molecule increases due to the activation of inducible nitric oxide synthase through neutrophils. Increased nitric oxide interacts with the superoxide radical to form the peroxynitrite radical, which causes greater oxidative damage. While the SOD enzyme should catalyze the conversion of superoxide radical to hydrogen peroxide, inflammation-mediated free radicals cannot meet the substrate requirement for SOD activity. Although SOD activity was found to be decreased in the colitis group in our study, the most important reason why it was not found to be significant is oxidative stress mediated by inflammation damage [75].

In the current study, the content of *O. onites* EE was analyzed qualitatively and quantitatively using TPC, TFC, and TTC assays. The blue, red, and violet colors in the tests indicated the presence of phenolic, flavonoid, and triterpene compound groups, respectively (Data not shown) [76-78]. It was also found that these compounds were present in very high amounts in the extract. Previous studies have revealed that TPC (such as phenolic acids) and flavonoids have antioxidant [79,80] and anti-inflammatory [81] activity, while triterpenes have anti-inflammatory activity [82,83]. It has also been reported that phenolic compounds [84-87] and triterpenes [83] are effective against UC. Therefore, phenolic (e.g. phenolic acids), flavonoid and triterpene compounds may have been responsible for the healing effect of *O. onites* EE against UC since it contains a good amount of these compounds.

5-LOX is an enzyme for the production of leukotrienes, which play a role in many inflammatory conditions. Hence, 5-LOX inhibitors are often used to investigate UC, and a 5-LOX inhibitor exerted curative effects in UC [88]. Injury of colonic tissue was lowered in 5-LOX-deficient mice with colitis when compared with wild-type ones [89]. In a previous study, *O. onites* essential oil was shown to have anti-LOX activity [90]. Our study showed *that O onites EE* has also anti-LOX activity.

Our macroscopic and microscopic scores data demonstrated that AA treated rats had severe colon tissue destruction, whereas *O. onites* or Sulfasalazine treatments significantly ameliorated this condition. Furthermore, our findings were very similar to the biochemical results stated above. Previously, it was reported that MMP-3 increased in UC [91,92]. Our results were consistent with previous studies that reported that MMP-3 was a potential marker of UC in terms of morphology and histopathology [91, 92]. Moreover, *O. onites* EE or Sulfasalazine treatments diminished MMP-3. Previously, a terpenic phenolic compound, carvacrol, suppressed the protein expression levels of MMP-3 in human chondrocytes [93] so we think our finding is due to the terpenoid content of the extract.

In our study, the potential therapeutic effects of *O. onites* EE in treating UC were evaluated based on antioxidant, anti-inflammatory, oxidative stress, and apoptotic markers. Our findings suggest that *O. onites* EE treatment can significantly alleviate colonic damage and decrease inflammation. In conclusion, *O. Onites* EE may be effective for treating UC.

AUTHOR CONTRIBUTIONS

Concept: D.O., A.S.; Design: D.O., A.S.; Control: A.S., L.B., A.D.; Sources: A.S., L.B, N.O.Y., A.N.H.Y., D.M.T., M.Y.; Materials: A.S., L.B., N.O.Y., D.M.T., M.Y.; Data Collection and/or Processing: D.O., A.S., N.O.Y., A.N.H.Y., D.M.T., A.D.; Analysis and/or Interpretation: D.O., A.S., A.N.H.Y., N.O.Y., M.Y. A.D.; Literature Review: D.O., A.S., A.A.; Manuscript Writing: D.O., A.S., N.O.Y., A.A.; Critical Review: D.O., A.S., M.Y., A.A.; Other: -

CONFLICTS OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

All procedures for experimental protocols of the present study involving animals were performed following the ethical standards of the institution or practice at which the studies were conducted. Approval was granted by the Ethics Committee of University Marmara (Date: 2023, No:50.2023mar). All experimental practices concerning rats were executed following "The Guide for the Care and Use of Laboratory Animals" (www.nap.edu/ catalog/5140.html) and the principles of ARRIVE guidelines.

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