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RESEARCH ARTICLE

Sideritis perfoliata Ethanolic Extract Mitigates Acetic Acid-Induced Ulcerative Colitis in Rats

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ABSTRACT

Objective: This study evaluated the antioxidant and anti-inflammatory effects of *Sideritis perfoliata* (SP) ethanolic extract *in vitro* and its impact on anti-inflammatory, antiapoptotic, oxidative stress, and tissue damage-related parameters in an ulcerative colitis (UC) rat model.

Materials and Methods: Dried and ground aerial parts of SP were extracted with 90% ethanol using maceration. Then, the SP extract's radical scavenging, anti-5-lipoxygenase (LOX) activities, and total secondary metabolic quantities were evaluated *in vitro*. In *in vivo* experiments, following acetic acid (AA)-induced colitis, physiological saline (PS) (1 mL, og), sulfasalazine (SS) (100 mg/kg bwt, 1mL, og), and SP extract (200 mg/kg bwt, 1mL, og) treatments were administered to AA, SS, and SP groups, respectively, once daily for three consecutive days. PS was given to the control group. At the 72nd hour, the rats were euthanized. Analyses were performed on colon tissues using ELISA, Chemiluminescence assay, Haematoxylin and Eosin staining.

Results: SP extract exhibited good antioxidant activity against 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and strong anti-5-LOX activity. The total amounts of phenol, flavonoid, and triterpene compounds in the extract were determined. Compared with the AA group, SS or SP extract treatments reduced tumour necrosis factor-α, interleukin-1β, interleukin-17, tool-like receptor-9, matrix metalloproteinases-3, caspase-9, caspase-3, luminol, and lucigenin chemiluminescence levels, macroscopic and microscopic morphological scores in colon.

Conclusion: Due to its flavonoids, phenolic acids, triterpene content, anti-5-LOX, and free radical scavenging activities, SP extract attenuates proinflammatory cytokines, mitochondrial apoptotic caspases, and oxidative stress. Our findings support the ethnopharmacological use of SP for colitis.

Keywords: *Sideritis perfoliata*, Ulcerative colitis, Cytokines, Apoptosis, Oxidative stress.

INTRODUCTION

Among the most prevalent signs of inflammatory bowel disease (IBD), a recurrent, chronic illness mediated by the immune system, are abdominal pain and bloody diarrhoea. Ulcerative colitis (UC) is one of the two most prevalent types of IBD.[1,2] It is more common in Europe and North America than globally in Africa and South America.[3] In contrast, the prevalence of the disease has increased in low-incidence countries, whereas the pace of increase in incidence has declined in high-incidence countries, according to recent studies. These results underscore the potential for IBD to emerge as a worldwide health issue in the future and stress the need to promptly identify the underlying pathophysiology of the disease and determine suitable treatments.[2,3] In the hope of reducing both the physical and psychological effects of IBD, many people with IBD (≅ 40%) use complementary and alternative medicines/products, the vast majority of which are herbal, in addition to their treatment.[4-6] Preclinical research highlights the therapeutic benefits of plants, which have been utilised for

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millennia in conventional medicine.[7,8] According to published research, nutraceuticals high in polyphenols and antioxidants are beneficial because they can scavenge free radicals, trigger anti-inflammatory reactions, and control the gut microbiota's homeostasis.[9,10]

The most common sign of UC is inflammation of the colon's mucosal lining. The inflammatory pathway causes an increase in pro-inflammatory eukaryotic transcription factors such as activator protein-1 and nuclear factor-κβ, an increase in the production of pro-inflammatory cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and a decrease in anti-inflammatory cytokines such as IL-10. Increased levels of pro-inflammatory cytokines exacerbate inflammation and oxidative stress while decreasing antioxidant levels.[11-13] All these processes lead to increased inflammation, epithelial cell damage, neutrophil infiltration, and malfunction of the intestinal barrier.[14,15]

Among more than 140 species, *Sideritis perfoliata* (SP) is a wild plant primarily found in the Mediterranean region.[16,17] In Türkiye, the genus *Sideritis* comprises 45 species (53 taxa, more than 40 of which are endemic).[18] They are often referred to as "mountain tea" plants. Since the Dioscorides era, *Sideritis* species have been used in traditional medicine as a stimulant, carminative, appetite stimulant, and stomach stimulant.[19] *Sideritis* plants are used as tea, feed, and sweeteners. Due to its anti-inflammatory, antibacterial, anti-ulcerative, antirheumatic, wound-healing, antioxidant, antispasmodic, analgesic, stomachic, and carminative qualities, *Sideritis* species are widely utilised as traditional remedies. It is also traditionally used in Türkiye for stomach illnesses, colds, bronchitis, coughs, flu, sore throat, diabetes, and as a digestive aid.[20-24] Research reports on species of *Sideritis* have attributed these pharmacological properties to the genus due to the flavonoids, terpenoids, coumarins, sterols, and iridoids found in its chemical profile.[23,25] Previously, it procured diterpenoids, flavonoids, and phenylpropanoid glycosides from the aerial parts of SP[26,27] and SP exerted significant antioxidant and anti-inflammatory activity.[20] On the other hand, there is no information in the literature about the effects of SP on ulcerative colitis.

In light of the above information, this study investigated the antioxidant and anti-5-lipoxygenase (anti-5-LOX) effects of SP ethanolic extract *in vitro* and its impacts on anti-inflammatory, antiapoptotic, oxidative stress, and tissue damage-related parameters in acetic acid (AA)-induced UC in rats.

MATERIALS AND METHODS

Chemicals

AA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), indomethacin, potassium persulfate, ascorbic acid, linoleic acid, trolox, lipoxidase from Glycine max (soybean), 3-Aminophthalhydrazide (luminol), N, N'-Dimethyl-9,9'-biacridinium dinitrate (lucigenin), Folin-Ciocalteu reagent, formaldehyde, dithiothreitol (DTT), glycerol, Tris-HCl, Triton X-100, Ethylenediaminetetraacetic acid (EDTA), $NaNO_2$, $AlCl_3 \cdot 6H_2O$, NaOH, methanol, ethanol, and aether were supplied by Sigma (Sigma-Aldrich, St. Louis, MO, USA). Sodium pentobarbital was obtained from IE Ulagay (Istanbul, Turkiye). Sulfasalazine (SS-Salazopyrin) was purchased from Pfizer (Istanbul, Turkiye). All other chemicals were of the highest quality commercially available.

Sourcing and Harvesting of Plant Materials

In July 2023, Dr. Bitis purchased the aerial parts of SP from a local plant market in the Bayramiç district of Çanakkale province and identified by Dr. Dogan. Some plant specimens were deposited at Marmara University, Faculty of Pharmacy Herbarium (International Code: MARE) (under herbarium no. 23481). The dried and ground aerial parts of SP (20.05 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 yield an ethanolic extract with 12.67% yield (g/g).

Validation of SP Extract's Radical Scavenging and Anti-Inflammatory Qualities *In Vitro*

The radical scavenging effects (represented by anti-ABTS and anti-DPPH radicals) of the SP extract were described by the method previously insisted on by Zou et al. (2011) as follows: 10 µL of the extract (stock solutions in the range 5000-9.77 µg/mL) dissolved in dimethyl sulfoxide (DMSO) was added to the well and mixed with ABTS or DPPH radical solution until the total volume was 200 µL.[28] After incubation (for 30 min in the dark), the mixtures on the plates (three times for each sample) were measured using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) or ascorbic acid as reference standards using a spectrophotometer (517 or 734 nm).

SP extract, water (distilled), ethanol, sodium borate buffer (SBB, 0.1 M), and soybean 5-lipoxygenase (5-LOX) dissolved in SBB (10 µL, 10 µL, 20 µL, 20 µL, 25 µL, and 20,000 U/mL, respectively) were added to each well, incubated (for 5 min) and finally, linoleic acid solution (0.6 mM, 100 µL) was added to the mixture and stirred.[29] The absorbance of all samples was read at 234 nm to determine the amount of SP extract and indomethacin (reference standard) required to inhibit 5-LOX activity by 50% as IC_{50} (µg/mL).

Assessment of the Total Secondary Metabolic Quantities of SP Extracts

The total phenolic content (TPC) of SP extracts was determined using the Folin-Ciocalteu colorimetric test by comparing the TPC of gallic acid (GA) (stock solutions in the range 500 - 15.63 µg/mL) (mg GA equivalent/g extract) used as the reference

standard.[30,31] The total flavonoid content (TFC) was calculated as quercetin (QUE) equivalent (mg QUE equivalent/g extract) for the SP extracts.[31,32] The determination of total triterpene content (TTC) for SP extract, which was carried out by adapting the method determined by Chang et al. (2012) to microplate, was carried out briefly with the following steps: before heating the plate at 60°C for 45 min, SP (10 µL, stock solution prepared at 5000 µg/mL concentration), vanillin-glacial AA solution (5% w/v), and perchloric acid solution (15 µL and 50 µL, respectively) were mixed in the plate.[33] The absorbance of the solutions in a plate cooled to room temperature with the help of an ice bath was evaluated at 548 nm after adding 225 µL of glacial AA. For the standard curve plot, absorbances corresponding to each concentration were determined using oleanolic acid (OLE) (1200-37.5 µg/mL). This graph was used to calculate the extract's TTC which was then expressed as milligrams of OLE equivalent per g of the SP extract.

Grouping of Rats in Experiments Based on Treatments

After obtaining approval from the Marmara University Local Ethics Committee for all experimental protocols, the rats (n=24) were obtained from the Marmara University Experimental Animal Application and Research Centre (Protocol number: 51.2023mar, Date: 2023). The male and female Wistar rats (age 3 months, 200-250 g) were randomly divided into 4 groups, with 3 males and 3 females in each group[34]: I) PS group: Sham and vehicle (Physiological saline-PS) treatment control group (n=6), II) AA group: AA-induced colitis+PS treatment group (n=6), III) SS group: AA-induced colitis+100 mg/kg bwt, SS treatment group (n=6),[35] and IV) SP group: AA-induced colitis+200 mg/kg bwt, SP extract treatment group (n=6).[36]

After an 8-hour fasting period, 5% AA (in 0.9% NaCl, pH: 3 total volume: 1 mL) was administered intrarectally (ir) with a 30% inclination using an 8-cm-long soft silicone catheter (6G) to rats in the AA, SS, and SP groups under aether anaesthesia (by inhalation). After waiting 30 s, the animals were placed back in the cage.[37] In the PS group, PS was applied to the rats in an equal volume (1 mL) and in a similar manner (ir) instead of AA. Following colitis induction, the treatment regimens (PS, SS, or SP extract) were administered via orogastric gavage (og) with a metal cannula (1 mL) and administered once a day for 3 days at the same time (at 24-hour intervals) and dosage. SS is a drug used for the treatment of active ulcerative colitis and for remission. Therefore, it is a treatment given to the positive control group in experimental studies. PS was similarly administered to the PS and AA groups (1mL/og). (Figure 1). In this model, signs of healing and regeneration of the mucosa were seen on the 7th day; therefore, we applied the treatments for 3 days in parallel with the practises in the literature.[38,39] At the end of the 72nd hour, the rats were euthanized with sodium pentobarbital (50 mg/kg bwt, ip).

In Vivo Assessment of SP Treatment Intervention in Rat Tissue from the Colon with AA-Induced UC

Lucigenin and luminol chemiluminescence levels, indicators of oxidative stress in rat colon tissue, were recorded using Mini Lumat. LB 9509 luminometer (EG&G Berthold, Germany). Counts were obtained at 1-min intervals for 5 min, as described by Haklar et al.[40]

In the colonic tissues obtained from the rat; cytokines (TNF-α, IL-1β, IL-10, IL-17), tool-like receptor-9 (TLR-9), mitochondrial-mediated apoptotic markers [caspase-3 and caspase-9], superoxide dismutase enzyme (SOD), and tissue damage indicator matrix metalloproteinase-3 enzyme (MMP-3) levels were measured using ELISA test kits which catalogue numbers are given respectively (E0764Ra, E0119Ra, E0108Ra, E0115Ra, E0082Ra, E1648Ra, E1898Ra, E1376Ra, E031Ra). These assay procedures were conducted using an Agilent Technologies Bio Tek Epoch Microplate spectrophotometer (450 nm) in compliance with the manufacturer's instructions (Bioassay Technology Laboratory (BT LAB, China).

Assessment of UC Severity and Response to Treatment Utilising Macroscopic and Microscopic Analysis

We graded the lesions that developed after harvesting and washing the 8-cm distal region of the colon using macroscopic scoring.[41] The samples were then photographed. For light microscopic evaluation, rat colonic tissue samples were fixed in 10% neutral-buffered formalin, dehydrated in a graded alcohol series, cleared with xylene, and embedded in paraffin wax. Then, 4 µm thickness sections were obtained and stained using Haematoxylin and Eosin (H&E) to study the general histological structure. The criteria for scoring and scale ranging were: damage/necrosis (0, none; 1, localised; 2, moderate; 3, severe); submucosal oedema (0, none; 1, mild; 2, moderate; 3, severe); inflammatory cell infiltration (0, none; 1, mild; 2, moderate; 3, severe); vasculitis (0, none; 1, mild; 2, moderate; 3, severe); perforation (0, absent; 1, present). The total score was 13.[42] Images were captured using a light microscope (Olympus CX21, Tokyo, Japan) and photographed using a camera (Olympus, Tokyo, Japan, BX51).

Statistical Analysis

Normality was assessed using the One-sample Kolmogorov-Smirnov test. Normally distributed data were analysed using Tukey's multiple comparison tests after analysis of one-way variance (ANOVA). Prism 6.0 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Results were expressed as mean ± S.E.M., and p-values of less than 0.05 were considered significant.

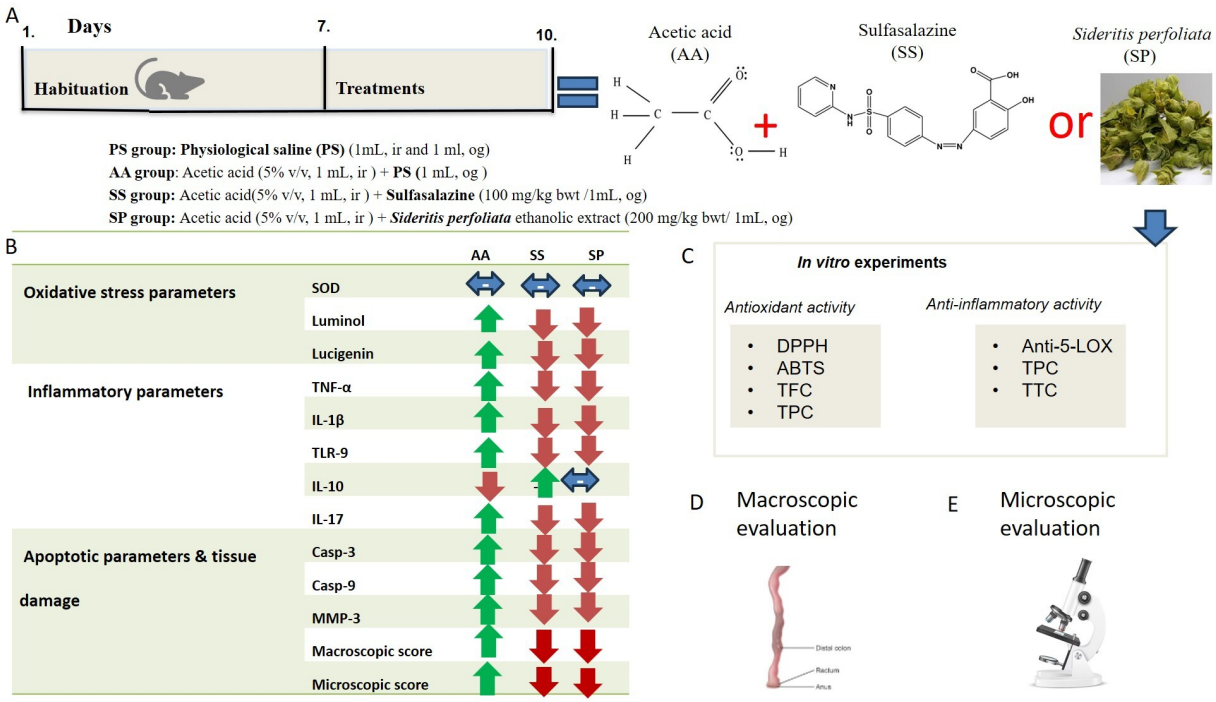

Figure 1. Experimental design. A. Following the habituation phase, the rats were randomly assigned to four experimental groups, each with 6 rats. I) PS group: Sham and vehicle (PS) treatment control group; II) AA group: AA-induced colitis+PS treatment group; III) SS group: AA-induced colitis+100 mg/kg bwt, SS treatment positive control group; IV) SP group: AA-induced colitis+200 mg/kg bwt, SP extract treatment group. After 8 h of fasting, acetic acid [1 mL 5% (v/v) in 0.9% saline] was administered intrarectally for colitis induction through a cannula. Treatments were applied for three days. B. Biochemical analyses. At the end of the 72nd h, the colon tissues of the euthanized rats were dissected for biochemical (ELISA) and other analyses. C. The radical-scavenging and anti- inflammatory properties of SP extract were verified in vitro. D. Macroscopic evaluation. E. Microscopic evaluation. **PS:** Physiological saline, **AA:** acetic acid, **SS:** Sulfasalazine, **SP:** *Sideritis perfoliata*, **ir:** intrarectally, **og:** orogastric.

RESULTS

In Vitro Evaluation of Antioxidant, Anti-Inflammatory, and Total Secondary Compounds in SP Extract

The IC_{50} values obtained because of DPPH radical analysis were determined to be 100.5±0.42 µg/mL for SP extract and 40.23±2.08 µg/g/mL for ascorbic acid used as a reference. The ABTS test results showed that the IC_{50} values for Trolox and SP extract, the reference standard, were 4.54±0.08 and 90.45±0.53, respectively. The antioxidant activity of the extract was evaluated as highly active when $IC_{50} \leq 10$ µg/mL, active when $10 < IC_{50} \leq 150$ µg/mL, moderately active when $150 < IC_{50} \leq 500$ µg/mL, and inactive when $IC_{50} > 500$ µg/mL according to the criteria proposed by Moga et al. (2021).[41] According to this criterion, SP extract was found to be active (good antioxidant activity) against DPPH and ABTS radicals.

The IC_{50} value of the extract against the 5-LOX enzyme was 17.64±0.45 µg/mL (compared to 21.42±0.48 µg/mL for conventional indomethacin), and the SP extract exhibited potent anti-inflammatory activity.

The TPC of the extract was calculated using the formula [y = 0.093x + 0.062 (R^2: 0.9987)] derived from the calibration curve defined as GA equivalent. The TPC of the extract was obtained at 140.8±1.21 mg GA equivalent per g of dried extract. The TFC of the extract was determined using the equation [y= 0.003x + 0.015 (R2: 0.9644)] obtained from the calibration curve as the QUE equivalent. The TFC of the extract was determined at 407.2±0.46 mg QUE equivalent per g of the dried extract. The TTC of the SP extract was determined using the equation [y = 0.027x + 0.016 (R2: 0.9981)] obtained from the calibration curve as the OLE equivalent. The TTC of the extract was determined at 85.97±1.66 mg OLE per g of dried extract.

In Vivo Results of Oxidative Stress, Inflammation, Apoptotic Tissue Damage, and SP Extract

The analyses revealed no statistically significant difference between the experimental groups in terms of SOD levels in colon tissue (Figure 2A). Colon luminol and lucigenin CL levels exhibited a notable increase in the AA group compared with the PS group ($p<0.001$). This elevation in luminol and lucigenin CL was diminished in both the SS and SP groups ($p<0.001$). The lucigenin level in the SS group did not deviate from the value found in the PS group, although the luminol value did not entirely revert to the PS value in the SS group ($p<0.05$; Figures 2B and 2C).

Compared with the PS group, colonic tissue levels of TNF-α, IL-1β, and TLR-9 in the AA group were considerably greater

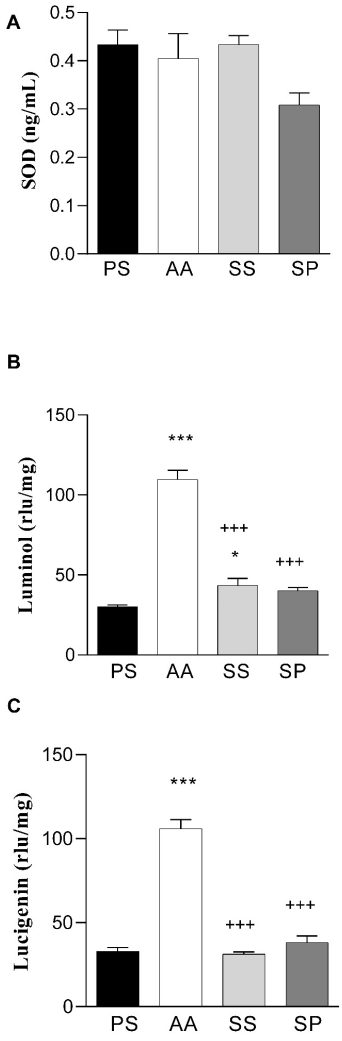

Figure 2. Evaluation of the levels of superoxide dismutase (SOD) activity (A), using ELISA method, and luminol (B), and lucigenin using CL assay (C) in colon tissue after treatments. PS, Sham, and vehicle (PS) treatment control group; AA, AA-induced colitis + PS treatment group; SS, AA-induced colitis + SS treatment positive control group; SP, AA-induced colitis + SP extract treatment group. Results were presented as mean ± S.E.M. *p<0.05, ***p<0.001 control vs. acetic acid; +++p<0.001 acetic acid vs. treatment group. **PS:** Physiological saline, **AA:** acetic acid, **SS:** Sulfasalazine, **SP:** *Sideritis perfoliata*.

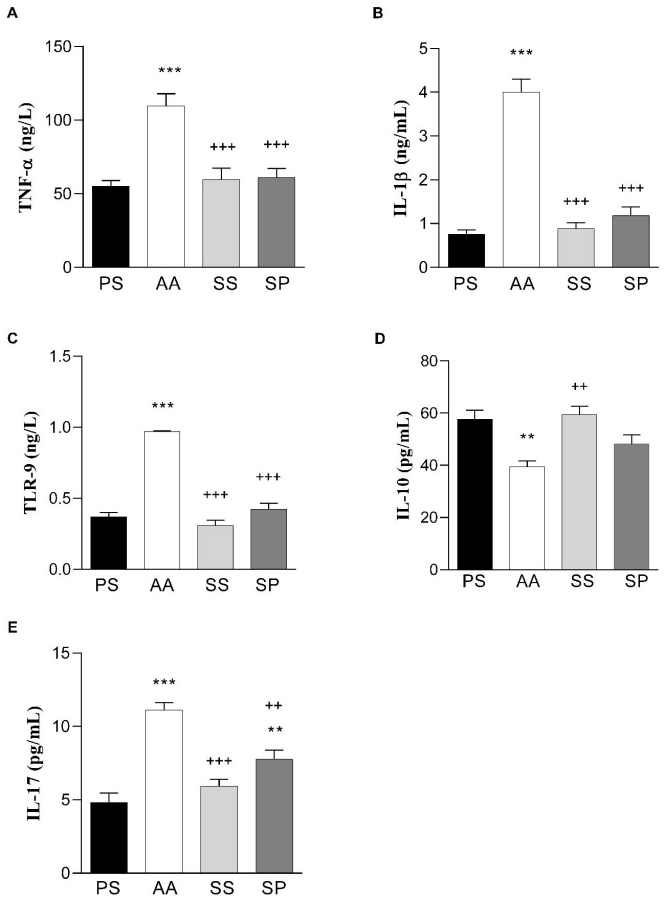

Figure 3. Evaluation of the levels of TNF-α (A), IL-1β (B), TLR-9 (C), IL-10 (D), and IL-17 (E) in colon tissue after treatments in AA-induced UC rat model, using ELISA method. PS, Sham, and vehicle (PS) treatment control group; AA, AA-induced colitis+PS treatment group; SS, AA-induced colitis+SS treatment positive control group; SP, AA-induced colitis+SP extract treatment group. Results were presented as mean ± S.E.M. **p<0.01 and ***p<0.001 control vs. acetic acid; $^{++}$p<0.01 and $^{+++}$p<0.001 acetic acid vs. treatment group. PS: Physiological saline, AA: acetic acid, SS: Sulfasalazine, SP: *Sideritis perfoliata*.

(p<0.001 for three markers), and the levels of these cytokines were reduced in the SS and SP groups (p<0.001) (Figure 3A-3C). IL-10 levels were significantly lower in the AA group than in the PS group (p<0.01), but increased IL-10 levels were found in the SS group than in the AA group (p<0.01) (Figure 3D). On the other hand, there were no significant differences in IL-10 levels between the SS or SP groups and the PS group (Figure 3D). Compared with the PS group, the IL-17 level was higher in the AA group (p<0.001) (Figure 3E). While a significant decrease in IL-17 levels was detected in the SS and SP groups compared with the AA group (p<0.001-p<0.01, respectively), the IL-17 level of the SP group was still higher than that of the PS group (p<0.05) (Figure 3E).

Caspase-3 and caspase-9 levels were elevated (p<0.001-p< 0.05 respectively) in the AA group; however, these elevations were attenuated in the SS (p<0.001 for both caspases) and SP (p<0.001 for both caspases) groups. There was a significant decrease in caspase-9 levels in the SP group compared with the PS group (p<0.01), while caspase-3 levels were still higher than in the PS group (p<0.01) (Figures 4A and 4B). MMP-3 activity was higher in the AA and SS groups compared with the PS group (p<0.001-p<0.05), while MMP-3 levels in the SS and SP groups were decreased compared with the AA group (p<0.05-p<0.01). In addition, no significant difference was observed between the SP and PS groups (Figure 4C).

The AA group had a higher overall macroscopic score (5.17±0.3) compared to the PS (0.00±0.1), SS (0.83±0.3), and SP (0.67±0.2) groups. The rats in the AA group had significantly higher macroscopic lesion ratings than those in the PS

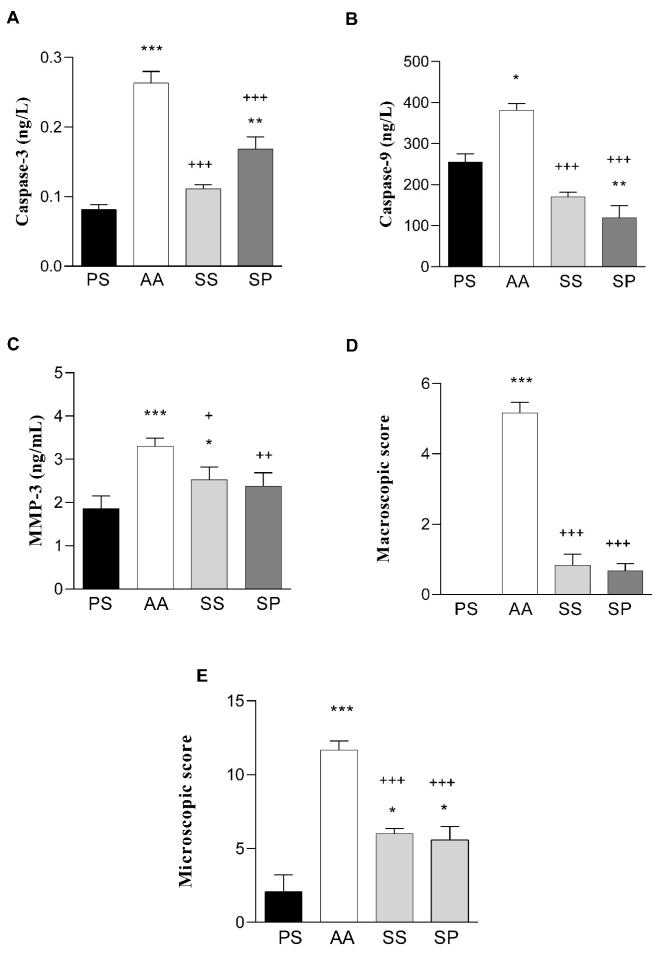

Figure 4. Evaluation of the levels of caspase-3 (A), caspase-9 (B), and MMP-3 (C), macroscopically (D), and microscopically (E) scores in colon tissue after treatments in AA-induced UC rat model. PS, Sham, and vehicle (PS) treatment control group; AA, AA-induced colitis+PS treatment group; SS, AA-induced colitis+SS treatment positive control group; SP, AA-induced colitis+SP extract treatment group; Results were presented as mean ± S.E.M. *, **: p<0.05, *** p<0.001 control vs. acetic acid; +: p<0.05, ++: p<0.01, +++p<0.001 acetic acid vs. treatment group. **PS:** Physiological saline, **AA:** acetic acid, **SS:** Sulfasalazine, **SP:** *Sideritis perfoliata*.

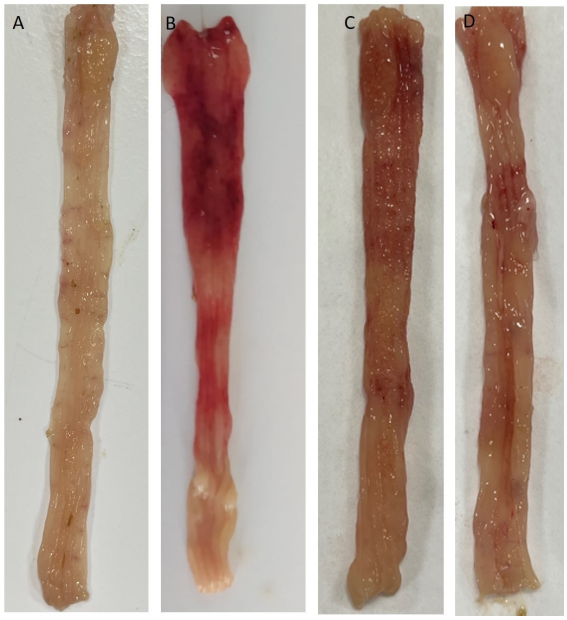

Figure 5. Effect of *Sideritis perfoliata* or sulfasalazine treatment on macroscopic images of rat colons with acetic acid-induced ulcerative colitis. A. PS Sham, and vehicle (PS) treatment control group; B. AA-induced colitis+PS treatment group; C. AA-induced colitis+SS treatment positive control group; D. AA-induced colitis+SP extract treatment group. **PS:** Physiological saline, **AA:** acetic acid, **SS:** Sulfasalazine, **SP:** *Sideritis perfoliata*.

group (p<0.001) (Figure 4D). The rats in the SS or SP groups showed a significant decrease in the overall macroscopic damage score compared with the AA group (p<0.001) (Figure 4D). The PS group's colon tissue showed standard macroscopic architecture with no signs of mucosal layer injury (Figure 5A). In contrast, the AA group (Figure 5B) exhibited significant ulcerative and oedematous mucosal lesions. Additionally, a significant reduction in mucosal damage and lesions was observed in the SS (Figure 5C) and SP (Figure 5D) groups compared with the AA group.

When colon tissues were examined for microscopic damage, the AA group was found to have a higher microscopic damage score than the control group (p<0.001) (Figure 4E). However, microscopic damage was significantly reduced in the SS or SP (p<0.001) groups (Figure 4E). On the other hand, the microscopic scores of the SS and SP groups were higher than those of the control group (p<0.05) (Figure 4E). The Haematoxylin and eosin (H&E) stained sections of the control group revealed typical colonic histology with intact epithelium, well-preserved glands, and regular submucosal layers (Figure 6A). In contrast, the AA group exhibited pronounced damage, including a marked loss of the surface epithelial lining, glandular destruction, considerable submucosal oedema, and inflammatory cell infiltration (Figure 6B). The SS group exhibited a histological improvement with more regular epithelial surfaces and maintained glandular structures (Figure 6C). Compared with the AA group, the SP group exhibited improvements, with reduced mucosal and glandular damage, decreased submucosal oedema, and moderate inflammatory cell infiltration (Figure 6D).

DISCUSSION

In this study, SP ethanolic extract showed good antioxidant activity against ABTS and DPPH radicals and strong anti-5-LOX activity. The TPC, TFC, and TTC levels of the SP extract were also determined. Treatment with SP extract reduced macroscopic and microscopic injury scores associated with UC. The SP extract diminished TNF-α, IL-1β, IL-17, TLR-9, MMP-3, caspase-9, caspase-3, luminol, and lucigenin CL levels.

SP ethanolic extract was qualitatively proven to carry phenolic, flavonoids, and triterpene compounds with blue, orange-red, and purple colours seen in total secondary compound

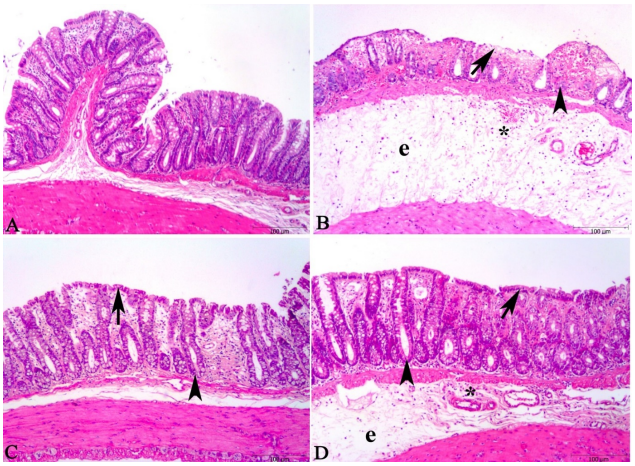

Figure 6. Light microscopic images of colonic tissue sections stained with H&E are representative examples. **A.** The PS group exhibited well-structured colonic mucosa, submucosa, and muscularis. **B.** In the AA group, there was a significant loss of colonic crypts (indicated by arrow) and glands (indicated by arrowhead), along with notable submucosal oedema (e) and inflammatory cell infiltration (marked by an asterisk). **C.** The SS group exhibited a more consistent appearance of epithelial surfaces (arrow) and preserved glandular structures (arrowhead). **D.** SP group exhibited improvements, with reduced mucosal damage (arrow), diminished glandular degeneration (arrowhead), oedema (e), and inflammatory cell infiltration (asterisk). Scale bar, 100 µm. **PS:** Physiological saline, **AA:** acetic acid, **SS:** Sulfasalazine, **SP:** *Sideritis perfoliata*.

determination tests. In addition, our quantitative analysis revealed that the compound contains high numbers of flavonoids, triterpenes, and phenolic compounds. *Sideritis* species are rich in flavonoids, terpenoids, coumarins, sterols, and iridoids, and these secondary metabolites are responsible for their pharmacological effects.[23] In addition, the extract exhibited good free radical scavenging activity against DPPH and ABTS radicals and robust anti-inflammatory activity against 5-LOX. Sarikurkcu et al. (2020) also reported that methanol and water extracts have high DPPH and ABTS radical scavenging and reducing power activities.[44] Although SP's effect on colitis is unknown, Charami et al. (2008) reported the antioxidant activity (using DPPH spectrophotometric and thiobarbituric acid lipid peroxidation measurements) and anti-soybean lipoxygenase activity of SP extracts *in vitro*.[20] Naturally derived compounds, such as flavonoids, triterpenes, and phenolic acids, have been reported to be promising agents against colitis because of their antioxidant (phenolic acids and flavonoids) and anti-inflammatory (phenolic acids, flavonoids, and triterpenes) effects.[43,45,46] Therefore, the antioxidant and anti-inflammatory properties of these compounds may be primarily responsible for the therapeutic effect of SP ethanolic extract. The consequences of our study also pointed to the 5-LOX inhibitory potential of the polar ethanolic extract. Therefore, the extract's improving impact on the UC could be attributed to the synergistic action of its constituents.

Oxidative stress causes mucosal damage accompanied by pro-inflammatory mediators in humans and animals. The AA leads to the upregulated production of free radicals and reactive oxygen species (ROS).[47] Hence, free radical scavenging and ROS elimination are essential in diminishing colon inflammation in the UC.[13] In the present study, treatment with SS and SP extract repressed the AA-induced oxidative stress, as affirmed by the declining luminol and lucigenin levels. On the other hand, no significant differences were observed between the groups in terms of SOD activity. Our *in vitro* analysis revealed that SP extract has good DPPH/ABTS radical scavenging activity, which may cause a reduction in the amount of luminol-lucigenin-enhanced CL. In addition, the antioxidant effect of flavonoids and phenolic substances in the extract also explains the low amounts of luminol and lucigenin. Therefore, reducing oxidative stress promotes biochemical and morphological colonic recovery. The antioxidant activity of *Sideritis* species was previously attributed to their high phenolic compound content, and a limited number of *in vivo* studies have been conducted to determine the anti-radical effects of extracts obtained from SP.[23] The ethanolic extract of SP displayed moderate antioxidant potential and antibacterial activity against *Prevotella intermedia*. In that study, the authors linked the plant's moderate antibacterial and antioxidant activity to the traditional use of SP for treating wounds and inflammatory conditions.[23]

The critical mediators of mucosal inflammation are cytokines and small cell-signalling molecules produced by numerous immune cells. Previous studies have demonstrated a direct correlation between cytokine levels and UC inflammation.[48,49] Colonic TNF-α, IL-1β, and IL-17 levels were upregulated as inflammation markers in male mice with UC.[50] The mucosal concentrations of pro-inflammatory cytokines IL-1β[51], TNF-α[51], and IL-17[52,53] are high in patients with UC. In the present study, AA administration increased these cytokines in the colon. Moreover, SS and SP extract treatments caused a decrease in the levels of these cytokines. Previously, SS treatment has shown a decreased effect of TNF-α, IL-1β, and IL-17 in the rat colon.[54] In our study, SP extracts reduced these cytokines similarly to SS even though TNF-α and IL-1β levels were no different from the control (PS group). This result indicates that SP mitigates cytokine responses. Only SS treatment increased the anti-inflammatory cytokine IL-10 levels, whereas SP did not cause a significant increase. We think SP's effect on IL-10 should be investigated in different colitis models.

TLR-9 levels are elevated in the colon with UC rats.[55] A relationship between the cruelty of inflammation and TLR-9 levels was shown in humans.[56,57] Our study found consistency between colonic TLR-9 levels and inflammation severity in UC rats, consistent with previous studies.[55-57] Our results suggest that SS and SP extract treatment inhibits TLR-9 levels. On the other hand, some researchers have reported that TLR-9 activation suppresses inflammation.[58,59] Therefore, more work is needed on this subject.

Suppression of caspase-9 and caspase-3 and prevention of apoptosis are potential therapeutic targets for UC.[60,61] Our

results indicated that SS and SP treatment diminished AA-induced apoptosis, partly because of reduced caspase-3 and caspase-9 activity levels. Moreover, we observed that SP extract treatment significantly improved caspase-9 levels and even reduced them to lower levels in the control group. Our findings demonstrate that SP attenuates mitochondria-associated apoptosis. Previously, medicinal or nutraceutical-based herbs have been shown to have antiapoptotic activity and to improve colitis and ulcerative lesions.[62,63] However, there is no research on the apoptotic effect of SP.

In this study, AA caused significant deterioration of macroscopic and microscopic tissue morphology and increased MMP-3 levels. Treatment with SP extract improved MMP-3 and macroscopic scoring at a level similar to control while partially improving microscopic scoring. Our *in vitro* and *in vivo* biochemical data results were reflected in the tissue and supported morphological improvements. MMPs play an essential role in connective tissue degradation and are fundamental in redundant tissue demolition in colitis.[64] MMPs are proteases that degrade all components of the extracellular matrix. Under physiological conditions, they are produced at low levels and play a role in normal tissue homeostasis. Specific inhibitors (TIMPs) inhibit their functions. However, in inflamed tissues of patients with IBD, MMPs are overproduced and TIMPs are deficient. Therefore, they contribute significantly to the mucosal degradation associated with IBD.[65] The specific inhibition of these MMPs may provide novel therapeutic options against IBD.

CONCLUSION

In conclusion, thanks to its flavonoids, phenolic acids, triterpene contents, anti-5-LOX, and free radical scavenging activities, SP extract attenuates pro-inflammatory cytokines, mitochondrial apoptotic caspases, and oxidative stress. Our findings support the ethnopharmacological use of SP for colitis.

Ethics Committee Approval: This study was conducted following the principles of the guidelines published by the International Council for Laboratory Animal Science (ICLAS), and the Regional Ethics Committee of Marmara University approved it (51.2023.mar; date 2023).

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