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Original Article

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Evaluation of Active Compounds of *Sideritis Trojana* Ethanolic Extract and Their Protective Effects in Rats with Acetic Acid-Induced Ulcerative Colitis

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Abstract

Objective: In this study, we aimed to examine the effects of *Sideritis trojana* (ST) ethanolic extract in acetic acid (AA)-induced rat model of colitis. ***Design:*** Firstly, anti-DPPH and anti-ABTS free radical scavenging, anti-5-lipoxygenase (LOX) activities, and total secondary metabolite quantities of ST extract were measured. Secondly, while the sham control group (C) and 5% AA-infused (intrarectally) colitis group were treated with saline, other colitis groups were treated with sulfasalazine (100 mg/kg/orally) or ST (200 mg/kg/orally). At the end of the 72-h colonic myeloperoxidase (MPO), glutathione (GSH), malondialdehyde (MDA), TNF-α, IL-1β, IL-17, IL-10 levels, and macroscopic and histopathologic evaluations were done. ***Results:*** Results: ST had high total phenolic, total flavonoid, and total triterpene content, while it showed high antioxidant activity against ABTS or DPPH radicals and anti-5-LOX enzyme activity. Increased macroscopic and microscopic scores in the colitis group were reduced by treatments. TNF-α, IL-1β and IL-17 levels were increased in the colitis group compared with the control group, while these levels were decreased in the ST and sulfasalazine groups. Moreover, increased MPO, MDA and decreased GSH levels in the colitis group were reversed by ST and sulfasalazine treatments. ***Conclusion:*** ST extract alleviates ulcerative colitis, attributed to its rich composition of total flavonoids, phenolics, and triterpenes, as well as its anti-5-lipoxygenase (5-LOX) activity and potent free radical scavenging properties.

Keywords: ***Sideritis trojana, Anti-5-LOX, IL-17, Radical scavenging, Ulcerative colitis.***

Introduction

Aiming to reduce the effects of ulcerative colitis (UC), an inflammatory bowel disease, individuals suffering from this disease benefit from complementary and alternative medicine/products, most of which are herbal, in addition to their medical treatments [1]. The therapeutic benefits of plants used in traditional medicine for thousands of years are highlighted by preclinical studies [2]. Nutraceuticals with elevated polyphenol and antioxidant content are reported by preclinical research results to be beneficial for health because they can scavenge free radicals, have anti-inflammatory effects, and regulate the gut microbiota's homeostasis [3].

With a total of 46 species and an endemism rate of 79%, Türkiye leads the world with *Sideritis* species after Italy [4]. *Sideritis trojana* (ST), known as ''mountain tea'' among the public, is generally used as tea, feed, and flavouring. This species is endemic to the Ida Mountains and constitutes one of the 46th *Sideritis* species in the flora of Turkey [5]. Previous phytochemical studies reported the occurrence of diterpenes [4], essential oil [6], iridoids, phenylethanoid glycosides, and flavonoids [7] in the aerial and underground parts of this species. Moreover, the in vitro antimicrobial [7] and antioxidant activities [8] of different extracts and/or the secondary metabolites purified were reported. In

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traditional treatments, it has been used for abdominal pain, kidney disorders, laxatives, stomach aches, and sore throat [9].

In light of the above literature, the current investigation intended to assess the antioxidant and anti-inflammatory [(anti-5-lipoxygenase (anti-5-LOX)] impact of ST ethanol extract *in vitro* and the effect of the treatment on anti-inflammatory, oxidative stress, and tissue damage-related parameters in acetic acid (AA)-induced UC rat colon tissue.

Materials and Methods

The plant material

The aerial parts of the ST were procured from the Bayramiç district of Çanakkale province (July 2023) and were identified. Some of the specimens of the plant were placed at Marmara University (M.U.), Faculty of Pharmacy Herbarium (International Code: MARE, under herbarium no. 23481). The dried and ground aerial parts of ST (20.10 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 no higher than 45 °C using a rotary evaporator to yield an ethanolic (Et-OH) extract with a 15.52% yield (g/g).

Verification of the anti-inflammatory and radical scavenging abilities of ST extract *in vitro*

The radical scavenging effects anti 2,2- diphenyl-1-picrylhydrazyl (DPPH), anti-2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals of the ST extract (stock solutions in the range of 5000-9.77 µg/mL) were evaluated by the method previously insisted on by Zou et al. (2011) [10]. Ascorbic acid or Trolox was utilized as reference standards in the spectrophotometric evaluation (517 or 743 nm). The determination of the ST extract required to inhibit LOX activity by 50% (IC_{50}, µg/mL) was performed as follows: 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 put into each well and incubated (for 5 min), and then, linoleic acid (0.6 mM, 100 µL) was added and mixed again [11]. The absorbance values of the samples and the reference standard, indomethacin, were read at 234 nm.

Evaluation of total secondary metabolites of ST extracts in terms of flavonoid, triterpene, and phenolic content

Experiments using gallic acid (GA, stock solutions in the range of 500- 15.63 µg/mL) as a reference standard to measure the total phenolic (TP) content of ST extracts (mg GAE/g extract) were performed using the Folin-Ciocalteu colorimetric test [12,13]. Utilizing the methodology of Zhang et al. (2013) and Yıldırım et al. (2019), total flavonoid (TF) content analysis, calculated as quercetin equivalent (mg QUE/g extract), of ST extract was performed [13,14]. The total amount of triterpenes (TT) contained in the ST extract was quantified using the method of Chang et al. (2012) adapted to the microplate method [15]. The absorbance values of the specimens were determined by reading at 548 nm after the addition of 225 µL of GA and then using the standard curve graph oleanolic acid (OLE, 1200-37.5 µg/mL).

Grouping rats in treatment-based experiments

M.U. DEHAMER provided the rats for the study, following approval of all experimental protocols by the M.U. Animal Investigations Local Ethics Committee (protocol number: 52.2023mar, date: 2023). Six rats (equal number of males and females, 250-300 g) each were at random assigned to one of four groups: I) control group (C), treated with saline; II) colitis group, acetic acid (AA)-induced + saline treatment [16]; III) S treatment group: AA-induced colitis + Sulfasalazine (S, Salazopyrin, Pfizer, Turkiye) treatment (100 mg/kg bwt) [17] and IV) ST treatment group: AA-induced colitis + ST (200 mg/kg bwt) [18].

In vivo evaluation of the treatment interventions in rat colon tissues

A luminometer (Mini Lumat LB 9509, EG & G Berthold, Germany) was used to measure the quantities of lucigenin and luminol chemiluminescence (CL), two oxidative stress markers, in rat colon tissue. The counts were taken at 1-minute intervals for 5 minutes [19].

ELISA tests were used to measure changes in the levels of cytokines (TNF-α, IL-1β, IL-10, and IL-17) (Cat. No. E0764Ra, E0119Ra, E0108Ra, E0115Ra, respectively). BioTek Epoch Microplate spectrophotometer at 450 nm (Agilent Technologies) was used for these, following the manufacturer's instructions [Bioassay Technology Laboratory (BT LAB), China].

To assess reduced glutathion (GSH) and malondialdehyde (MDA) levels in colon tissue, samples were homogenized using KCl (ice-cold, 150 mM), following the protocol of Beuge and Aust (1978) [20]. The GSH content of the tissue was determined by the use of an adjustment of the Ellman procedure [21]. Lipid peroxidation was computed and presented regarding MDA equivalents [20]. To evaluate the MPO activity of the samples, absorbance was measured at 460 nm using the Hillegeas technique [22].

We graded the lesions that developed after harvesting and washing the 8-cm distal region of the colon using macroscopic scoring [23]. Rat colon tissue samples were fixed within 10% neutral-buffered formalin, dehydrated within a graded alcohol series, clarified with xylene, embedded in paraffin wax, and evaluated by light microscopy. Sections obtained at 4 µm thickness were stained using H&E for evaluation of general histological structures such as damage/necrosis, submucosal oedema, inflammatory cell incursion, vasculitis, and perforation. The scoring and scale range criteria used in the histological evaluation were as follows: 0, none; 1, limited; 2, medium; 3, extreme [24]. Images saved using a light microscope (Olympus CX21, Tokyo, Japan) were photographed using a camera (Olympus BX51, Tokyo, Japan).

Statistical analysis

Normality was verified via the One-Sample Kolmogorov-Smirnov test, and normally distributed data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Prism 6.0 (GraphPad Software, USA) was used for statistical analysis. The outcomes are stated as mean ± SEM, and p-values less than .05 were considered significant.

Results

In vitro results of antioxidant, anti-inflammatory, and total secondary compounds in ST extracts

The IC_{50} values established by DPPH analysis and ABTS of ST extract were 118.7 ± 0.42 µg/mL, and 116.1 ± 0.35 µg/mL, and the IC_{50} values established for the reference standards utilized in the analyses were 40.23 ± 2.08 µg/mL (ascorbic acid) and 4.54 ± 0.08 (Trolox), respectively. According to the criteria suggested by Moga et al. (2012), the antioxidant activity of the extract is considered 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. In line with this criterion, it was discovered that the ST extract exhibited good antioxidant activity against DPPH and ABTS radicals [25].

ST extract showed that it has strong anti-inflammatory activity against the LOX enzyme with an IC_{50} value of 16.65 ± 0.22

µg/mL compared to 21.42 ± 0.48 µg/mL for conventional indomethacin.

Utilizing the equation [y = 0.003x + 0.015 (R^2: 0.9644)] derived from the calibration curve as defined QUE (mg/g extract), the TF content of the extract was calculated to be 143.7 ± 0.92 mg QUE. The TP content of the ST extract, counted using the formula [y = 0.093x + 0.062 (R^2: 0.9987)] derived from the calibration curve defined as GA equivalent, was obtained as 68.28 ± 1.16 mg GA (per g dried extract). The TT content of the ST extract was determined using the equation [y = 0.027x + 0.016 (R^2: 0.9981)] obtained from the calibration curve as the OLE equivalent. The TT content of the extract was determined at 119.50 ± 0.59 mg OLE.

Treatment interventions' effects on parameters related to inflammation, oxidative stress, morphology, and histology

Tissue levels of TNF-α ($p < 0.001$), and IL-1β ($p < 0.001$) in the AA group were considerably greater than C group (**Figure 1a and 1b**), and following S or ST treatment the levels of these cytokines were reduced ($p < 0.001$ for both markers in S treatment; $p < 0.01$, report as $p = 0.0027$) for TNF-α and $p < 0.001$ for IL-1β in ST treatment). It was determined that the reduction in IL-1β level brought about by ST treatment did not entirely restore the values seen in the control level. IL-10 level was significantly decreased in the AA group compared to the C group ($p < 0.01$, report as $p = 0.0083$; **Figure 1c**). Both S and ST treatments resulted in a significant increase in IL-10 levels of the treatment groups compared to the AA group ($p < 0.01$, report as $p = 0.0040$ for S treatment and $p < 0.01$, report as $p = 0.0024$ for ST treatment). IL-17 levels in colon tissue were significantly increased in the AA group compared to the C group ($p < 0.001$, **Figure 1d**). Both S and ST treatments resulted in a significant decrease in IL-17 levels compared to the AA group ($p < 0.001$ for both treatments).

GSH levels decreased in the AA group ($p < 0.001$) compared to the C group, while MPO ($p < 0.001$) and MDA ($p < 0.001$) levels increased. GSH levels increased significantly in the S ($p < 0.01$, report as $p = 0.0013$) and ST treatment groups ($p < 0.01$, report as $p = 0.0011$) compared to the AA group (**Figure 2a**). The MPO and MDA levels for the S and ST treatment groups were compared with the AA group; both parameter levels decreased significantly in the S ($p < 0.01$, report as $p = 0.0033$ and $p < 0.001$, respectively) and ST ($p < 0.01$, report as $p = 0.0033$) and $p < .001$, respectively) treatment groups and returned to control levels (**Figure 2b and 2c**). Colon luminol and lucigenin levels were significantly increased in the AA group compared to the C group ($p < 0.001$ for both CLs; **Figure 2d and 2e**). This rise in luminol- or lucigenin-induced CL was diminished in both treatments ($p < 0.001$, in the S and ST treatment groups). The lucigenin CL level in the S-treated group did not deviate from the value found in the C group. However, the luminol CL value did not entirely revert to the control value in the S-treated group ($p < 0.05$, report as $p = 0.036$).

The AA-induced colitis group had a higher overall macroscopic score (5.33 ± 0.2) compared to control (0.18 ± 0.2), S (0.83 ± 0.3), and ST (2.33 ± 0.2) groups. The colitis group rats had significantly higher macroscopic lesion ratings than those in the control group ($p < 0.001$, **Figure 3a**). A significant decrease in the overall macroscopic damage score was found when rats treated with S or ST after AA induction were compared with AA group rats ($p < 0.001$ for both treatments). Still, the macroscopic damage score did not return to control values after ST treatment ($p < 0.001$). The results of histopathological assessments conducted on the colon tissues of the experimental groups to calculate the total microscopic damage score showed that the colitis group ($p< 0.001$) had significantly more total microscopic damage than the control, that total microscopic damage was decreased with S ($p < 0.001$) and ST ($p < 0.05$, report as $p = 0.012$) treatments. Still, that total microscopic damage could not be brought back to the values of the control following S and ST ($p < 0.01$, report as $p = 0.0018$ and $p < 0.001$, respectively) treatments (**Figure 3b**).

Microscopic evaluation revealed well-organized structures in the C group (**Figure 4a**). In contrast, the AA-induced colitis group exhibited surface and glandular epithelial loss, severe oedema, and extensive inflammatory cell infiltration (**Figure 4b**). In the S-treated group, significant improvement in the surface and glandular epithelium was observed (**Figure 4c**). In the group treated with ST, focal degeneration of the surface epithelium, regeneration of the glandular epithelium, marked oedema in the submucosal area, and extensive inflammatory cell infiltration were observed (**Figure 4d**).

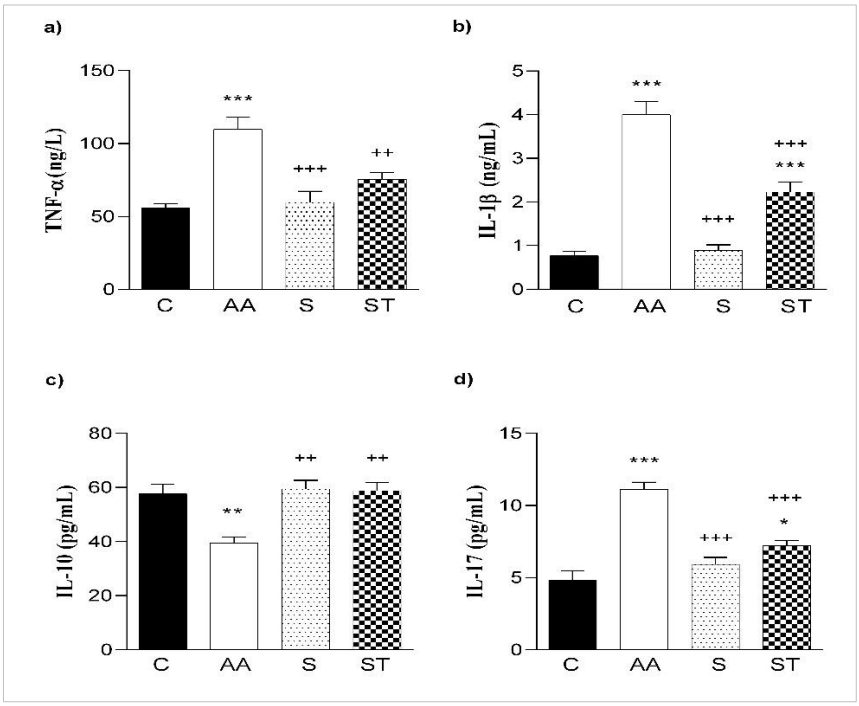

Figure 1: Evaluation of the TNF-α (a), IL-1β (b), IL-10 (c), and IL-17 (d), using the ELISA method in colon tissue after treatments (saline, AA, S, or ST).

C, control group; AA, acetic acid-induction group; S, sulfasalazine treatment group; ST, *Sideritis trojana* treatment group. Results were presented as mean ± SEM. *, **, *** $p < 0.05$-0.001 control vs. acetic acid; $^{++}$, $^{+++}$ $p < 0.01$-0.001 acetic acid vs. treatment group.

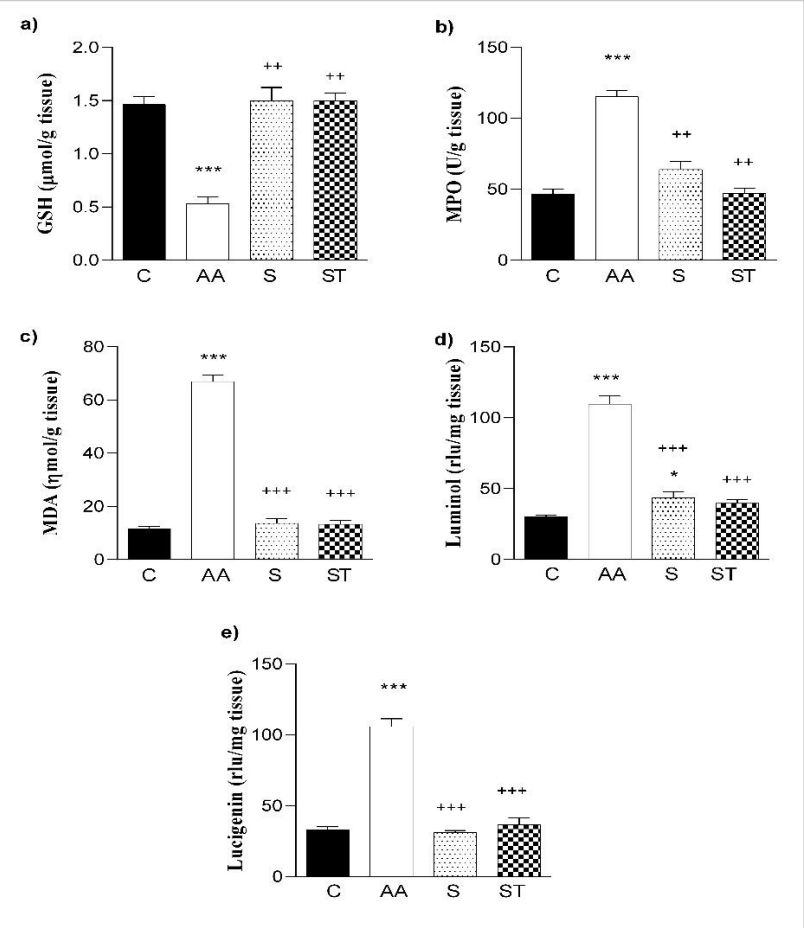

Figure 2: Evaluation of the levels of GSH (a), MPO (b), MDA (c), using ELISA method, and luminol (d), and lucigenin (e) using CL assay in colon tissue after treatments (saline, AA, S or ST).

GSH, glutathione; MPO, myeloperoxidase; MDA, malondialdehyde; C, control (saline treated) group; AA, acetic acid-induction group; S, sulfasalazine treatment group; ST, *Sideritis trojana* treatment group. Results were presented as mean ± SEM. *, *** $p < 0.05$-0.001 control vs. acetic acid; $^{++}$, $^{+++}$ $p < 0.01$-0.001 acetic acid vs. treatment group.

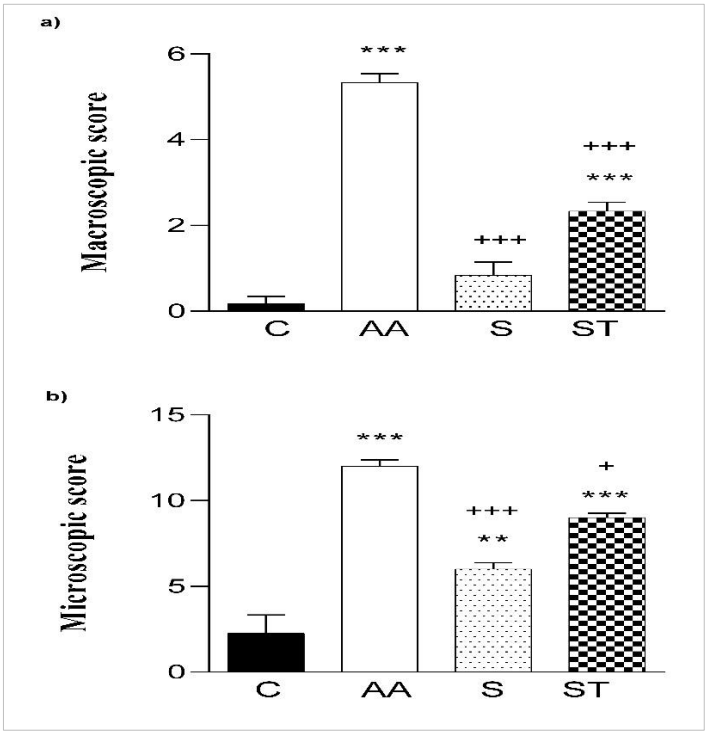

Figure 3: Effect of treatments (C, AA, S, ST) on macroscopic (a) and microscopic (b) score of rat colons with AA-induced ulcerative colitis.

C, control (saline treated) group; AA, acetic acid-induction group; S, sulfasalazine treatment group; ST, *Sideritis trojana* treatment group. Results were presented as mean ± SEM. **, *** $p < 0.05$ and 0.001 control vs. acetic acid; $^{+}$, $^{+++}$ $p < 0.01$-0.001 acetic acid vs. treatment group.

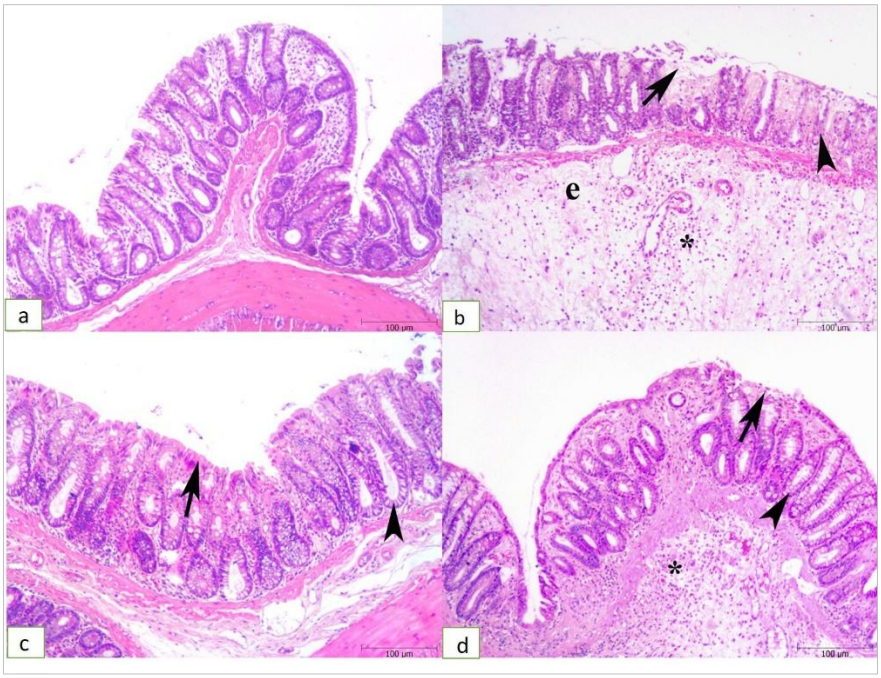

Figure 4: a. Regular colon tissue structure in the control group. b. Surface (arrow) and glandular epithelial (arrowhead) loss, severe edema (e), and extensive inflammatory cell infiltration (*) in the acetic acid-induced group. c. Regeneration of the surface (arrow) and glandular epithelium (arrowhead) in the Sulfasalazine-treated group. d. Area of degeneration in the surface epithelium (arrow), regeneration of the glandular epithelium (arrowhead) and extensive inflammatory cell infiltration (*) in the *Sideritis trojana* group. Hematoxylin and Eosin stain, scale bar, 100 µm.

Discussion

In the current study, ST ethanolic extract exhibited strong antioxidant activity against ABTS and DPPH radicals and has strong anti-inflammatory (anti-5-LOX) activity. Also, the levels of total phenolic, flavonoid, and triterpene contents were evaluated for the ST extract. Treatment with ST extract partially mitigates colonic damage by reducing macroscopic and microscopic injury scores related to UC. The ST extract decreased TNF-α, IL-1β, IL-17, luminol, and lucigenin CL levels, while increasing the levels of GSH and IL-10.

AA contributes to the increased generation of free radicals and reactive oxygen species (ROS). Therefore, scavenging free radicals and eliminating ROS are critical processes in mitigating colonic inflammation associated with UC [17]. The strong antioxidant activity of ST ethanolic extract against ABTS and DPPH radicals indicates its ability to cope with oxidative stress contributing to the development of inflammatory bowel diseases. Previously, ST was found as a crucial antioxidant source in *in vitro* tests like DPPH [26,27]. Studies on medicinal plants have shown a strong correlation between phenolic compounds, flavonoids, and antioxidant activity [28,29]. With their strong antioxidant capacity, flavonoids effectively scavenge ROS and prevent oxidative damage. Phenolic compounds directly neutralize free radicals, thanks to the hydroxyl groups in their structure. This reduces oxidative damage at the cellular level [30]. Due to the toxic effects of synthetic derivatives, people have turned to plants with high phenolic and flavonoid content and strong antioxidant capacity [26]. Moreover, after measurements of cytotoxicity analysis, Altun et al. (2023) have suggested that ST can be used as an antioxidant agent in the pharmaceutical industry [27]. In the present study, decreased luminol and lucigenin CL, MDA levels, and increased GSH levels with ST may be due to free radicals scavenging activities of ST. Our study supports previous studies indicating that ST ethanolic extract exhibits strong antioxidant activity.

5-LOX is an enzyme responsible for leukotriene production, which contributes to various inflammatory conditions. Previously, colonic tissue damage was reduced in 5-LOX-deficient mice with colitis compared to their wild-type counterparts. Additionally, 5-LOX increased neutrophil infiltration in experimental colitis by inducing the expression of adhesion molecules [31]. The decreased MPO levels in our study, indicating decreased infiltration of neutrophils into the tissue, may also be due to the anti-5-LOX activity of ST. Phenolic compounds by inhibiting the activity of inflammation-associated enzymes such as lipoxygenase can reduce the production of pro-inflammatory mediators (e.g. TNF-α, IL-1β, IL-6) [30].

In UC, the intestinal tissues exhibit elevated expression of IL-1β, IL-6, and TNF-α, as well as cytokines associated with Th17 responses in both humans and animals [32,33]. In a previous study by our group, we showed that a different type of S*ideritis* (*S. perfoliata*) had a significantly decreasing effect on IL-1, TNF-α, IL-17 and elevating IL-10 levels [34]. The active components of ST, which we have shown to be present, such as phenolic, flavonoid, and triterpene, can reduce the production of pro-inflammatory cytokines and also increase anti-inflammatory cytokines such as IL-10. Furthermore, it has been previously shown that these secondary compounds can reduce the inflammatory response and cytokine production (e.g., TNF-α, IL-1β, and IL-6) through inhibition of signalling pathways that play critical roles in inflammatory processes, such as NF-κβ and MAPK [35]. In the current study, decreased levels of IL-1, TNF-α, IL-17, and increased IL-10 levels support this. In addition, thanks to its antioxidant effect, it can alleviate oxidative stress by contributing to the elimination of ROS and thus control the inflammatory response. We see the reflections of this in the decreased macroscopic and microscopic damage scores.

However, the limitation of our study is that signalling pathways such as NF-κβ and MAPK as well as cyclooxygenase activity, were not examined in the current study. In addition, if the study duration had been longer, the reflection of biochemical parameters on tissue morphology could have been better observed.

Our findings give support to the ethnopharmacological use of ST for UC by attenuating inflammation and oxidative stress thanks to its flavonoids, phenolic acids, and triterpene contents and also anti-5-LOX and free radical scavenging activity.

Declarations

Ethics approval

This study was conducted following the principles of the guidelines published by the International Council for Laboratory Animal Science (ICLAS), and the Animal Investigations Local Ethics Committee of M.U. approved it (52.2023.mar; date 2023).

Source of funding

No funds, grants, or other support was received.

Data Availability

All data is included in the article.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

None

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