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Potential therapeutic effects of escin in mitigating inflammation and oxidative stress in experimentally induced ulcerative colitis in mice by acetic acid

Mohamed A. El-Sayed¹, Gamal Ramadan^{1*}  and Hend A. Mohammed¹

Abstract

Background Ulcerative colitis (UC) is a long-term immune-triggered disorder marked by persistent inflammation, oxidative stress, and lesions in the colonic mucosal layer. Escin, a triterpenoid saponin derived from the horse chestnut (*Aesculus hippocastanum*) tree, has been experimentally demonstrated to exhibit anti-inflammatory and antioxidant activities. Therefore, the current work aimed to assess the potential effects of escin in managing UC induced by intrarectal (I.R.) of acetic acid (AA) in mice in comparison with the customary drug sulfasalazine (SSZ).

Materials and methods Escin (20 or 40 mg/kg body weight "b.w") or SSZ (500 mg/kg b.w) was administered I.R. one hour (h), 6 h, and 12 h following the enema administration of 6% AA solution to provoke UC. Eventually, colon tissues were harvested at the final stage of the study for morphological and histological analysis, in addition to the assessment of biochemical markers.

Results The UC-mice treated with escin exhibited a significant decline in macroscopic and microscopic lesions of colon tissues, colon weight-to-length ratio, mortality rate, and colonic degranulated mast cells. Additionally, escin reduced substantially malondialdehyde and nitric oxide, whereas it elevated the content of reduced glutathione and the activities of superoxide dismutase and catalase in colon tissues of UC-mice. Moreover, escin successfully declined the nuclear factor kappa B and cyclooxygenase-2 concentrations in colon tissues, and suppressed the colonic expression of interleukin (IL)-23 and IL-17 A.

Conclusion Escin may offer a novel therapeutic strategy for treating UC conditions *via* suppressing the inflammatory process and inhibiting oxidative stress, as shown here in the experimental UC-mice.

Keywords Escin, NF- κ B, Oxidative stress, Proinflammatory cytokines, Ulcerative colitis

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Background

Ulcerative colitis (UC), a type of inflammatory bowel disease (IBD), is an immune-mediated disorder marked by persistent inflammation of the colonic mucosal layer with alternating periods of relapse and remission (Radziszewska et al., 2022). UC is distinguished by continuous inflammation that affects the mucosal and submucosal layers and is limited to the rectum and colon, whereas Crohn's disease is distinguished by patchy inflammation that can extend through all layers of the digestive tract and can affect any part of it (Neurath, 2019). IBD is a global disease; it is expected that the number of IBD patients will surge in North America from 2 million to 4 million by 2030. In 2018, IBD incurred significant Canadian and American healthcare expenses, with annual expenditures of 7.2 billion American dollars in the USA and 2.57 billion Canadian dollars in Canada (Peery et al., 2019). UC is most common among people aged 30 to 40; however, it can occur at any age. The global prevalence of UC is about 120 per 100,000 persons, while the global incidence is approximately 5 per 100,000 persons/year (Heydari et al., 2025). The World Health Organization (WHO) has listed UC as an intractable disease (Liu et al., 2022).

While multiple models, such as dextran sulfate sodium, 2,4,6-trinitrobenzene sulfonic acid, oxazolone, carrageenan, and peptidoglycan polysaccharide, were used to study UC, the acetic acid (AA)-induced colitis model remains a well-established and widely used experimental approach (Randhawa et al., 2014). This model exhibits histopathological features and an inflammatory profile similar to human UC. It is characterized by the simplicity of induction, a short experimental timeline, and low operational cost. In addition, it effectively induces colitis with key pathological features, including epithelial damage, mucosal inflammation, and ulceration, which are relevant for evaluating potential therapeutic interventions (Tie et al., 2024; Roychowdhury et al., 2025). Given these considerations, this model provides a valuable and practical approach to investigating potential treatments for colitis.

The exact reason for UC is still ambiguous; however, it is suggested to involve environmental circumstances, genetic vulnerability, psychological health, and other factors. In addition, it is considered that oxidant/antioxidant system disturbance and immune system dysfunction are involved in the onset of UC (Wan et al., 2022). Dysregulation of nuclear factor kappa B (NF- κ B), the primary controller of gene transcription that modulates the equilibrium between proinflammatory and anti-inflammatory cytokines, is related to the onset and progression of UC (Atreya et al., 2008). NF- κ B activation stimulates the generation of inflammatory cytokines, including interleukin (IL)-23, IL-17 A, and cyclooxygenase (COX)-2 (McDaniel

et al., 2016; Kałużna et al., 2022). IL-23 engages with both the innate and adaptive immune systems and seems to have an impact on the progression of chronic colonic inflammation (Korta et al., 2023). It is believed that IL-17 A, a cytokine with potent proinflammatory activities, contributes to the progression of UC (Park et al., 2020). The inflammatory enzyme COX-2 is implicated in producing prostaglandins, which aggravates colonic inflammation. Also, oxidative stress is considered as a major factor involved in the occurrence of UC (Sheibanie et al., 2007). An Imbalance between reactive oxygen species (ROS) and the antioxidant system causes oxidative stress; accumulated ROS like superoxide anion free radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO $\cdot$), which stimulates DNA damage, membrane lipid peroxidation, and protein dysfunction, causes cell death (Wang et al., 2016).

Many different medications are currently approved to treat UC, including 5-aminosalicylates, glucocorticoids, thiopurines, antitumor necrosis factor antagonists, immunosuppressors, and biological agents. Medications for UC are selected based on several factors such as the severity of symptoms and the degree of inflammation (Li et al. 2022a; Jarmakiewicz-Czaja et al. 2023). These traditional medications have many side effects, may not be able to cure UC completely, become less effective over time, and some of them are very expensive. Therefore, it is urgent to develop new therapies for UC that are more affordable and have fewer adverse impacts than conventional medications; the natural products are promising candidates, as they also have anti-inflammatory and antioxidant properties (Cao et al. 2019; Li et al. 2022a). Escin is a triterpenoid saponin natural product obtained from the horse chestnut (*Aesculus hippocastanum*) tree. It has anti-inflammatory, antioxidant, and antiedematous effects. It has been proven that escin was used as a treatment for hemorrhoids, chronic venous insufficiency, and edema (Gallelli, 2019). Therefore, the current work was established to evaluate the anti-inflammatory and antioxidant impacts of different doses of escin (20 and 40 mg/kg body weight "b.w"), compared to a customary drug sulfasalazine (SSZ), *versus* AA-induced UC in mice.

Materials and methods

Chemicals and dyes

AA was offered by El-Nasr Pharmaceutical Chemical Company (Cairo, Egypt). A white powder escin (Cat No. OA07948) with a purity of $\geq 98\%$, which was dissolved in 0.9% saline, was characterized and provided by Biosynth Ltd (Compton, Berkshire, UK). A customary drug SSZ was provided by ACDIMA International Trading Company (Cairo, Egypt) and dissolved in 0.9% saline to be intrarectal (I.R.) injected. Formamide was provided by Nice Chemicals Pvt. Ltd. (Kerala, India). Isoflurane

was provided by Sedico Pharmaceutical (Giza, Egypt). Toluidine blue (Cat No. 89640), gentian violet (Cat No. 48770), and Evans blue (Cat No. E2129) dyes were provided by Sigma-Aldrich Company (St Louis, MO, USA). The other used reagents were of high purity.

Animals

Seventy-five male Swiss albino mice (*Mus musculus*), CD 1 strain and aged between 8 and 10 weeks with b.w ranging from 25 to 30 g, were delivered from Theodor Bilharz Research Institute in Giza, Egypt. Mice were placed in appropriate polypropylene cages with regular availability to water and standard rodent diet (Cairo Feed Company, Giza, Egypt), which consists of maize, bean-hay, sunflower-hay, methionine, and salt, along with a mix of minerals and vitamins that meet international standards, during the experiment period, while they were subjected to 24 h/s (h) fast prior to UC induction. Mice were maintained in typical laboratory circumstances at 26 °C, 57% humidity, and a 12-hour light/dark cycle. Mice were adapted to the laboratory surroundings for two weeks.

AA-induced UC in mice

Mice were lightly anesthetized with isoflurane after 24 h fasting. Then, mice were I.R. injected with diluted AA (200 μ L, 6% volume/volume with 0.9% saline) using

a polyurethane cannula that was inserted 4 cm into the mice's anus (Oliveira et al., 2019). For two minutes, the mice were held in a head-down position to inhibit AA leakage. Applying the exact same procedure, mice of the control group were only administered 200 μ L saline (Oliveira et al., 2019).

Study plan and schedule

Randomly, five groups of mice (each containing 15 mice in order to measure the mortality rate, alongside excluding 5 mice out of the 15 to evaluate colonic vascular permeability) were designated following the acclimation period (Fig. 1): (1) the control group were I.R. injected with 200 μ L of 0.9% saline at 0 h, 1 h, 6 h, and 12 h; (2) the UC group were I.R. injected with 200 μ L of AA (6% volume/volume with 0.9% saline) at zero time, and then they were I.R. injected with 200 μ L of 0.9% saline after 1 h, 6 h, and 12 h; (3) the AA + SSZ group were I.R. injected with 200 μ L of AA (6% volume/volume with 0.9% saline) at zero time, and then they were I.R. injected with 200 μ L of SSZ (500 mg/kg b.w) after 1 h, 6 h, and 12 h; (4) the AA + escin 20 group were I.R. injected with 200 μ L of AA (6% volume/volume with 0.9% saline) at zero time, and then they were I.R. injected with 200 μ L of escin (20 mg/kg b.w) after 1 h, 6 h, and 12 h; (5) the AA + escin 40 group were I.R. injected with 200 μ L of AA (6% volume/

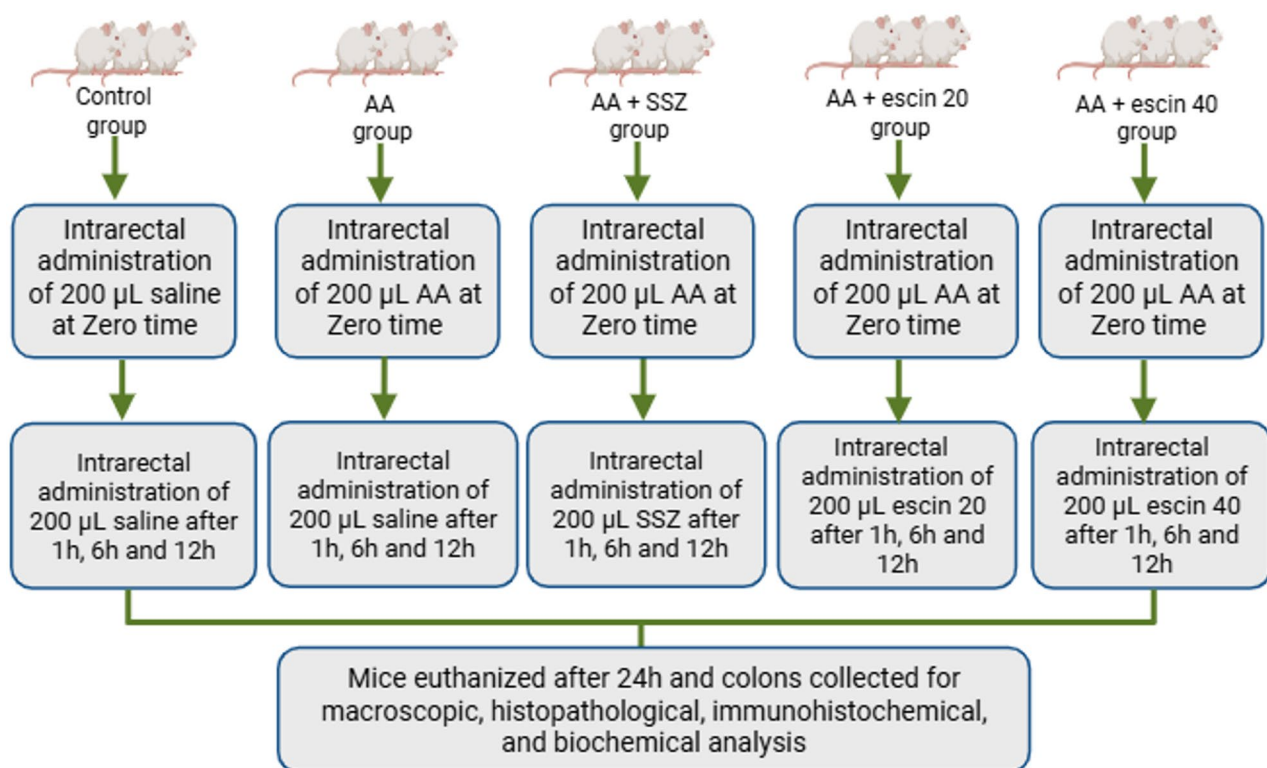


Fig. 1 Diagrammatic representation of the experimental design. AA acetic acid (6% volume/volume with 0.9% saline), escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, dissolved in 0.9% saline, h hour/s, SSZ sulfasalazine (500 mg/kg body weight, dissolved in 0.9% saline)

volume with 0.9% saline) at zero time, and then they were I.R. injected with 200 μ L of escin (40 mg/kg b.w) after 1 h, 6 h, and 12 h. The dosages utilized in the study were determined based on our preliminary studies on escin and previously published studies conducted on escin or SSZ (Li et al., 2015; Chen et al., 2021; Zhang et al., 2022).

Preparation of colon tissue homogenate extraction

Following anesthesia with isoflurane inhalation, the animals' cervical dislocation was humanely carried out at the final stage of the study. Then, the colon was collected, washed with saline, weighed, and grossly examined. Exactly 0.2 g of colon (distal part) was thoroughly homogenized in 2 mL phosphate buffer saline (PBS) for biochemical analysis.

Assessment of b.w change, mortality rate, spleen weight, and weight-to-length ratio of the colon

The b.w and spleen weight, as well as the colon weight and length, were measured at the final stage of the study using a digital precise balance and measuring ruler. The b.w change, mortality rate, and colon weight/length ratio were calculated as follows:

- The b.w change (%) = [(b.w at the final day of the study – b.w at the first day of the study) / b.w at the first day of the study] $\times$ 100.
- The mortality rate (%) = [(mice number at the start of the study – mice number at the termination of the study) / mice number at the start of the study] $\times$ 100.
- Colon weight/length ratio = weight of colon (g) / length of colon (cm).

Macroscopic examination of colon injury

Briefly, the macroscopic lesions of mice colons were assessed based on Mei et al. (2005) scale ranging from 0 to 4: 0=no mucosal damage; 1=erythematous mucosa; 2=mild swelling, and slight erosion or bleeding in the mucosa; 3=moderate mucosal swelling, and minor hemorrhagic ulcer or abrasions; 4=severe colonic ulcers, edema, and necrosis.

Determination of antioxidants and oxidative biomarkers

Colon tissue homogenates were used to determine reduced glutathione (GSH) levels, and the activities of superoxide dismutase (SOD) and catalase (CAT), along with concentrations of malondialdehyde (MDA) and nitric oxide (NOx), using kits provided by Biodiagnostic (Cairo, Egypt), according to guidelines provided by the manufacturers.

Assessment of NF- κ B and COX-2 concentrations

Mouse NF- κ B and COX-2 ELISA kits (Cat. No. CSB-E12108m and CSB-E12910m, respectively) were provided

by CUSABIO (Houston, TX, USA) to determine colonic NF- κ B and COX-2 concentrations, according to the manufacturer's recommendations.

Histological assessment of colonic lesions

Samples of colon mice were embedded in 10% neutral-buffered formalin, dehydrated using a sequence of increasing alcohol concentrations, cleared in terpineol, and submerged in paraffin. The paraffin blocks were sliced into 5 μ m sections and stained with hematoxylin and eosin (Waheed et al., 2021). Finally, these sections were investigated to evaluate the fundamental structural changes in the tissue. According to Khan et al. (2005), the scores of crypt destruction, edema, severity of ulceration, and infiltration of inflammatory cells were recorded on a scale (0–4): 0 = no lesion; 1 = weak lesion; 2 = mild lesion; 3 = moderate lesion; 4 = severe lesion.

Immunohistochemical evaluation of IL-17 A and IL-23 expression in colon tissue

Avidin-biotin-peroxidase complex (ABC) method was applied to assess mouse colonic IL-17 A and IL-23. The following kit and antibodies were used: Vectastain ABC-HRP kit (code: PK-4001; Vector Laboratories, Oxfordshire, UK), IL-17 A (code: A12454; ABclonal, Woburn, MA, USA), and IL-23 (code: orb10907; Biorbyt, Durham, NC, USA). The staining intensity was assessed using ImageJ software (NIH, USA).

Assessment of degranulated mast cells (MCs) in colon tissues

After mounting tissue sections onto the slides, deparaffinization and washing with distilled water were performed. For thirty minutes at ambient temperature, the tissue sections were then submerged in a solution of 0.5 N HCL and 0.5% powdered toluidine blue at pH 0.5. Following a distilled water rinse, tissue sections were left to dry before being coverslipped. Eventually, a Leica light microscope was used to examine the specimens (Karaca et al., 2010).

Assessment of colonic vascular permeability

Animals received an intravenous injection of 200 μ L Evans blue (2%, diluted in saline) precisely 24 h after UC induction. The animals were euthanized after 30 min, and the colon was removed from the body, washed, and allowed to dry. After that, the colon samples were immersed in 700 μ L formamide and left to incubate for the entire night at 60 $^{\circ}$ C. After collecting formamide extract, the concentration of Evans blue was measured using spectrophotometry at 630 nm (Tolstanova et al., 2009).

Assessment of peritoneal leukocyte count

The peritoneal cavity was injected with 2 mL of extremely cold PBS, followed by an abdominal massage for 30 s. Exactly 1.5 mL of peritoneal lavage fluid was withdrawn. The lavage fluid was centrifuged, the supernatant was removed, and the remaining solid pellet was mixed with 1.0 mL ice-cold saline. The Neubauer chamber and gentian violet staining were used to count the leukocytes as follows:

Exactly, 20 μ L from cells suspended in 1.0 mL ice-cold saline + 180 μ L of gentian violet solution.

Leukocytes count (cells/mm³) = [A $\times$ (dilution factor = 20)] / (volume of 4 chambers = 0.4).

Where "A" is the cell count in 4 large corner squares.

Statistical analysis

Data analysis was carried out utilizing Graph Pad Prism (GraphPad, San Diego, CA, USA). Kolmogorov-Smirnov test was utilized to assess data's normality. The data were expressed as means $\pm$ standard errors of mean. For parametric data, one-way analysis of variance (ANOVA) was followed by Tukey's multiple comparison test to identify group differences. For data not following a parametric distribution, Kruskal-Wallis and Dunn's multiple comparison tests were used. *P*-values < 0.05 were deemed statistically significant.

Results

Impact of escin on b.w change, spleen weight, and mortality rate of UC-mice

Figure 2 demonstrated that mice given AA injections had a substantial reduction (*P* < 0.001) in b.w opposed to the control mice. However, mice given SSZ after receiving an AA injection had a substantial rise (*P* < 0.001) in b.w opposed to the AA-treated mice. In addition, in comparison with the AA group, mice given varying dosages of escin (20 and 40 mg/kg b.w) following AA injection exhibited a substantial increase (*P* < 0.001) in b.w depending on the dosage. Furthermore, the high dose of escin showed a powerful effect (*P* < 0.001) in increasing b.w in mice administered with AA more than SSZ.

Mice given AA had a substantial rise (*P* < 0.001) in spleen weight opposed to the control mice. However, in comparison with the AA results, animals treated with SSZ after being administered AA had a significant reduction (*P* < 0.001) in spleen weight. By comparing with the AA group, mice treated with varying dosages of escin (20 and 40 mg/kg b.w) after being administered AA exhibited a significant reduction (*P* < 0.01–0.001) in spleen weight in a dose-dependent manner (Fig. 2). Additionally, the administration of AA resulted in a 40% mortality rate. Conversely, the high dosage of escin (40 mg/kg b.w.) therapy highly decreased the mortality and was sufficient to prevent 90% of mice from dying, outperforming the low

doses of escin (20 mg/kg b.w.) and SSZ, which only prevented 80% of mice from dying (Fig. 2).

Impact of escin on the colon weight-to-length ratio and macroscopic lesions of colon in UC-mice

According to the findings in Fig. 3, animals exposed to AA had a considerable increase (*P* < 0.001) in colon weight-to-length ratio than the control mice. Conversely, there was a substantial reduction (*P* < 0.001) in the colon weight-to-length ratio by comparing the SSZ-treated mice with the AA-treated mice. Additionally, animals given varying doses of escin (20 and 40 mg/kg b.w) following AA exposure exhibited a considerable reduction (*P* < 0.001) in the colon weight-to-length ratio, as compared with the AA group. In addition, macroscopic examination of the colons demonstrated that mice given AA showed major morphological abnormalities (*P* < 0.001) as indicated by severe necrosis, erythema, and thickness in contrast to the control group, which showed a normal colonic appearance (Fig. 3). However, in comparison with the AA results, only animals given the high dosage (40 mg/kg b.w) of escin following AA exposure demonstrated a substantial colonic lesion recovery (*P* < 0.05).

Escin mitigated oxidative stress in colon tissues of UC-mice

The findings shown in Table 1 demonstrated that, in comparison with the control mice, the animals administered AA exhibited substantial reductions (*P* < 0.001) in GSH concentrations, along with the activities of SOD and CAT, whereas they exhibited substantial increases (*P* < 0.001) in MDA and NOx concentrations in colon tissues. Conversely, as opposed to the AA-treated mice, the mice administered with SSZ after receiving AA exhibited significant elevations in GSH concentrations, along with the activities of SOD and CAT, in addition to notable reductions in MDA and NOx levels in colon tissues (*P* < 0.05–0.001). Additionally, by comparing with the AA group, mice given varying doses of escin (20 and 40 mg/kg b.w) exhibited substantial increases in GSH concentrations, along with the activities SOD and CAT (in a dose-dependent manner), in addition to marked reductions in MDA and NOx levels (*P* < 0.05–0.001). Moreover, the low dose of escin exhibited greater capacity (*P* < 0.05) in reducing colonic NOx in mice administered with AA compared with SSZ (Table 1).

Impact of escin on NF- κ B and COX-2 levels in colon tissues of UC-mice

Table 2 showed that, in contrast to the control mice, a considerable rise (*P* < 0.001) in the colonic levels of NF- κ B and COX-2 was seen after AA injection. Conversely, as compared with the AA group, SSZ therapy after AA instillation reduced substantially (*P* < 0.05–0.001) colonic

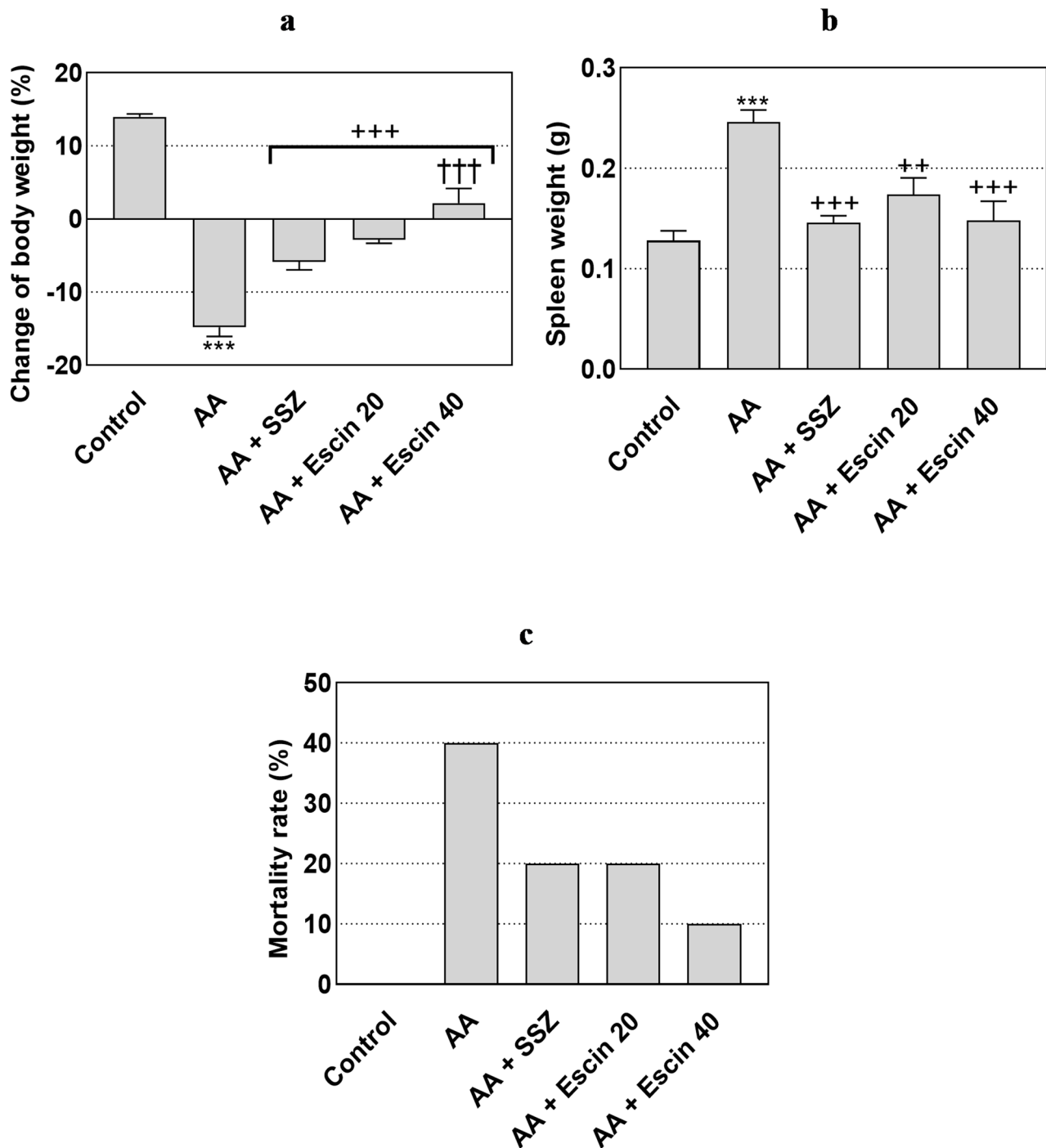


Fig. 2 Impact of escin on b.w change **a**, spleen weight **b**, and mortality rate **c** in UC-mice. Data in **a** and **b** were presented as means $\pm$ their standard errors ($n=5$). AA acetic acid, b.w body weight, escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, SSZ sulfasalazine, UC ulcerative colitis. ***: $P < 0.001$ against the control mice; †† and †††: $P < 0.01$ and $P < 0.001$, respectively, against the AA-treated mice; †††: $P < 0.001$ against AA + SSZ-treated mice

levels of NF- κ B and COX-2. Moreover, in comparison with the AA results, animals given varying doses of escin (20 and 40 mg/kg b.w) following AA instillation showed a substantial drop ($P < 0.001$) in NF- κ B and COX-2 concentrations in colon tissues. Additionally, by comparing with the SSZ group, both doses of escin showed a greater

capacity ($P < 0.01 - 0.001$) in reducing colonic COX-2 concentration in mice injected with AA. Only the high dose of escin outperformed ($P < 0.001$) SSZ in diminishing colonic NF- κ B in mice injected with AA.

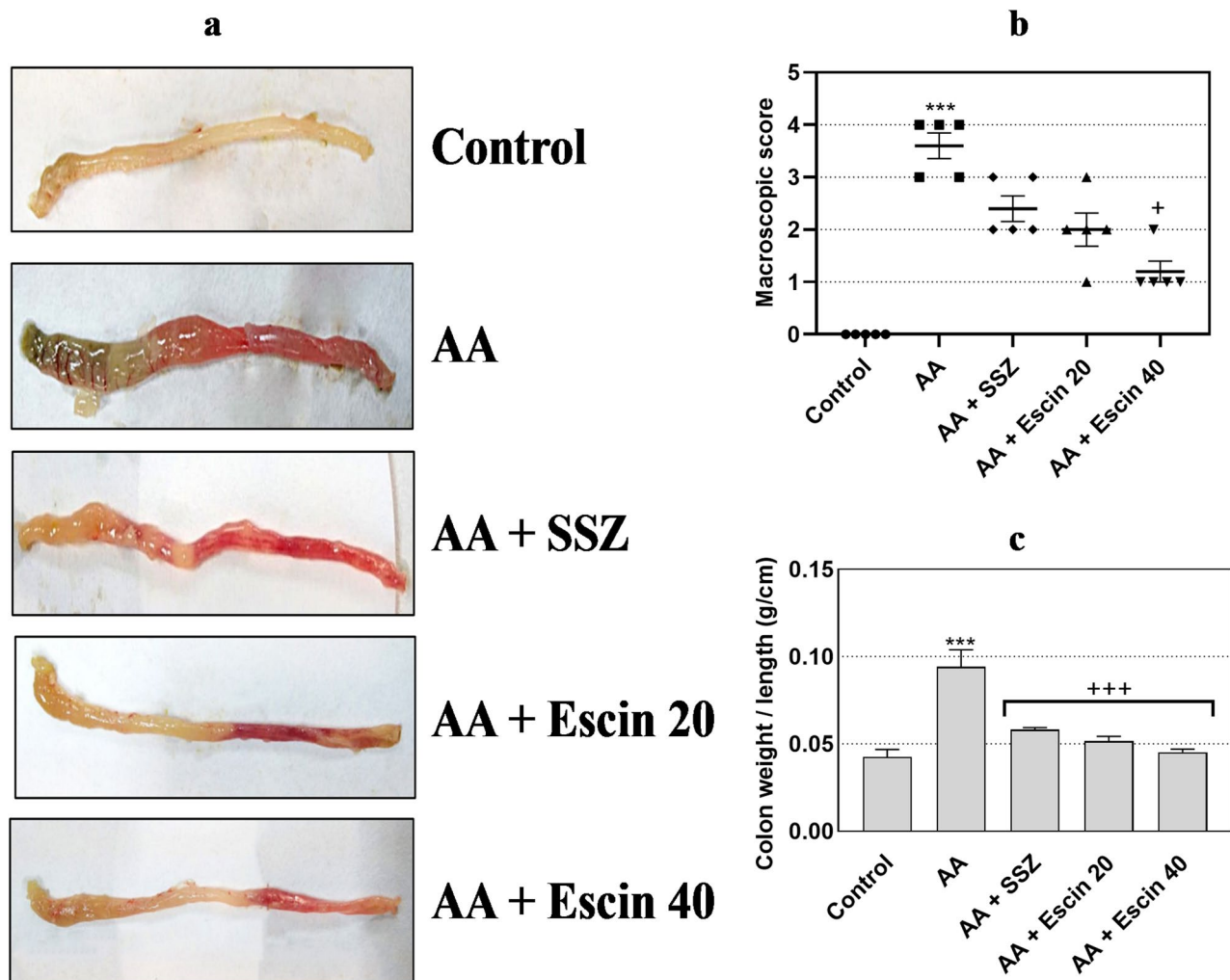


Fig. 3 Impact of esцин on macroscopic lesions of the colon and colon weight-to-length ratio in UC-mice. **a** Macroscopic images of mice colons; **b** macroscopic assessment score; **c** colon weight-to-length ratio. Data were presented as mean $\pm$ their standard errors ($n=5$). AA acetic acid, esцин 20 and 40 20 and 40 mg of esцин/kg body weight, respectively, SSZ sulfasalazine, UC ulcerative colitis. ***: $P < 0.001$ against the control mice; + and +**: $P < 0.05$ and $P < 0.001$, respectively, against the AA-treated mice

Table 1 Impact of esцин on GSH, SOD, CAT, MDA, and NOx in colon of UC-mice

Control	GSH (mmol/g tissue)	SOD (U/g tissue)	CAT (U/g tissue)	MDA (nmol/g tissue)	NOx (μ mol/g tissue)
	0.86 $\pm$ 0.04	1294 $\pm$ 84.48	4.23 $\pm$ 0.14	7.54 $\pm$ 0.33	1.45 $\pm$ 0.35
AA	0.20 $\pm$ 0.08***	363.8 $\pm$ 37.43***	1.07 $\pm$ 0.22***	34.59 $\pm$ 1.03***	5.04 $\pm$ 0.14***
AA + SSZ	0.50 $\pm$ 0.03 ⁺	645 $\pm$ 5.47 ⁺	2.04 $\pm$ 0.08 ⁺⁺	9.52 $\pm$ 0.63***	3.44 $\pm$ 0.27 ⁺⁺
AA + Escin 20	0.71 $\pm$ 0.08***	703.1 $\pm$ 111.1 ⁺	1.90 $\pm$ 0.14 ⁺	11.54 $\pm$ 0.98***	2.14 $\pm$ 0.19*** [†]
AA + Escin 40	0.73 $\pm$ 0.06***	753.8 $\pm$ 31.32***	2.35 $\pm$ 0.21***	11.47 $\pm$ 0.69***	2.75 $\pm$ 0.22***

AA acetic acid, CAT catalase, esцин 20 and 40 20 and 40 mg of esцин/kg body weight, respectively, GSH reduced glutathione, MDA malondialdehyde, NOx nitric oxide, SOD superoxide dismutase, SSZ sulfasalazine, UC ulcerative colitis

Data were presented as means $\pm$ their standard errors ($n=5$). ***: $P < 0.001$ against the control mice; +, ++ and +**: $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, against the AA-treated mice; [†]: $P < 0.05$ versus AA + SSZ-treated mice

Impact of esцин on histological changes in colon tissues of UC-mice

Fig. 4a demonstrated that the colons of the control group revealed a normal mucosal layer with normal crypts and goblet cells. However, in contrast to the control mice,

the UC-mice exhibited notable histological changes, signified by ulcers, crypts atrophy, edema, and a considerable inflammatory cell influx (Fig. 4b). On the flip side, as opposed to the AA-treated mice, mice administered with SSZ following AA administration showed a notable

Table 2 Impact of escin on NF- κ B, COX-2, IL-17 A, and IL-23 levels in colon of UC-mice

Control	NF- κ B (pg/mg tissue)	COX-2 (ng/mg tissue)	IL-17 A expression	IL-23 expression
	242.6 $\pm$ 25.10	3.04 $\pm$ 0.39	17.28 $\pm$ 0.31	14.49 $\pm$ 0.73
AA	815.7 $\pm$ 28.61 ^{***}	10.43 $\pm$ 0.34 ^{***}	38.13 $\pm$ 0.87 ^{***}	33.39 $\pm$ 0.80 ^{***}
AA + SSZ	637.3 $\pm$ 15.47 ⁺⁺⁺	9.02 $\pm$ 0.22 ⁺	31.76 $\pm$ 0.80 ⁺⁺⁺	26.29 $\pm$ 0.88 ⁺⁺⁺
AA + Escin 20	554.8 $\pm$ 17.24 ⁺⁺⁺	7.14 $\pm$ 0.24 ⁺⁺⁺ , ^{††}	25.49 $\pm$ 1.26 ⁺⁺⁺ , ^{†††}	24.66 $\pm$ 0.75 ⁺⁺⁺
AA + Escin 40	462.2 $\pm$ 11.81 ⁺⁺⁺ , ^{†††}	6.54 $\pm$ 0.23 ⁺⁺⁺ , ^{†††}	22.71 $\pm$ 0.47 ⁺⁺⁺ , ^{†††}	22.27 $\pm$ 0.44 ⁺⁺⁺ , ^{††}

AA acetic acid, COX-2 cyclooxygenase 2, escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, IL interleukin, NF- κ B nuclear factor kappa B, SSZ sulfasalazine, UC ulcerative colitis

Data were expressed as means $\pm$ their standard errors ($n=5$). ^{***}: $P < 0.001$ against control mice; ⁺ and ⁺⁺⁺: $P < 0.05$ and $P < 0.001$, respectively, against the AA-treated mice. ^{††} and ^{†††}: $P < 0.01$ and $P < 0.001$, respectively, against AA + SSZ-treated mice

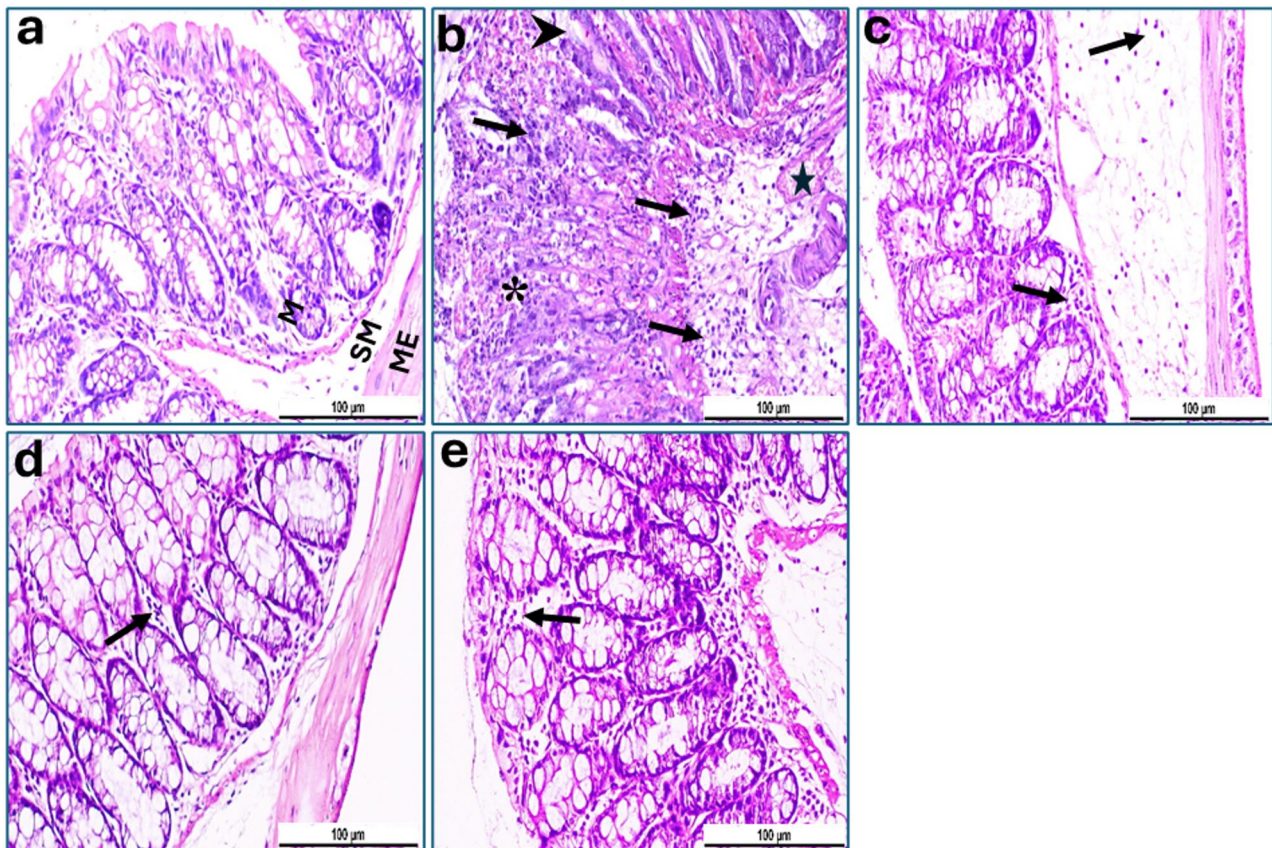


Fig. 4 Impact of escin on histological alterations in colon tissues of UC-mice. **a** The control group exhibited a normal colon tissue structure showing intact muscularis externa (ME), submucosa (SM), and mucosa (M). **b** The AA group exhibited substantial colonic lesions signified by ulceration (arrowhead), edema (star), significant inflammatory cell infiltration (arrows), and crypt atrophy (asterisk). **c** The AA + SSZ group showed a marked reduction in mucosal inflammatory signs with mild inflammatory cell infiltration (arrows). **d** The AA + escin 20 group showed a considerable recovery of colon sores with a low inflammatory cell infiltration. **e** The AA + escin 40 group showed a high degree of similarity with the control mice with a considerable decline in inflammatory cell infiltration. Hematoxylin and eosin stain; magnification power: 200 $\times$; bar size: 100 μ m. AA acetic acid, escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, SSZ sulfasalazine, UC ulcerative colitis

reduction in crypts atrophy, edema, and inflammatory cell infiltration (Fig. 4c). In addition, Fig. 4d showed that the injection of escin (20 mg/kg b.w.) following AA exposure enhanced considerably sore recovery, whereas a mild influx of inflammatory cells persisted. Additionally, Fig. 4e demonstrated that the injection of escin (40 mg/kg b.w.) after AA exposure healed substantially the majority of the colon damage; however, a lower influx

of inflammatory cells was observed. The data presented in Fig. 5 demonstrated a substantial increase ($P < 0.001$) in inflammatory cell infiltration (Fig. 5a), edema (Fig. 5b), crypt atrophy (Fig. 5c), and ulcer formation (Fig. 5d) in mice injected with AA, as opposed to the control mice. On the contrary, mice administered with SSZ or escin 20 following AA administration showed a reduction, to an extent, in all previous inflammatory signs compared

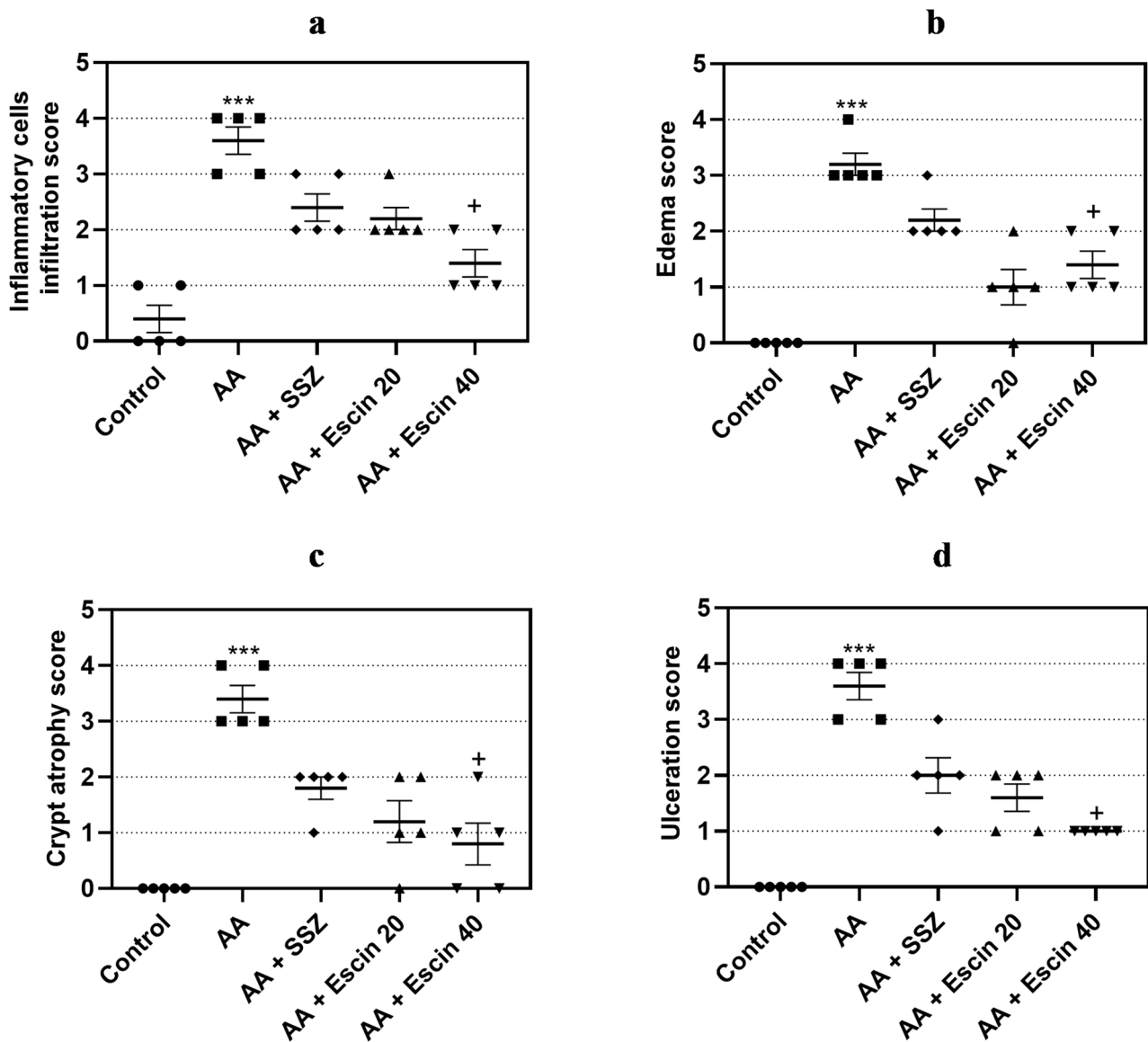


Fig. 5 Histopathological assessment of **a** inflammatory cells infiltration; **b** edema; **c** crypt atrophy; **d** ulceration in colon of UC-mice. Data were expressed as means $\pm$ their standard errors ($n=5$). AA acetic acid, escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, SSZ sulfasalazine, UC ulcerative colitis. ***: $P < 0.001$ against the control mice; +: $P < 0.05$ against the AA-treated mice

with the AA-treated mice. Moreover, as opposed to the AA-treated mice, only UC-mice treated with escin 40 following AA instillation exhibited a considerable decline ($P < 0.05$) in colonic inflammatory cell infiltration, edema, crypt atrophy, and ulcer formation (Fig. 5).

Impact of escin on IL-17 A and IL-23 expression in colon tissues of UC-mice

According to the data presented in Table 2, animals given the AA had significant increases ($P < 0.001$) in colonic IL-17 A and IL-23 expression, as evidenced by extremely strong immunostaining of colon tissues (Supplementary Figs. 1b and 2b, respectively) compared with extremely faint immunostaining in the control group

(Supplementary Figs. 1a and 2a). In contrast, comparing the SSZ group with the AA group, there was a considerable reduction ($P < 0.001$) in colonic IL-17 A and IL-23 expression, as signified by moderate to strong immunostaining of colon tissues (Supplementary Figs. 1c and 2c). In addition, in comparison with the AA results, administration of varying doses of escin (20 and 40 mg/kg b.w) following AA exposure decreased significantly ($P < 0.001$) IL-17 A and IL-23 expression in colon tissues based on the dose, as evidenced by weak to mild immunostaining of colon tissues (Supplementary Figs. 1d, e and 2d, e). Moreover, by comparing with the SSZ group, both doses of escin demonstrated a higher ability ($P < 0.001$) in reducing colonic IL-17 A expression, while only the

high dose of escin showed a higher capacity ($P<0.01$) in reducing IL-23 expression in the colons of animals given AA.

Escin suppressed MCs degranulation in colon tissues of UC-mice

As demonstrated in Table 3, AA induced a substantial elevation ($P<0.001$) in degranulated MCs, which were stained pale blue as they released their granules (Supplementary Fig. 3b), compared with the control mice that displayed nearly no degranulated MCs (Supplementary Fig. 3a). However, animals treated with SSZ after AA injection showed a substantial drop ($P<0.001$) in colonic degranulated MCs, as seen by a small number of cells-stained pale blue (Supplementary Fig. 3c), compared with the AA group. Furthermore, escin (20 and 40 mg/kg b.w) treatment diminished considerably ($P<0.05-0.001$) degranulated MCs in colon tissues of the UC group based on the dosage, as evidenced by a few to moderate numbers of cells-stained pale blue (Supplementary Fig. 3d, e), as compared with the AA group.

Escin modulated vascular permeability of the colon and leukocytes in the peritoneal cavity of UC-mice

According to the findings shown in Table 3, mice exposed to AA had a considerable increase ($P<0.001$) in colonic vascular permeability and peritoneal leukocytes opposed to the control mice. On the contrary, animals treated with SSZ after being exposed to AA showed a substantial reduction ($P<0.001$) in colonic vascular permeability and peritoneal leukocytes compared with the UC group. Additionally, in comparison with the AA group, mice that received varying doses of escin (20 and 40 mg/kg b.w) after being exposed to AA showed a noticeable reduction ($P<0.001$) in colonic vascular permeability and peritoneal leukocytes, depending on the dose. Both doses

of escin outperformed ($P<0.05-0.001$) SSZ in reducing colonic vascular permeability and peritoneal leukocytes in mice exposed to AA.

Discussion

UC, a chronic, complicated, and persistent inflammatory disorder, has grown to be a major worldwide public health concern and is related to significant financial strain. Severe disability and substantial mortality are common among UC-patients (Zhang et al., 2024). Although the exact pathophysiology of UC is still unknown, it has been found that oxidative strain, inflammation, genetics, as well as intestinal microbiota are the primary causes of UC (Peng et al., 2023; Liu et al., 2024). Searching for substitutes that mainly offer significant recovery and minimize the recurring relapse phases that characterize UC has become crucial due to the numerous side effects and potential drug interactions of the available chemical medications used to manage UC (Liang et al., 2024). One of the natural medicinal substances is escin, which is obtained from a plant source and has anti-inflammatory, antioxidant, and antiallergic properties (Wang et al., 2009; Lindner et al., 2010; Yang et al., 2020). It has been found that escin has limited oral bioavailability (often $<2-12.5\%$) owing to inefficient absorption and significant first-pass metabolism in the gastrointestinal tract and liver. Escin specifically targets inflamed areas by reducing vascular permeability and edema, demonstrating localized anti-inflammatory effects rather than widespread systemic distribution (Lang and Menicke, 1972; Lang, 1974; Wu et al., 2011). Moreover, it has been proven that escin at a dose of 2000 mg/kg b.w was fatal to animals, whereas at a dose up to 300 mg/kg b.w there were no symptoms of toxicity. These findings declared that escin is generally safe and almost has no side effects (Suryavanshi & Kulkarni, 2021). The current work explored escin's ability to treat AA-induced UC. A well-established model of AA-induced UC exhibited symptoms that resemble those of UC in people, including bloody diarrhea, colonic ulcers, shortening and thickening of the colon, and weight loss (Shahid et al., 2022).

The current work indicated that the AA administration decreased considerably b.w and increased significantly the colon weight-to-length ratio, spleen weight, mortality rate, and colonic macroscopic lesions and histological alterations showing severe ulcers, hemorrhage, crypt abscesses, epithelial erosion, and inflammatory cell infiltration. These findings align with those of earlier research indicating that AA injection induced UC, accompanied by the previously mentioned symptoms (Niu et al., 2013; Gupta et al., 2018). Surprisingly, mice treated with escin following AA demonstrated a considerable rise in b.w and a substantial decline in the colon weight-to-length ratio, spleen weight, mortality rate, and colonic macroscopic

Table 3 Impact of escin on colonic vascular permeability and degranulated MCs, and peritoneal leukocytes number in UC-mice

Control	Colonic vascular permeability	Colonic degranulated MCs (%)	Peritoneal leukocytes (cells/mm ³)
	0.34 ± 0.03	2.80 ± 0.37	2900 ± 139.6
AA	2.87 ± 0.15***	27.20 ± 2.13***	7470 ± 145.4***
AA + SSZ	2.01 ± 0.09***	14.80 ± 1.32***	4340 ± 135.5***
AA + Escin 20	1.46 ± 0.14***, †	20 ± 1.27 [†]	3440 ± 226.6***, ††
AA + Escin 40	1.34 ± 0.15***, ††	15.4 ± 1.57***	3110 ± 128.8***, †††

AA acetic acid, escin 20 and 40 20 and 40 mg of escin/kg body weight, respectively, MCs mast cells, SSZ sulfasalazine, UC ulcerative colitis

Data were expressed as means ± their standard errors (n=5). ***: $P<0.001$ against control mice; + and ***: $P<0.05$ and $P<0.001$, respectively, against the AA-treated mice. †, ††, and †††: $P<0.05$, $P<0.01$, and $P<0.001$, respectively, against AA + SSZ-treated mice

lesions and histological alterations. These impacts could be attributed to the antioxidant and anti-inflammatory activities of escin, which were confirmed by a notable elevation in GSH, SOD, and CAT, as well as a marked reduction in MDA, NO_x, NF- κ B, COX-2, IL-17 A, and IL-23 in colon tissues of escin-treated UC-mice.

The major triggers of UC are oxidative and nitrative stress, in addition to an impaired free radical elimination system, which regulates the production of ROS (Nakajima et al., 2024). Antioxidants such as GSH, SOD, and CAT are depleted as a result of the elevation of ROS and reactive nitrogen species (RNS), which stimulate reactive metabolite production and the lipid peroxidation process that increases MDA levels. In our investigation, AA administration induced a substantial decrease in GSH content and the activity of SOD and CAT, as well as a marked increase in MDA and NO_x in colon tissues. These findings are along with the preceding study that showed how AA elevated ROS generation and disrupted antioxidant-oxidant balance, inducing UC (Sepehrmanesh et al., 2018). Strikingly, when the UC group is treated with escin, oxidative markers MDA and NO_x are significantly decreased in colon tissues, whereas antioxidants GSH, SOD, and CAT are considerably increased in colon tissues, which play a crucial role in colon recovery. Escin displayed a potent antioxidant effect in a variety of disease models other than UC, this impact was ascribed to its capacity to donate hydrogen and chelate ions owing to the presence of hydroxyl groups (Vašková et al., 2015). Also, Suryavanshi and Kulkarni (2020) indicated that escin improves antioxidant capacity in diabetic rats by raising the concentration of GSH and the activity of SOD and CAT. In addition, Wang et al. (2014) proved that escin has great efficacy in the amendment of oxidative stress by diminishing MDA levels in mice orally administered with indomethacin. Xin et al. (2011) indicated that the deleterious effects of lipopolysaccharide, which are related to NO_x generation, could be mitigated by escin through its ability to eliminate RNS including NO_x. Moreover, previous in vitro studies indicated that escin has the ability to reduce oxidative stress by increasing the SOD, while decreasing NO_x production (Maghsoudi et al., 2018; Qiao et al., 2022).

The immune system imbalance triggers the development of UC and a relapse of colonic inflammation (Uthaman et al., 2024). NF- κ B is a key modulator of the gene expression of several inflammatory mediators associated with the onset of colitis (Wang et al., 2017). NF- κ B is stuck to the inhibitor of NF- κ B (I κ B) protein that keeps it inactive, which must be degraded by phosphorylation to activate NF- κ B. Moreover, its activation depends on various stimuli such as ROS, cytokines, and other signaling pathways (Schreck et al., 1991). The induction of *NF- κ B gene* promotes the generation of cytokines like IL-23,

IL-17 A, and COX-2, stimulating tissue inflammation and damage (McDaniel et al., 2016). It is well-established that elevated NF- κ B expression is associated with serious harm to colon tissue (Li et al. 2022b). The current work showed that AA induced NF- κ B elevation in the colon tissues, which is consistent with Tunc et al. (2022) findings. In the current work, the escin-treatment suppressed considerably the NF- κ B levels in the colon tissues of UC-mice. Previous research by Harikumar et al. (2010) mentioned that escin impaired *NF- κ B* gene activation by preventing I κ B kinase complex (IKK) alpha activation through inhibition of its phosphorylation and degradation.

IL-23, a heterodimeric cytokine that is a member of the IL-12 cytokine family, comprises a common p40 subunit and a distinct p19 subunit. IL-23, generated mainly from macrophages and dendritic cells, is a key activator of the Th17 cell pathway (Hanžel & D'Haens, 2020). It promotes the differentiation of naïve CD4⁺ Th cells into Th17. In addition to its effect regarding Th17 cells, IL-23 may activate several immune cells like type 3 innate lymphoid cells, gamma delta T cells, mucosal-associated invariant T cells, and invariant natural killer T cells (Navarro-Compán et al., 2023). Th17 cells and other immune cells, which are stimulated by IL-23, secrete IL-17 A and other proinflammatory cytokines. Our findings indicated that AA treatment raised markedly the expression of colonic IL-23. Noviello et al. (2021) also indicated a substantial increase in IL-23 level in UC-mice. The findings of the present study established that escin reduced significantly IL-23 expression, which may be attributed to the ability of escin to suppress *NF- κ B* gene activation and/or the impact of escin on glucocorticoid receptors.

IL-17 has been extensively studied because of its critical role in inflammatory diseases. IL17A through IL17E, make up the cytokine family IL17. IL-17 A is a potent inflammatory cytokine that increases the release of proinflammatory cytokines involving TNF- α and IL-6, which intensifies the inflammation. Moreover, it promotes the expression of genes that regulate neutrophil invasion, which exacerbates inflammation. Active neutrophils may use positive feedback to initiate the production of IL-17 A (Chen et al., 2016; Moschen et al., 2019; Noviello et al., 2021). In addition, IL-17 A triggers the COX-2 activation, inducing prostaglandin E2 generation that exacerbates inflammation by promoting vasodilation and thereby increasing vascular permeability and immune cells migration to the site of inflammation (Țiburcă et al., 2022). Based on current results, AA treatment increased significantly the colonic expression of IL-17 A. Lin et al. (2018) also demonstrated a substantial increase in IL-17 A expression in mice colon tissues of dextran sulfate sodium-induced colitis. According to the current investigation, escin diminished colonic expression of

IL-17 A in UC-mice. Elshal and Hazem (2022) declared that escin decreased the production of IL-17 A by diminishing the infiltration and differentiation of CD4⁺ T cells and blocking NF- κ B in concanavalin A-induced autoimmune hepatitis.

An inducible enzyme COX-2, which is stimulated by inflammatory conditions, contributes to the aggravation of UC (Li et al., 2018). It stimulates the production of prostaglandin E2, which is essential for exacerbating inflammation, because it influences the release and activity of proinflammatory cytokines (Sheibanie et al., 2007). As the previous study of Elhefnawy et al. (2023), the present investigation exhibited that the level of COX-2 was considerably elevated in the colons of mice given AA. In our investigation, mice treated with escin following AA injection exhibited a considerable decrease in colonic COX-2. Li et al. (2015) concluded that escin reduced the production of COX-2, which is attributed to escin's impact on glucocorticoid receptor levels.

Proinflammatory cytokines and chemokines induce the migration of inflammatory cells including macrophages and neutrophils to the inflamed colon, thereby these cells stimulate the disruption of the intestinal barrier and immunity *via* secreting more inflammatory mediators (Lissner et al., 2015; Wéra et al., 2016). The intestinal permeability is regulated by both the epithelial barrier and vascular endothelial barrier (Sun et al., 1998). A compromised endothelial barrier is implicated in the onset of UC *via* increased colonic vascular permeability (Tolstanova et al., 2012). The outcomes of the current investigation showed that AA treatment increased vascular permeability, which in turn caused immune cell migration to the colon. This might be explained by endothelial cell degeneration and endothelial barrier deformity. Sakthivel and Guruvayoorappan (2014) also reported that rats given AA exhibited increased vascular permeability along with immune cell migration to the colon. More notably, the outcomes of the current investigation indicated that escin administration reversed substantially the increased colonic vascular permeability in the UC group. Fu et al. (2005) also reported that escin has the ability to prevent the vascular permeability induced by AA.

Additionally, MCs have been involved in the onset of UC, as they release chemical and inflammatory mediators including histamine, proteases, chymase, and TNF- α upon their interaction with microorganisms penetrating the epithelial barrier. MC-chymase may participate in increasing colitis severity by reducing intestinal barrier integrity (Groschwitz et al., 2009; Boeckxstaens, 2015). In addition, MC-derived cytokines perpetuate inflammation by triggering neutrophil infiltration (Bischoff, 2009). Our findings indicate that mice given AA exhibited a higher percentage of degranulated MCs in the colon tissues of mice. Adakudugu et al. (2020) also indicated that

AA increased the percentage of degranulated MCs in the colon tissues of rats. The current results showed that escin therapy suppressed colonic MCs degranulation in AA-treated mice. A previous study indicated that escin reduced MCs degranulation by inhibiting immunoglobulin E (IgE) binding to high-affinity IgE receptors (Fc ϵ RI) on the MCs, stimulating their activation (Lindner et al., 2010).

It has been suggested that leukocytes may infiltrate from the inflammatory colon to the peritoneal cavity by an unidentified mechanism. The mediators of inflammation produced by the colon's inflamed regions may excite the immune cells in the peritoneum. The inflammation may worsen as a result of these inflammatory mediators' potential to attract more inflammatory cells to the abdominal cavity. Consequently, peritoneal inflammatory cells may act as an additional source of cytokines, potentially exacerbating the UC disease (Murthy & Biondi, 1992). The current study indicated that opposed to the control mice, the peritoneal cavity of the UC-mice had more leukocytes after receiving an AA injection. This outcome agrees with a prior research reported that UC-mice had more leukocytes in their peritoneal cavity (Rojas-Cartagena et al., 2005). Surprisingly, the current findings showed that escin was effective in reducing the number of white blood cells in the abdominal cavity in mice. This impact might be associated with escin's capacity to decrease colonic vascular permeability, which lowers the number of immune cells that invade the colon and attenuates colonic inflammation. Thereby reducing the possibility and the amount of WBCs that recruit to the abdominal cavity from the inflamed colon.

Conclusion

The present study demonstrated that escin (especially the high dose) ameliorated the colonic lesions induced by AA administration by suppressing the inflammatory mediators, as well as amending the oxidant-antioxidant system, thereby attenuating the colonic inflammation and oxidative stress (Fig. 6). Moreover, the high dose of escin was more effective than SSZ in modulating some parameters in UC-mice. Consequently, escin may represent a revolutionary advancement in the management of UC in the near future. The present study did not evaluate the signal transduction cascades regarding NF- κ B/IL-23/IL-17 A/COX-2 pathway; this will explain more the anti-inflammatory mechanisms of escin in UC-mice. In addition, it is important to conduct additional clinical trials to determine the efficacy of escin in treating UC-patients.

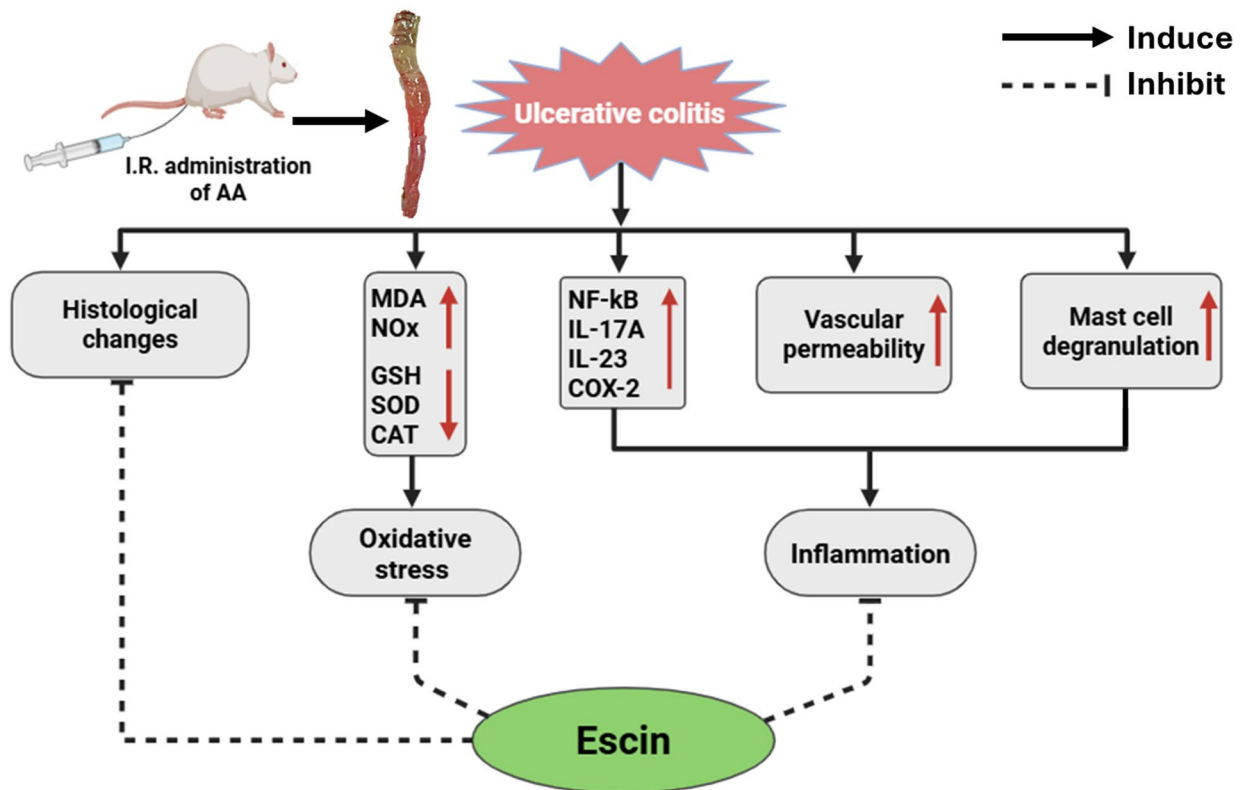


Fig. 6 Diagram demonstrating potential pathways that elucidate ameliorative impacts of escin against AA-induced UC in mice. AA acetic acid, CAT catalase, COX-2 cyclooxygenase 2, GSH reduced glutathione, IL interleukin, I.R. intrarectal, MDA malondialdehyde, NOx nitric oxide, NF-κB nuclear factor kappa B, SOD superoxide dismutase, UC ulcerative colitis

Abbreviations

AA	Acetic acid
ABC	Avidin-biotin-peroxidase complex
ANOVA	One-way analysis of variance
b.w	Body weight
CAT	Catalase
COX	Cyclooxygenase
ELISA	Enzyme-linked immunosorbent assay
FcεRI	High-affinity IgE receptors
GSH	Reduced glutathione
h	Hour/s
H ₂ O ₂	Hydrogen peroxide
HO [•]	Hydroxyl radical
I.R.	Intrarectal
IBD	Inflammatory bowel disease
IgE	Immunoglobulin E
IKK	IkappaB kinase complex
IL	Interleukin
IκB	Inhibitor of NF-κB
MCs	Mast cells
MDA	Malondialdehyde
NF-κB	Nuclear factor kappa B
NOx	Nitric oxide
O ₂ ^{•-}	Superoxide anion free radical

PBS	Phosphate buffer saline
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SSZ	Sulfasalazine
TNF-α	Tumor necrosis factor-alpha
UC	Ulcerative colitis
WHO	World Health Organization

Supplementary Information

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Supplementary Figs. 1-3

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Author contributions

Mohamed A. El-Sayed: Data curation, Formal analysis, Visualization, Investigation, Resources, Writing –original draft. Gamal Ramadan: Conceptualization, Methodology, Supervision, Data curation, Formal analysis,

Validation, Software, Writing – review & editing. Hend A. Mohammed: Methodology, Supervision, Data curation, Formal analysis, Investigation, Writing – original draft.

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Data availability

Data will be made available on request.

Declarations

Ethics approval and consent to participate

The mice were cared for and gently treated in compliance with the National Institutes of Health standards for the humane handling of laboratory animals (NIH publication No. 86 – 23, modified 1985). All experiments and sample size adhered to ARRIVE guidelines and followed the ethical principles and standards permitted by the Research Ethics Committee, Faculty of Science, Ain Shams University, Egypt (Approval code: ASU-SCI/ZOOL/2025/1/7).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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