

Umbelliferone ameliorates ulcerative colitis induced by acetic acid *via* modulation of TLR4/NF- κ B-p65/iNOS and PPAR γ /SIRT1 signaling pathways in rats

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Abstract

Ulcerative colitis (UC) is a common chronic, idiopathic inflammatory bowel disease associated with inflammatory perturbation and oxidative stress. Umbelliferone (UMB) is a potent anti-inflammatory and antioxidant coumarin derivative. Depending on the possible mechanisms, we aimed to explore and elucidate the therapeutic potential of UMB on UC-inflammatory response and oxidative injury-induced *via* intrarectal administration of acetic acid (AA). Rats were assigned into four groups: control group, UMB (30 mg/kg) treated group, colitis model group (2 ml of AA; 3% v/v), and colitis treated with UMB groups. Our results exhibited that UMB improved macroscopic and histological tissue injury caused by the AA. Mechanistically, UMB reduced the elevated TNF- α , IL-6, MPO and VCAM-1 *via* effective downregulation of TLR-4, NF- κ B and iNOS signaling pathway, thereby mediated potent anti-inflammatory effects. Moreover, UMB administration resulted in effective up-regulation of both PPAR γ and SIRT1 signaling pathways, thereby inhibited both oxidative injury and inflammatory response. Conclusively, UMB protected against AA-induced UC in rats through suppressing of the TLR4/NF- κ B-p65/iNOS signaling pathway and promoting the PPAR γ /SIRT1 signaling. Indeed, our data proved the effectiveness of UMB in UC and introduced it as a potential therapeutic beneficial applicant for clinical application.

1. Introduction

Ulcerative colitis (UC) is one of the inflammatory bowel disease (IBD) that represents a global increasingly widespread health problem [1]. UC may cause idiopathic, recurrent inflammation in the colorectal area, as well as the entire gastrointestinal tract [2]. There is evidence that the risk of colorectal cancer is increased by chronic UC [3]. Poor outcomes and the incidence of adverse effects of the currently available treatments [4]. Therefore, novel and effective therapeutic alternatives are urgently needed.

While the exact cause of UC remains unclear, its pathogenesis involves several processes that include neutrophil infiltration, excessive proinflammatory cytokines, and reactive oxygen species (ROS) production [5]. Cytokines such as tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) are frequently linked with UC [6].

Nuclear factor kappa-B (NF- κ B) is a nuclear transcription factor, that plays a crucial role in gastrointestinal homeostasis. Disturbances in the NF- κ B pathway initiates inflammatory perturbation and immunity disturbances. NF- κ B plays a significant role in UC through the induction of inducible nitric oxide synthase (iNOS), proinflammatory cytokines, and COX-2 expressions in intestinal mucosae [7, 8]. Notably, iNOS and COX-2 activation resulted in obvious large intestinal mucosa damage through the ROS and reactive nitrogen species (RNS) production along with suppressing the antioxidative system [9]. Moreover, Toll-like receptors (TLRs) play an important role in the primary innate immune responses and inflammation-associated carcinogenesis in the gastrointestinal tract [10], where the up-regulation of TLR4 and induction of the uncontrolled inflammatory response in UC weakens regeneration of the mucosa, resulting in its damage, and consequently may later lead to colon cancer development [11]. Activation of TLRs stimulates the transcription of many inflammatory regulators such as NF- κ B and the mitogen-

activated protein kinase (MAPK) [12]. MAPKs are an important intracellular signal transduction factor family that regulates cell proliferation, division, and apoptosis [13]. The MAPK family members include p38 MAPK, and extracellular signal-regulated kinase (ERK) [14]. Interestingly, MAPK signal activation induce the cytokines release which play a critical role UC pathogenesis [15]. Therefore, targeting the TLR4/NF- κ B-p65/iNOS signaling pathway might be a reasonable strategy for management inflammatory diseases, including UC [16].

Additionally, peroxisome proliferator-activated receptor gamma (PPAR γ) participates in the regulation of mucosal inflammatory injury in UC [17]. Alternatively, PPAR γ activation effectively downregulates various pro-inflammatory cytokines expressions *via* potent inhibition of the stimulation of NF- κ B [18]. Another important potent inhibitor signaling is the silent information regulator-1 (SIRT1). SIRT1 suppresses the inflammation *via* MAPK and NF- κ B signals inhibition [19]. Recently, activators of SIRT1 have shown protective effects against chemically induced colitis [20].

Umbelliferone (7-hydroxycoumarin; UMB), a coumarin derivative, has a potent anti-inflammatory and antioxidant activities [21]. The therapeutic potentials of UMB in diverse models have been established in previous studies. UMB has shown antidiabetic effect against diabetes mellitus [22], and hepatoprotective activities against hepatic injury [23]. Moreover, UMB showed protective, anti-inflammatory, and antiapoptotic effects against colon cancer in rats [24].

In the present investigation, we aimed to assess whether UMB has a therapeutic beneficial protective effect against the UC induced by AA in rat model and to explore the involved underlying mechanisms *via* studying the impact of UMB on TLR4/NF- κ B-p65/iNOS and PPAR γ /SIRT1 in these protective effects.

2. Materials And Methods

2.1. Drugs and chemicals

Umbelliferone (UMB) was purchased from Sigma Aldrich (USA). Acetic acid AA was purchased from El-Naser Pharmaceutical Chemicals Company (Egypt). Rats TNF- α and IL-6 ELISA kits for were purchased from Glory Science Company (USA). Trizol reagent was purchased from Invitrogen Inc, Grand Island, NY (USA). Complementary DNA (cDNA) was purchased from Thermo Scientific Revert Aid (USA). SYBR green master mix was purchased from Bioline, myBio (Ireland) PCR primers for NF- κ B-p65, iNOS, TLR4, PPAR γ , SIRT1, and β -actin genes were synthesized by Vivantis Technologies (Malaysia). The antibodies used were mouse monoclonal anti-NF- κ B-p65, anti-iNOS, anti-TLR4, anti-PPAR γ , anti-SIRT1 anti-p38MAPK, anti-ERK1, anti-VCAM-1, and anti- β -actin antibodies and were purchased from Santa Cruz (USA). BCIP/NBT substrate detection kit was purchased from Genemed Biotechnologies, (USA). Alkaline phosphatase (ALP)-conjugated secondary antibody was purchased from Santa Cruz (USA).

2.2. Animals

Adult male Wistar rats, weighing 190–210 g, were obtained from the animal house of the Faculty of Medicine, Assiut University, Egypt. The rats were maintained on a 12 h dark/light cycle at 24 ± 2 °C with free access to food and water *ad libitum*. Experimental procedure was conducted in accordance with the ethical standards and was approved by the Animal Ethics Committee, Faculty of Medicine, Assiut University, Egypt. Ethics Committee, approval No. (AUN/MED/20/123).

2.3. Induction of ulcerative colitis

Animals were starved for at least 24 hours prior to colitis induction, with free access to tap water. The water was held two hours before the procedure on the day of the experiment, then anaesthetized with ketamine (100 mg/kg, i.p.). Ulcerative colitis was induced by administering 2 ml of acetic acid (AA) (3% v/v in saline) by intrarectal route using an elastic catheter (outer diameter of 2 mm) under ketamine anesthesia. The catheter was inserted up to 8 cm rectally in the colon, then rats were kept in a straight position for two min to prevent the outflow of AA. Then to spread the AA totally in the colon, 2 ml of air were injected before removing the catheter. Using the same methods, control rats were given 2 ml of saline instead of acetic acid [25].

2.4. Experimental protocol

Forty rats were randomly allocated into four groups (10 rats per group). Group 1: Control group received the vehicle only (The same volume saline instead of AA and the vehicle was delivered only once a day for seven consecutive days). Group 2: (UMB) received UMB suspended in 0.5 % carboxymethyl cellulose (CMC) at a dose of (30 mg/kg/day, orally) [26]. once daily for seven days plus the same volume of saline instead of AA solution. Group 3: (AA): UC model (+ve control group) rats were administered the vehicle orally for seven days, and a rectal AA that was given at the end of the 5th day. Group 4: (UMB+AA) received UMB once daily for five days before AA and two days and served as UMB+AA group.

2.5. Samples preparation

Twenty-four hours after the last treatment, rats were sacrificed under ketamine anesthesia, and blood samples were collected *via* cardiac puncture. Blood samples were subjected to centrifugation for obtaining the sera. Sera were stored at -20 °C for determination of TNF- α and IL-6 levels using ELISA. The 8 cm distal colon portions of each rat were removed and longitudinally opened, cleaned with cold saline and observed for macroscopic colitis assessment, then cut into three small pieces. One part of the colon was used for histopathological examination and the determination of NF- κ B-p65, iNOS, TLR4, PPAR γ , and SIRT1 proteins expression using immunohistochemical staining. Another part was used for preparation of 20% (w/v) homogenates and the supernatants used for assays of NO $_2^-$ content and MPO activity. Additionally, small part of colonic tissue was stored in RNeasy lysis buffer and used for assessment of NF- κ B-p65, iNOS, TLR4, PPAR γ , and SIRT1 genes using qRT-PCR. Finally, a small part stored in a lysis buffer for western blot analysis of p38MAPK, ERK1, and VCAM-1 proteins.

2.6. Macroscopic scoring:

2.6.1. Evaluation of Disease activity index (DAI):

Evaluation of the clinical severity of colitis was done through calculation of DAI according previously described method with criteria provided in Table 1 [27]. It was calculated according to this equation:

$$\text{DAI} = \frac{\text{Body weight loss score} + \text{diarrhea score} + \text{rectal bleeding score}}{3}$$

2.6.2 Colon mucosal injury score (CMIS):

Furthermore, the percentage of the affected area for which 8 cm of colon area proximal to the anus with criteria provided in Table 2 [28].

2.7. Histopathological study

Histopathological evaluation was done following the method described by Bancroft and Gamble, (2008) [30]. Colon biopsies were fixed with 10% neutral buffer formalin and embedded in paraffin. The specimens were washed, dehydrated by alcohol, cleared in xylene and embedded in paraffin. For histopathological assessment, 3µm thickness sections were cut and stained with hematoxylin and eosin (H&E). Histopathological assessment of specimens was performed by an experienced pathologist blindly under the light microscope to the identity of the tested samples.

2.8. Biochemical assessments

2.8.1. Assessment of serum cytokines; TNF-α and IL-6

Following the manufacturer's instructions and depend on the previously described principles, biochemical estimations of serum TNF-α and IL-6 levels were performed using ELISA kits [31].

2.8.2. Assessment of NO₂⁻ content and MPO activity

Determination of NO₂⁻ content and MPO enzymatic activity were done by methods described by Tasikas (2007) [32] and Krawisz *et al.* (1984) [33] respectively.

2.9. Western blot analysis

Colon tissues were homogenized in Tris lysis buffer and protease inhibitor cocktail (Biospes, China) at 4°C for 30 minutes. The residual tissue was discarded by centrifugation at 12000 xg for 10 min at 4 ° C for 10 minutes. The amount of total protein in each sample was measured according to Bradford's method [34]. A total 50 µg of total protein was loaded in each lane and resolved by 10% SDS-polyacrylamide gel electrophoresis at 150 V for 50 minutes. SDS-gel was transferred to a PVDF membrane after the completion of the electrophoresis using semi-dry transfer methods [35]. The membranes were blocked with 5% skimmed milk in TBST buffer at room temperature for 1 hour. The

membranes were then incubated at 4 °C with primary antibodies overnight. The membranes were then incubated with the ALP-conjugated secondary antibody for 1 hour. Bands were visualized by BCIP/NBT detection kit. The produced bands were subjected to analysis using image J® software (National Institutes of Health, Bethesda, USA) related to β -actin.

2.10. Immunohistochemical study

For immunohistochemical investigation, multiple consecutive sections were used. Tissue sections were deparaffinized then treated with 3% H₂O₂ for 10 minutes. Heated in 10 mM citrate buffer at 121 °C for 30 minutes for antigen retrieval in order to inactivate endogenous peroxidases, then blocked by 5 percent BSA in Tris-buffered saline. Then samples incubation with primary antibodies against NF- κ B-p65, iNOS, TLR4, PPAR γ , and SIRT1 overnight were done. After incubation with HRP-conjugated secondary antibodies for 30 minutes at room temperature, the slides were washed and stained with DAB staining and hematoxylin counterstaining. It was observed that the positive staining of brown color by light microscopy [36]. Using Image J® software, the percentage of area occupied by brown colour was evaluated and the mean area for each slide was obtained.

2.11. Real-time PCR analysis

Genes expression estimation of NF- κ B-p65, iNOS, TLR4, PPAR γ , and SIRT1 (Primers sequences were listed in Table (3)) by qRT-PCR. Briefly, Using the Trizol reagent, RNA was extracted from rat colon tissues, and subsequent RNA was transcribed in reverse to cDNA as instructed by the manufacturer. SYBR green was used to assay target genes' expression as previously described [37]. The β -actin is employed as a reference gene. After PCR amplification, according to the method described by Livak and Schmittgen (2001), the data obtained was analyzed to represent a fold change using the $2^{-\Delta\Delta CT}$ equation [38].

2.12. Statistical Analysis

All data are expressed as mean \pm SEM (standard error of the mean). Statistical analysis was performed using the Graph Pad Prism Software Version 6.00 (GraphPad Software, Inc. La Jolla, CA, USA). One-way analysis of variance (ANOVA), followed by Tukey's test, was used to evaluate the statistical significance of differences in each parameter among the different groups, and a *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Effect of UMB on disease activity index, colonic weight/length ratio, and macroscopic colonic examination

As shown in Figure 1A., rats that received only AA demonstrated a significant elevation in DAI and CMIS with respect to these of the control rats. Pretreatment with UMB (30 mg/kg) resulted in a remarkable decrease in the DAI scores and CMIS of the colon of relative to the AA group (Table 4).

3.2. Effect of UMB on AA-induced colonic histopathological examination

Normal control rat (Fig. 1 & 2A–B) and UMB-treated rats (Fig. 1 & 2C–D) showed the normal layers of rat's colon. Rats received intrarectal AA showed sever histopathological changes as the desquamated epithelial cells intermingled red blood cells in the colon lumen. The ulcers were observed as deep defects of the lamina epithelialis with its basement membrane. Also, showed severe hemorrhage and marked exhaustion and distortion of goblet cells of the intestinal glands. Well-expressed hyperemia of blood vessels and mixed inflammatory cellular reaction made of neutrophils, eosinophils, and few lymphocytes were observed in the submucosa (Fig. 1 & 2E–G). On contrast, rats taken UMB greatly attenuated these histopathological abrasions and showed intact mucosa with minimal cellular reaction in the submucosa. The absence of desquamated cells in the colon lumen and intact mucosa were observed with apparent healthy goblet cells with a minimal cellular reaction in the submucosa (Fig. 1 & 2H–I).

3.3. Effect of UMB on proinflammatory cytokines; TNF- α , IL-6 levels as well as MPO enzymatic activity and nitrite content.

Rectal administration of AA-induced colonic inflammatory response, colitis and significant increase in the proinflammatory cytokines; TNF- α (Fig. 3A) and IL-6 (Fig. 3B) as well as MPO enzymatic activity (Fig. 3C) in addition to the NO₂⁻ content (Fig. 3D) compared with the control rats. Pretreatment with UMB significantly reduced AA-induced upregulation of the proinflammatory cytokines; TNF- α , IL-6 levels and decreased MPO enzymatic activity and NO₂⁻ content relative to AA group.

3.4. Effect of UMB on colonic p38 MAPK, ERK1, and VCAM-1 level

To examine the possible role of p38 MAPK, ERK1, and VCAM-1 in AA-induced UC, the protein expressions of p38 MAPK (Fig. 4A), ERK1 (Fig. 4B), and VCAM-1 (Fig. 4C) using Western blotting assay. As represented in Figs. 4 rectal administration of AA resulted in a significant upregulation of p38 MAPK, ERK1, and VCAM-1 expressions in rat colon in comparison with the control group. Alternatively, UMB treatment resulted in remarkable downregulation of p38 MAPK, ERK1, and VCAM-1 compared with the AA group. Moreover, rats treated with UMB alone significant decrease in ERK1 expression compared with the control rats.

3.5. Effect of UMB on colonic iNOS, TLR4, and NF- κ B p65 in AA-induced UC

To explore the impact of iNOS, TLR4 and NF- κ B-p65 (Fig. 5A-D) in the pathological induction of the colonic inflammatory process and ulcerative colitis, the protein expression of these biomarkers was investigated immunohistochemically, and their gene expression was investigated using quantitative PCR analysis.

Results showed that rectal administration of AA-induced a significant increase in protein expression of iNOS (Fig. 5B), TLR4 (Fig. 5C), and NF- κ B-p65 (Fig. 5D) when compared with the normal

control group. In parallel with these results, the genes expression also significantly increased when compared with their gene expression levels in the control group. Pretreatment with UMB inhibited the AA-induced increase in both protein and gene expression of iNOS (Fig. 6A), NF- κ B-p65 (Fig. 6B) and TLR4 (Fig. 6C) when compared with the AA group. Moreover, animals treated with UMB only exhibited downregulation of these biomarkers compared with the control group.

3.6. Effect of UMB on colonic PPAR γ , and SIRT1 expression in AA-induced UC:

Animals given AA's rectal administration showed a significant decrease in protein and mRNA expression of PPAR γ (Fig. 7B & C) and SIRT1 (Fig. 7D & E), as illustrated in figure 7, in comparison with the normal rats. Conversely, pretreatment with UMB significantly up-regulated both protein and mRNA of PPAR γ and SIRT1 compared with the AA group.

4. Discussion

Lately, IBD could not be cured without treatment and available IBD treatment deals mainly with increasing remission periods and improving patient life quality [39]. Therefore, novel IBD drugs are urgently needed for effective treatment of IBD to replace existing traditional drugs, that cause many harmful side effects. The introduction of these new drugs will lead to more efficient treatment and increase the safety of IBD patients in the treatment process [40]. Accordingly, our investigation was designed on this principle of assessing the therapeutic beneficial effects of UMB in protecting against AA-induced colitis.

Acetic acid (AA)-induced UC is a reasonable widely used model for studying UC [41]. This model is accompanying neutrophil infiltration, inflammation, and oxidative stress that mimics human IBD's pathogenesis [42]. Upon assessing the effect of UMB on colitis manifestation, a significant decrease in the DAI score and CMIS. The results of H&E examination revealed that UMB markedly reduced the histopathological changes observed in colons of the AA-treated rats.

TNF- α , and IL-6 are key pro-inflammatory cytokines have a critical role in the colonic mucosa inflammation and mucosal barrier destruction [43]. TNF- α is a crucial cytokine linked with continuous immune dysregulation in the inflamed tissues of IBD and generates many chemical inflammatory mediators [44, 45]. Additionally, IL-6 contributes to neutrophil infiltration in chronic inflammatory diseases like colitis [45]. In this study, induction of UC was accompanied by an increase in TNF- α , and IL-6 levels and MPO activity relative to control rats, the effect that was significantly attenuated by UMB. Moreover, AA-treated rats showed significant increase in the MPO activity, an important marker of neutrophil infiltration, which was significantly attenuated by UMB. The reduction of the TNF- α , and IL-6 levels and MPO activity by UMB in our results indicates the anti-inflammatory effect of UMB, the effect previously reported [24]. Additionally, UC induction by AA is followed by the endothelial VCAM-1 upregulation that is regarded a main cause of leukocyte recruitment to the area of intestinal inflammation [46]. This effect was abrogated by UMB treatment. In the colonic inflammatory conditions, cells and macrophages release TNF- α , which stimulates adhesion molecules like VCAM-1 production, thus enhances leukocytes infiltration into the intestinal mucosa. Therefore, inhibition of these adhesion molecules can successfully

suppress inflammation [47], which another mechanism *via* which UMB could inhibit the inflammatory response in AA-induced UC.

Another crucial factor causing colon tissue damage in UC is the oxidative stress [48]. ROS and RNS negatively influence on colonic mucosal membrane organization and resulted in proteins and DNA damages as well as lipid peroxidation [16]. In this study, AA produced a significant increase in both mRNA and protein expression of iNOS that was greatly attenuated by UMB administration. Several experimental studies showed that iNOS expression level is increased in IBD [49, 50]. Also, iNOS expression and NO production are elevated in IBD patients' colonic tissue [51]. The increased level of NO and superoxide radicals during UC increases peroxynitrite production, which induces oxidative stress and membrane lipid peroxidation [52]. The ability of UMB to inhibit iNOS expression and activity was previously reported [53]. Expression of iNOS can be stimulated by various factors; among these factors is TNF- α , and NF- κ B [54].

NF- κ B plays a key role in gut homeostasis and its activation participate in the pathogenesis of IBD in humans [55]. NF- κ B activation promotes TNF- α and IL-6 production in UC [56]. In line with these facts, this study showed an increase in both gene and protein expression of NF- κ B in colons of animals exposed to AA, the effects that were markedly decreased by UMB treatment. The ability of UMB to suppress NF- κ B activation and its correlation with the UMB anti-inflammatory effect was previously reported [57].

To furtherly investigate the mechanism of the effect of UMB in colitis and its ability to inhibit NF- κ B transcription, we studied the effect on the TLR4/MAPKs signaling pathway, which control of the expression of cytokines and chemokines [58]. Our data demonstrated that AA resulted in significant upregulation in TLR4, p38 MAPK and ERK1. On the contrary, the expression levels of these markers significantly lowered in colitis rats treated with UMB. Thus, we speculated that UMB might protect against AA-induced colitis through the suppression of the TLR-4/MAPK/NF- κ B signaling pathway. MAPKs induce and activate cytokines and NF- κ B activation [59]. Pretreatment of animals with UMB downregulated expression of TLR-4 and its downstream p38 and ERK1 indicating that inhibition of NF- κ B p65 inflammatory response is mediated *via* TLR4/MAPK signaling pathway. Previous studies showed the ability of UMB to inhibit the TLR4/MAPK/ NF- κ B p65 signaling [60, 61].

For further examination of the possible mechanisms of the protective effect of UMB in UC, we studied the possible involvement of the PPAR γ /SIRT1 signaling pathway. This study showed both the mRNA and protein expression of PPAR γ and SIRT1 were significantly downregulated by the colonic instillation of AA while significantly restored in colons of animals pretreated with UMB. PPAR γ controls the expressions of various cytokines by regulating the activity of NF- κ B. PPAR γ expression is downregulated in the colonic epithelium of UC patients. PPAR γ agonist rosiglitazone effectively treated moderate active distal cases of UC [18, 62]. Additionally, SIRT1 plays an important role in UC where SIRT1 has been shown to inhibit UC inflammation through suppression of NF- κ B activation [63]. Also, SIRT1 suppressed MAPK activation which represents another pathway by which SIRT1 inhibits NF- κ B and corresponding inflammation [64]. Moreover, SIRT1 potently inhibited intracellular ROS [65] and stimulate antioxidants' expression [66]. There is an evidence that UMB as a coumarin compound has an antioxidant activity, which is linked to

the upregulation of SIRT1 [67]. Hence, upregulation of PPAR γ /SIRT1 signaling, shown in our results also contributes to its antioxidant effect of UMB, which represents additional mechanism for its protective effect against AA-induced UC.

Conclusions

UMB is a promising candidate for UC treatment. UMB potently mitigated AA-induced colonic injury and inflammation proved by a decrease in macroscopic colon damage score besides the microscopic features of inflammation and colonic tissue damage through its anti-inflammatory activity. UMB inhibited the inflammation through TLR4/NF- κ B-p65/iNOS signaling pathway, thereby effectively decreased the expression of cytokines; TNF- α , IL-6, and MPO enzymatic activity. Moreover, UMB upregulated the PPAR γ /SIRT1 signaling pathway, which inhibits NF- κ B-p65 activation and restored antioxidant status. Therefore, our data proved the beneficial efficacy of UMB and introduced it as a promising therapeutic applicant for UC.

Declarations

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Basel A. Abdel-Wahab¹], [Emad H.M. Hassanein], [Saad A Alkahtan] and [Abdulsalam A. Alqahtani]. The first draft of the manuscript was written by [Basel A. Abdel-Wahab] and [Emad H.M. Hassanein] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval: Not applicable

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Tables

Table 1. Criteria for DAI

Score	Weight loss (%)	Occult blood	Stool consistency
0	<0	Negative	Normal
1	1–5	+	
2	5–10	++	Soft
3	10–20	+++	
4	>20	Gross bleeding	Severe Diarrhea

Table 2. Criteria for colon mucosal injury score (CMIS)

Score	Criteria
0	No damage
1	Mild hyperemia with small edema and no corrosion or ulcer seen in the mucosal layer
2	Moderate hyperemia and edema with one corrosion site in the mucosal layer
3	Moderate hyperemia and edema with two corrosion sites in the mucosal layer
4	Severe hyperemia and edema and inflammation covering the mucosal layer and the size of the major inflammation does not exceed 1 cm
5	Severe hyperemia and edema with swelling, bleeding, and inflammation covering the mucosal layer, and the size of the major inflammation covers \geq 1 cm.

Table 3. The sequence of primers used for mRNA expression quantification by RT-PCR.

Target gene	Sequence (5– 3)
NF-kB p65	F: TTCCCTGAAGTGGAGCTAGGA R: CATGTCGAGGAAGACACTGGA
TLR4	F: CGAGCCAGAATGAGGACTGG R: TCCCACTCGAGGTAGGTGTT
iNOS	F: CAGGCTTGGGTCTTGTTAGC R: CTTGTGGTGAAGGGTGTCGT
PPARγ	F: GGGACGCTGAAGAAGAGACCTG R: CACAGTCCGGTCAGAAAGTGA
SIRT1	F: CGGTCTGTCAGCATCATCTTCC R: CGCCTTATCCTCTAGTTCCTGTG
β-actin	F: CCACCATGTACCCAGGCATT R: ACGCAGCTCAGTAACAGTCC

Table 4. Effect of REB on DAI and CMIS of AA-induced UC

	Control	UMB	AA	UMB+AA
DAI	0	0	3.83 \pm 0.24 ^a	2.4 \pm 0.17 ^{a,b}
CMIS	0	0	4.94 \pm 0.30 ^a	3.42 \pm 0.16 ^{a,b}

Figures

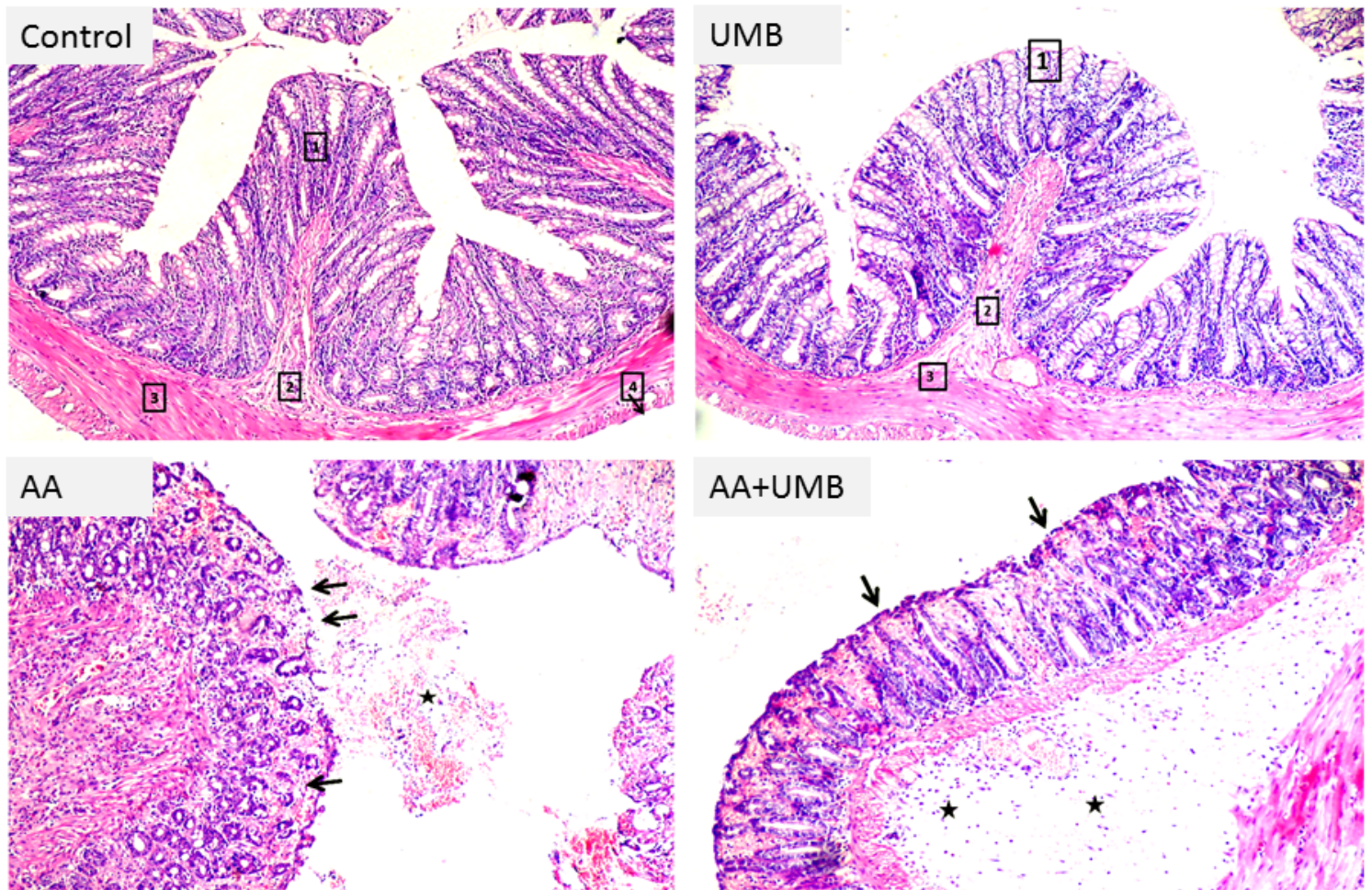


Figure 1

Effect of UMB in histopathological changes-induced by AA in UC at lower magnifications. Normal control colon and UMB showing the mucosa (1), submucosa (2), muscularis (3) and serosa (4). On the other hand, intrarectal administration showing desquamated epithelial cells intermingled red blood cells in the colon lumen (star) and ulcers (arrow). Alternatively, treatment with UMB exhibiting intact mucosa (arrow) minimal cellular reaction in the submucosa (star). (H&E x100)

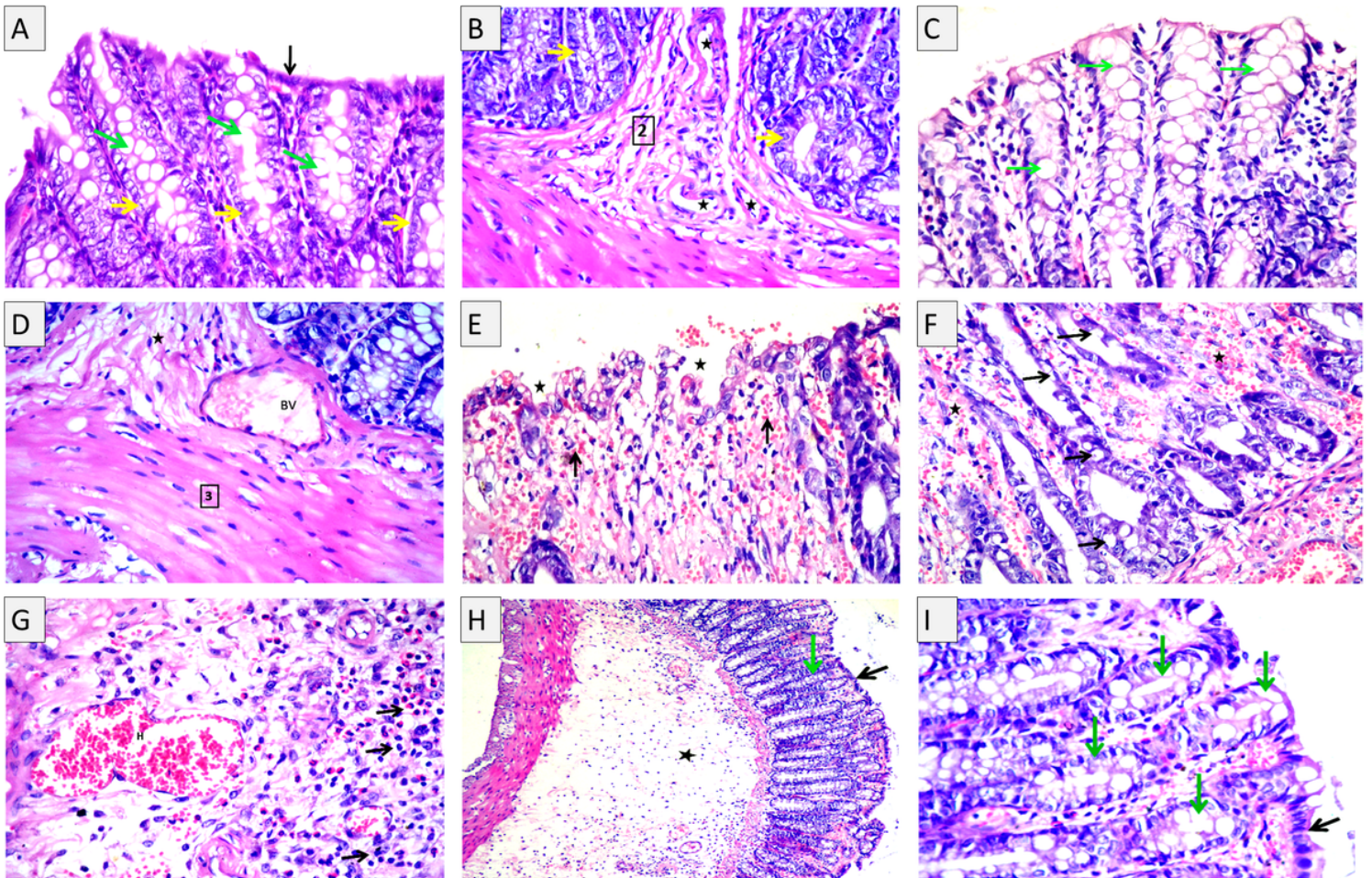


Figure 2

Effect of UMB in histopathological changes-induced by AA in UC at higher magnifications. Normal control colon showing the mucosa, simple columnar epithelium (black arrow), goblet cells (green arrow) and vertical intestinal glands (yellow arrow) (A). Also, showing the submucosa (2), made of dense connective tissue containing large diameter blood vessels (star) and vertical and horizontal intestinal glands (yellow arrow) (B). Regarded to UMB treatment, the results showing the normal layers of colon, mucosa (1), submucosa (2), muscularis (3) (C) as well as normal appearance of intestinal glands and goblet cells (B) as well as showing the normal appearance of submucosa (star) and blood vessels (BV), and muscularis (3) (D). On the other side intrarectal administration of AA resulted in desquamated epithelial cells intermingled red blood cells in the colon lumen (star) and ulcers (arrow) (E). Another view showing severe hemorrhage (star) and marked exhaustion of goblet cells (arrow) of the intestinal glands (F). Also, well-expressed hyperemia of blood vessels (H) and mixed inflammatory cellular reaction (neutrophils, eosinophils, few lymphocytes, arrow) in the submucosa (G). in contrast, UMB administration resulted in an intact mucosa (arrow), apparent healthy goblet cells (green arrow) minimal cellular reaction in the submucosa (star) (H & I). (H&E x400).

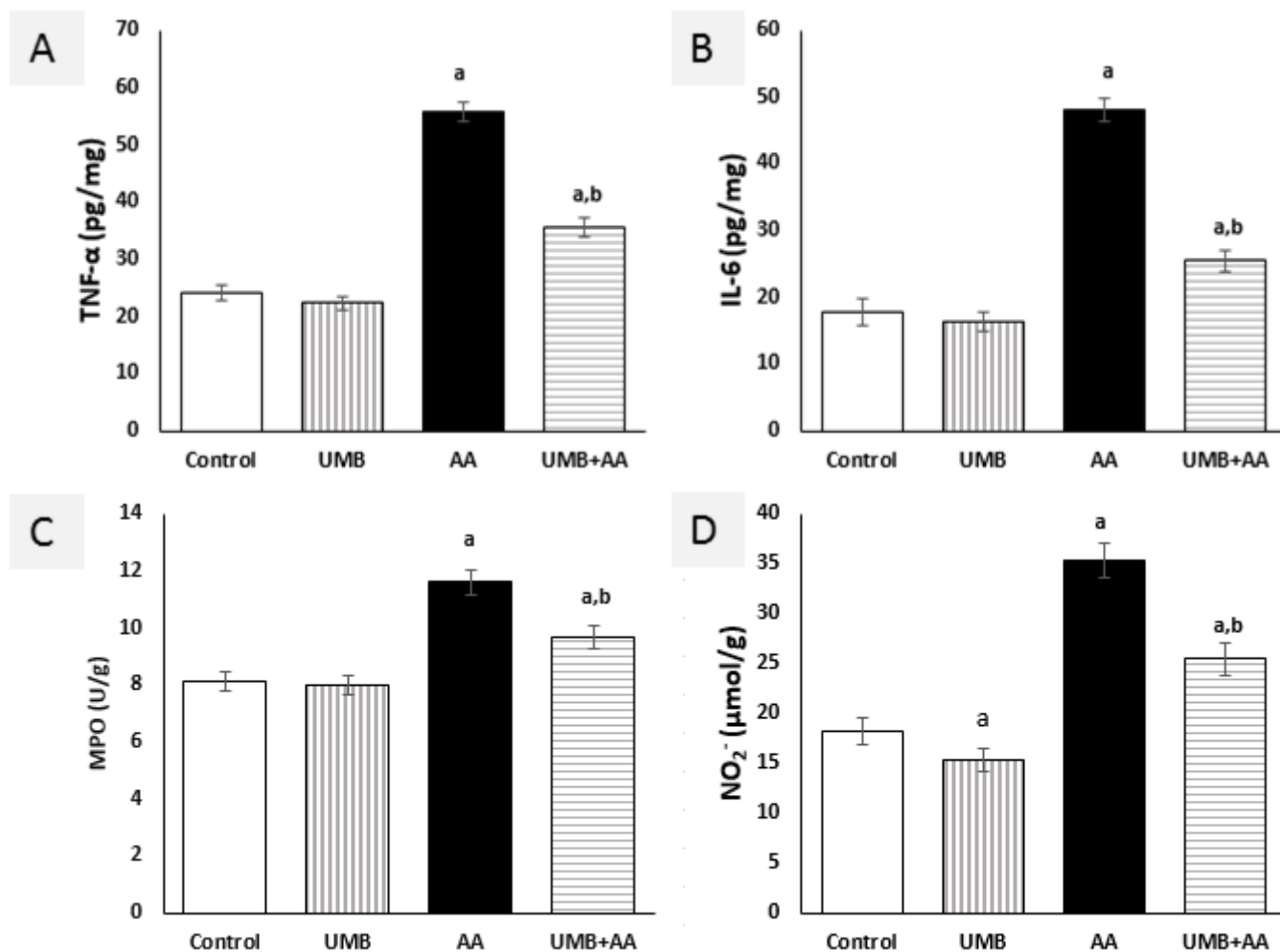


Figure 3

Effect of UMB on the inflammatory cytokines levels; TNF- α (A), and IL-6 (B) levels as MPO activity (C), and NO₂⁻ level (D) in AA-induced UC in rats. UMB: Umbelliferone; AA: Acetic acid; UC: Ulcerative colitis. Results represent mean \pm SEM (n = 10). aP < 0.05 vs. control rats, bP < 0.05 vs. AA-treated rats using Tukey's post hoc test.

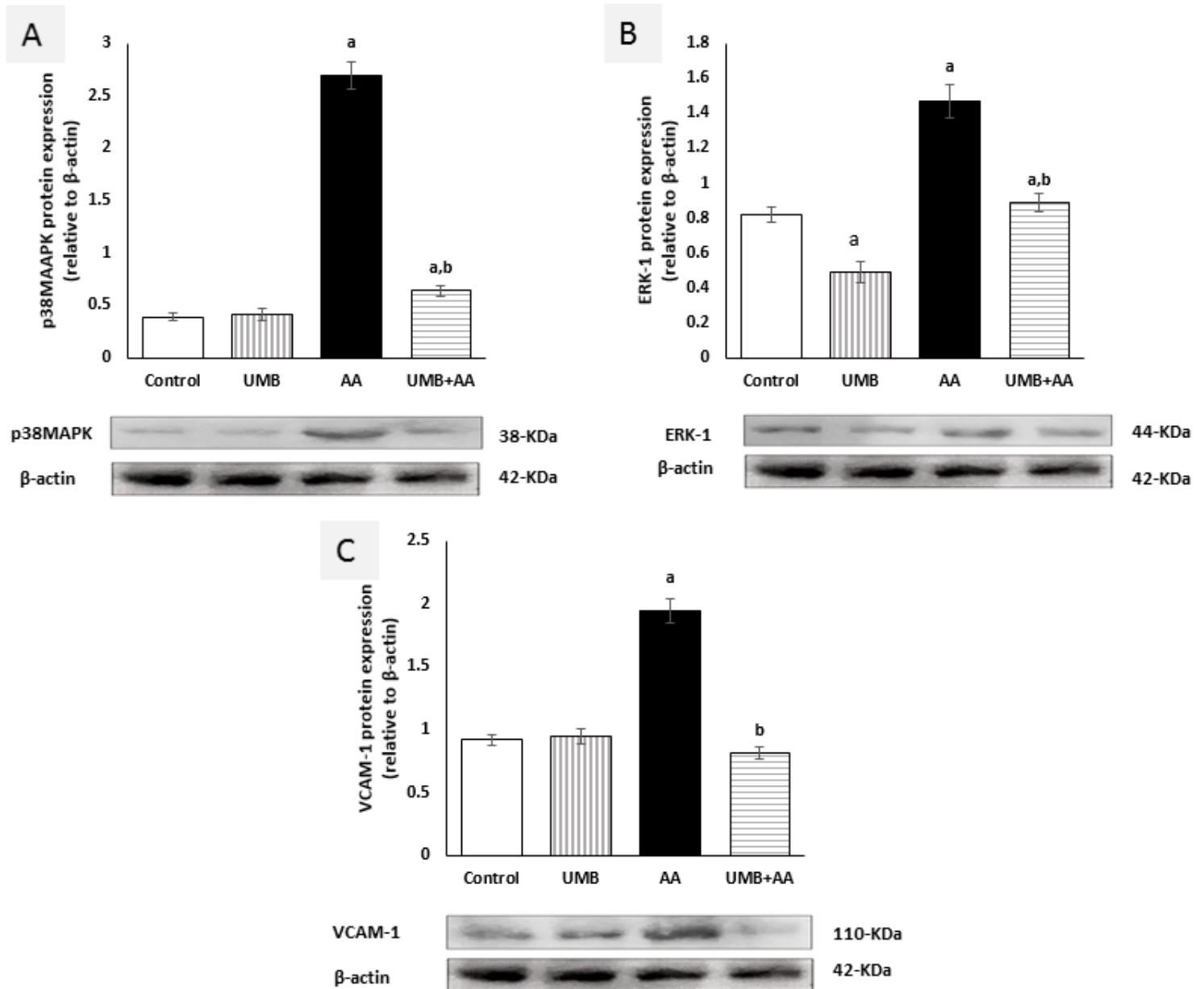


Figure 4

Effect of UMB on the protein expression levels of p38 MAPK (A), ERK1 (B), and VCAM-1 (C) in AA-induced UC in rats. UMB: Umbelliferone; AA: Acetic acid; UC: Ulcerative colitis. Results represent mean \pm SEM ($n = 10$). aP < 0.05 vs. control rats, bP < 0.05 vs. AA-treated rats using Tukey's post hoc test.

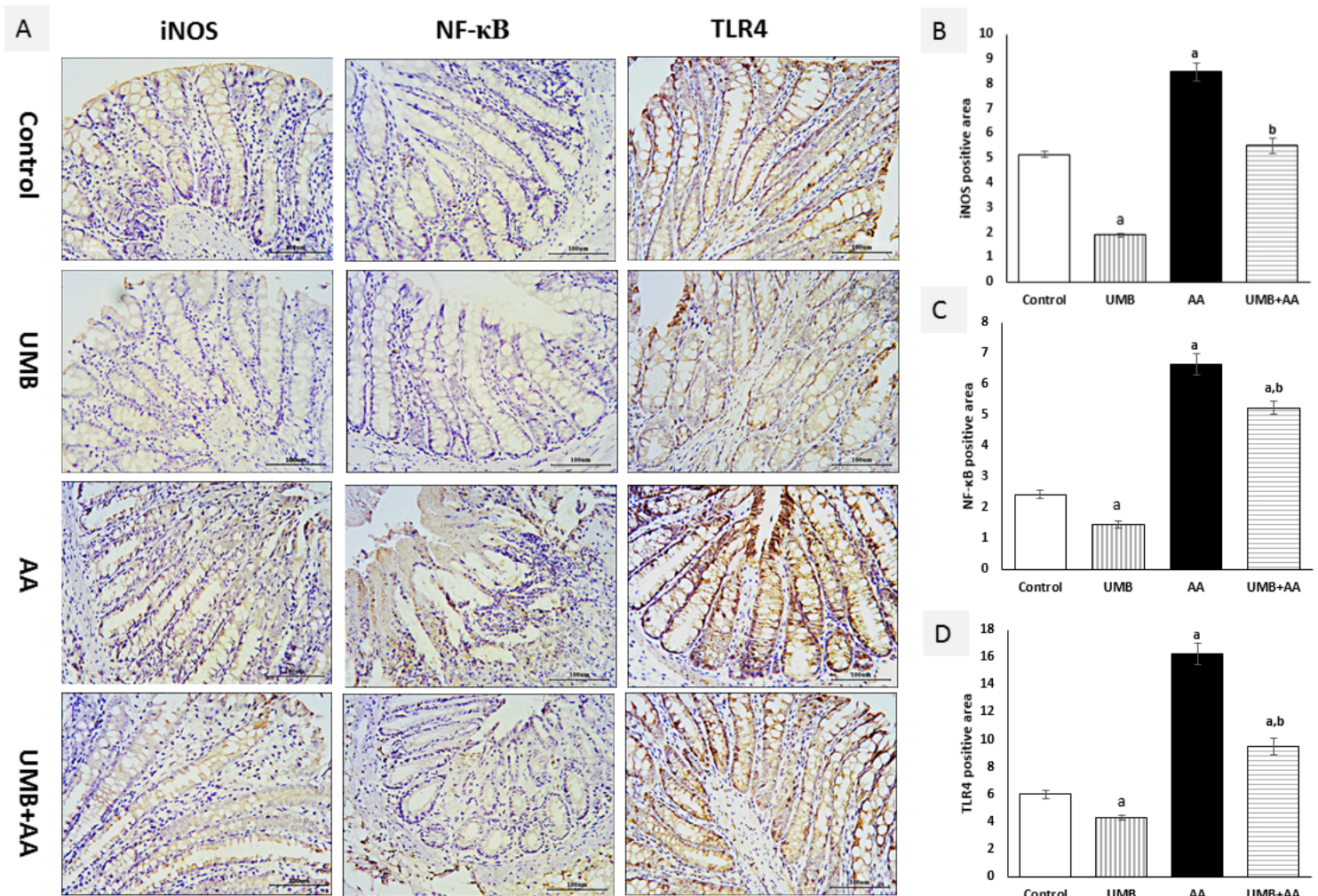


Figure 5

Effect of UMB on the immunohistochemically- assessed protein expression of iNOS (B), NF-κB(C) and TLR4(D) in AA-induced UC in rats. UMB: Umbelliferone; AA: Acetic acid; UC: Ulcerative colitis. Results represent mean \pm SEM (n = 10). aP < 0.05 vs. control rats, bP < 0.05 vs. AA-treated rats using Tukey's post hoc test.

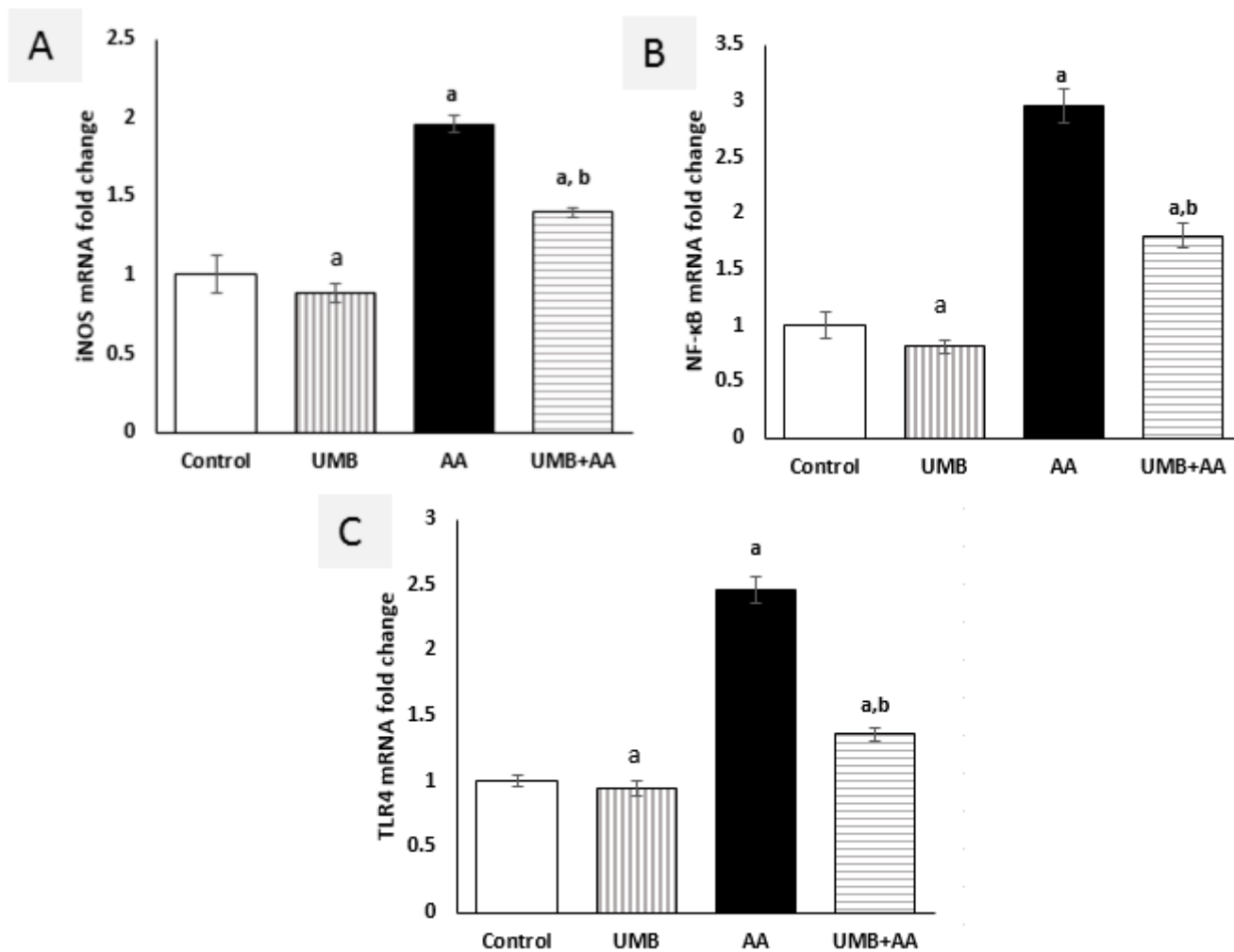


Figure 6

Effect of UMB on the mRNA expression of iNOS (A), NF-κB (B) and TLR4(C) in AA-induced UC in rats. UMB: Umbelliferone; AA: Acetic acid; UC: Ulcerative colitis. Results represent mean \pm SEM (n = 10). aP < 0.05 vs. control rats, bP < 0.05 vs. AA-treated rats using Tukey's post hoc test.

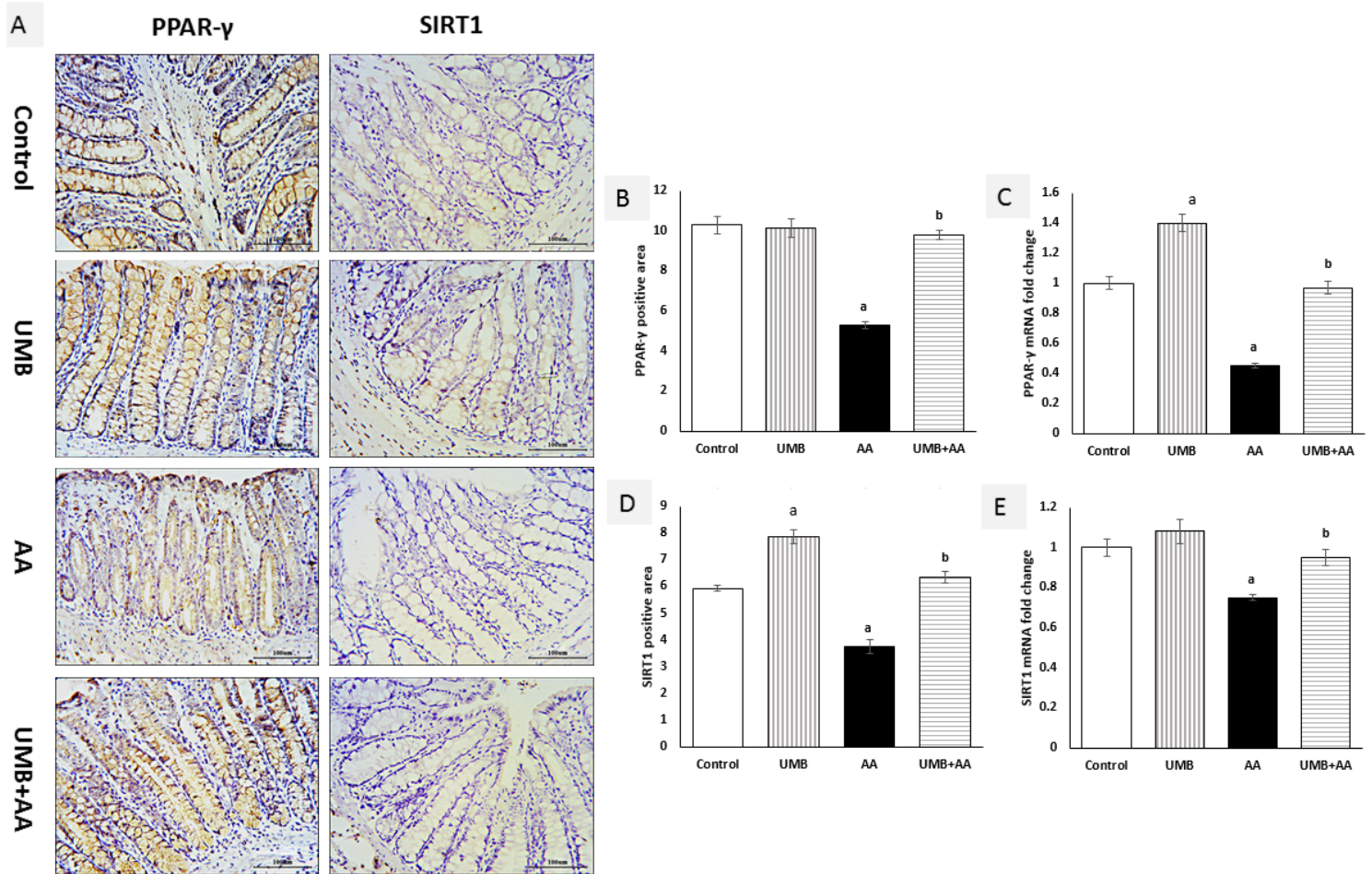


Figure 7

Effect of UMB on the immunohistochemically-assessed protein and mRNA expression of PPAR γ (B & C) and SIRT1 (D&E) in AA-induced UC in rats. UMB: Umbelliferone; AA: Acetic acid; UC: Ulcerative colitis. Results represent mean \pm SEM (n = 10). aP < 0.05 vs. control rats, bP < 0.05 vs. AA-treated rats using Tukey's post hoc test.

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