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The therapeutic and immunomodulatory effects of black pomegranate peel extract (from Iraq) plus to sirolimus in an experimental rat's model of ulcerative colitis

Sarah S. Abdel Abbas
*Correspond author. Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq. E-mail: Sara.sameer.ms12@gmail.com

Falah M. K. AL-Rekabi
Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq. E-mail: falah@covm.uobaghdad.edu.iq

Abstract

Objective

Ulcerative colitis is a chronic type of inflammatory bowel disease that is characterized by chronic colonic mucosa injury. Current treatments for the disease only provide symptomatic management rather than a cure. The present study aimed to investigate the therapeutic and antioxidant effects of ethanolic black pomegranate peel extract (BPPEE) used as monotherapy or in combination with sirolimus and compare the effects to those of standard sulfasalazine treatment in the 2,4-dinitrobenzenesulfonic acid-induced colitis rat model.

Materials and methods

Rats were randomly divided into eight groups: a negative control group, a positive control group, and treatment groups with different doses of BPPEE (200 and 300 mg/kg), sulfasalazine, and sirolimus, and the combination of BPPEE and sirolimus, for four weeks. Black pomegranate fruits were collected from Karbala orchards in Iraq in September 2024, and the extract was formulated using 70% ethanol supplemented with 1% acetic acid of this fruit. The extract was characterized by physical features of dark purple in color, thick and viscous nature, sour in taste, and aromatic smell, with the extraction yield being 42.5%. Therapeutic effects were determined on the basis of clinical observation, changes in body weight, histological and transmission electron microscopy study of colon tissues and the measurement of oxidative stress markers including myeloperoxidase and malondialdehyde.

Results

There was a marked improvement in clinical signs and a gradual recovery of body weight in all treated groups compared to the positive control group. The histological and electron microscopy analyses showed that there was a marked improvement in the integrity of the cellular structure of the colonic epithelium in comparison to the positive control group, especially in those animals

treated with the combination therapy. Moreover, these two groups showed a marked decrease in myeloid peroxidase (MPO) and myelondylaldehyde (MDA) levels.

Conclusion

These findings show that the black pomegranate peel extract has anti-inflammatory and antioxidant properties. Moreover, the use of the black pomegranate peel extract in conjunction with sirolimus has shown a significant synergistic effect in the improvement of clinical, histological, and biochemical parameters in the ulcerative colitis model. Thus, the use of the black pomegranate peel extract in conjunction with sirolimus could be regarded as a promising approach in the management of ulcerative colitis.

Keywords: black pomegranate peel extract, inflammatory bowel disease, rat, therapeutic effects, ulcerative colitis

Paper Type: Research Paper.

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Introduction

The intestines within the gastrointestinal system form a fundamental aspect of the body's homeostatic regulation via absorption, electrolyte balance, and autoimmunity via the gut microbiome. Disruption of this system causes complex disorders, most notably inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (Azzouz & Sharma, 2023). Epidemiological data suggest a global rise of IBD, especially in developing, rapidly industrialized regions. Thus, IBD has become an emerging global health issue that warrants novel therapeutic approaches (Hirsch et al., 2024). Ulcerative colitis (UC) is an autoimmune disease primarily affecting the colonic mucosa leading to its inflammation and ulceration. UC has clinically significant symptoms such as bloody diarrhea, abdominal pain, and weight loss (Boyer & Mortimore, 2023). As numerous studies have shown, an excess of immune response, oxidative stress, and intestinal barrier dysfunction are major contributors to disease development (Hirsch et al., 2024). Current therapeutic approaches mainly involve anti-inflammatory drugs, for instance, sulfasalazine, which inhibits leukotrienes and prostaglandins. However, prolonged usage of this drug leads to the existence of side effects that may deter patients from proper treatment adherence

(Patel et al., 2025). On the other hand, other innovative approaches to target specific cellular signaling pathways, such as mTOR pathway using sirolimus (rapamycin) as a highly effective immunosuppressant, have been developed. It is known that the inhibition of mTOR signaling improves epithelial barrier function and decreases immune cell infiltration during experimental colitis, which indicates that it could be a potential therapeutic strategy in refractory cases (Zhao et al., 2013; Gutiérrez-Martínez et al., 2019; Jia et al., 2026). In the quest for new safer and less toxic natural therapeutic alternatives, special emphasis has been placed on the use of phenolic-rich medicinal plants. Phytobiotics and medicinal plants have gained considerable attention in recent years due to their potential as natural alternatives to synthetic additives in nutrition (Amirteymoori et al., 2021; Mohammadabadi et al., 2022). These natural products are rich in bioactive compounds such as essential oils, alkaloids, flavonoids, and phenolic acids, which contribute to their antimicrobial, antioxidant, anticancer, and anti-inflammatory properties (Vahabzadeh et al., 2020; Mohammadabadi et al., 2025). Consequently, phytobiotics play a crucial role in improving health, performance, and immunity (Safaei et al., 2025). The use of phytobiotics and medicinal plants as natural antimicrobial growth promoters in place of antibiotics in feed offers numerous advantages (Khezri et al., 2025). These benefits include improved zootechnical efficiency parameters, suppression of specific diseases (Mohammadabadi et al., 2023), antimicrobial and antioxidant activities, hypocholesterolemic effects, enhancement of digestive enzymes, anticancer role, and improved liver function (Roudbar et al., 2015). Moreover, phytobiotics have been shown to modulate gut microbiota, which enhances nutrient absorption and supports overall immune function (Vahabzadeh et al., 2021). Studies have demonstrated that phytobiotics are associated with reducing stress-related impacts, improving meat quality, regulating gene expression involved in cancer, and decreasing the environmental impact of animal production systems by optimizing nutrient utilization (Mohammadabadi et al., 2024). Pomegranate (Punica granatum L.) is one of the most valuable plants due to its high content of anthocyanins, ellagic acid, and flavonoids (Bar-Ya'akov et al., 2019). The black pomegranate is one of the varieties with unique chemical characteristics. It contains the highest total phenolic compounds compared to common red varieties. The therapeutic effects of black pomegranate peel extract are mainly due to its higher ability to neutralise free radicals and alleviate oxidative stress related to inflammation (El Moujahed et al., 2022). Moreover, black pomegranate peel extract has anti-cancer and antibacterial effects and thus offers an appropriate dietary and therapeutic supplement without any harmful side effects (Khorrami et al., 2019). Recently, it has been reported that these compounds enhance the integrity of the intestinal barrier and balance the inflammatory cytokine response in experimental colitis models (Derosa et al., 2019; Gao et al., 2023). Although a large number of studies have been conducted on pomegranate, there are few studies that compare the effects of black pomegranate with the conventional medication (sulfasalazine) and the newest immunosuppressants (sirolimus) in the treatment of ulcerative colitis. Therefore, the aim of this study was to assess the therapeutic effects of black pomegranate peel extract (BPPEE) and compare it with sulfasalazine and sirolimus in the experimental model of DNBS-induced colitis in mice in terms of clinical, biochemical and histological aspects.

Materials and methods

Experimental procedures were performed in the University of Baghdad, Department of Physiology, Biochemistry and Pharmacology, in accordance with the Scientific Committee on Animal Welfare of the College of Veterinary Medicine.

Experimental design: Adult female Wistar rats 80 number were obtained from an Iraqi cancer research center. The rats were 150-225 g and 6-8 months old. They were kept in 8 cages (ten rats per cage) in the animal house of the Veterinary Medicine College University of Baghdad. They were exposed to light for 12 h/ day with free access to feed and water and were observed for 2 weeks before the experiment at 20-23 degrees C in an air-conditioned room to minimize the effect of stress.

Black pomegranate peel Fruits Collection and extract: The black pomegranate fruits were collected in September 2024 in Karbala. The peels were dried in air and then electro-ground. The powder was extracted with ethanol and acetic acid in a Soxhlet apparatus for 72 hours. The mixture was then filtered under negative pressure. The extract was concentrated with a rotary evaporator at 30-40°C and frozen until it was used. The ethanolic extraction percentage of the black pomegranate peel was calculated with the following equation: BPPEE % = (Weight of the extract / Weight of the crude powder)/100.

Black pomegranate peel extract (BPPEE) dosage rates: BPPEE was prepared in our earlier experiment (in press) and all the details of the characteristics yield extract and Phytoconstituent has been mentioned. Dosage rates of BPPEE were 200 mg/kg BW and 300 mg/kg BW orally in this experiment (Raesi Vanani et al., 2020).

Sulfasalazine dosage rate: Sulfasalazine (Salazopyrin) ® (Pfizer, USA) used at dose 25 mg/kg BW orally (Choi et al., 2024). The rats were randomly divided into eight equal groups of 10 rats each: Control positive (C +ve) induced colitis by 15 mg/kg BW. DNBS (BioDuly, China) 0.25 mL of 50% ethanol intrarectally. Negative control (C -ve) administered 0.25 mL of 50% ethanol intrarectally and given only a vehicle (distilled water) orally by stainless gastric gavage needle for 4 weeks. Group 1: After DNBS- induced colitis given 200 mg/kg BW of BPPEE for 4 weeks. Group2: After DNBS- induced colitis given 300 mg/kg BW of BPPEE for 4 weeks. Group3: After DNBS- induced colitis given, sulfasalazine 25 mg/kg three times daily (TID) for 4 weeks. Group4: After DNBS- induced colitis given 200 mg/kg BW of BPPEE plus 30 µg Sirolimus for 4 weeks. Group5: After DNBS- induced colitis given 300 mg/kg BW of BPPEE plus 30 µg Sirolimus for 4 weeks. Group 6: After DNBS- induced colitis given, only 30 µg Sirolimus.

Sirolimus dosage rate: Sirolimus was administered orally at a dose 30 µg/kg BW (Neef et al., 2006). A stock solution was prepared by finely pulverizing 1 mg tablet of Rapamune® and suspending it in 10 mL of 0.5% carboxymethyl cellulose sodium salt (CMC-Na). The CMC-Na vehicle was prepared by dissolving 5 g of CMC-Na in 100 mL of distilled water, yielding a viscous suspension suitable for oral delivery. From the stock solution, 3 mL was taken and diluted to final volume 10 mL using the same CMC-Na suspension to achieve the desired working concentration. The final formulation was administered orally at a volume of 1 mL per 100 g BW

of rat. once daily for 30 consecutive days. Treatment was initiated 1 week after BNBS -induced colitis to evaluated its therapeutic effect.

Colitis induction by 2,4-Dintrobenzen sulfonic acid (DNBS): Colitis was induced by DNBS (BioDuly, China) according ti the method described by (Barbara et al., 2000) with negligible changes. DNBS 15 mg/kg BW/0.25 mL of 50% ethanol was freshly prepared and administrated intrarectally by using polyethylene catheter (PE-90). The catheter was gently inserted intrarectally about 8 cm above the anus. Inject a small volume of solution into the catheter to lubricate and facilitate insertion. Inject 0.25 mL of DNBS or the same volume of 50% ethanol (controls) slowly. The animals were then placed in the Trendelenburg position for 2 minutes to prevent reflex action and loss of reagent. Animals feed with 8% sucrose water in 0.22% saline to avoid dehydration, Weight monitored to sign weight loss.

Tissue sampling: After completion of the various periods of experiment 4 weeks of treatment, rats were weighed, anesthetized with diethyl ether (Alpha Chemika, India) and sacrificed. The abdomen was opened and distal 8 cm of colon of each rat was removed and opened longitudinally and cleaned from their luminal contents with normal saline and observed for macroscopic evaluation for colitis and then were cut into two pieces. For Histological criteria of colonic tissue by transmission electronic microscopic (TEM) One specimen placed in glutaraldehyde 2.5% for 1 week before used and Malondialdehyde (MDA) and myeloperoxidase (MPO) are two oxidative stress biomarkers that were measured in the second frozen sample.

Macroscopic and transmission electronic of colitis: Macroscopic assessment scoring is determined directly by macroscopic observation of colonic samples. Damage is scored on a 0- to 4- point scale using the system proposed by (Fornai et al., 2006).

Transmission assessment scoring: The transmission electronic microscopic examination was used to evaluate the transmission damage and inflammation, stained with 20% uranyl acetate (BDH Laboratory Chemicals Division, England, No. 0148860) in pure methanol (E. Merck, D-6100 Darmstadt) for 45 min and in Reynolds solution (Reynolds, 1963).

Evaluation of oxidative stress biomarkers: Evaluation of tissue Myleloperoxidase tissue assay (Rat MPO ELISA Kit, Cat. No E0574Ra, Bioassay Technology Laboratory, USA). Myeloperoxidase (MPO) concentration in colonic tissue establishes and suppose as quantitative marker to determine the extent of mucosal infiltration by polymorph nuclear cells.

Determination of tissue Malondialdehyde: To determine the quantitatively degree of membrane lipid peroxidation, concentration of Malondialdehyde (MDA) in colonic specimens was examined using MDA ELISA Kit (cat no E0156Ra, Bioassay Technology Laboratory, USA).

Statistical analysis: Statistical analysis was carried out using one-way and two-way analysis of variance (ANOVA). Two-way ANOVA was used for parameters measured over time, for example, changes in body weight, to assess the effects of treatment, time, and their interaction. For single-time-point measurements, one-way analysis of variance (ANOVA) was employed. These include biochemical parameters like myeloperoxidase (MPO) activity, malondialdehyde (MDA) content, and. Macroscopic scoring of colonic tissue, with post hoc analysis where appropriate. Results were represented as mean ± SEM. A p value of less than 0.05 was considered statistically significant.

Results and discussion

Black pomegranate peel extract properties and yield: Once it is exposed to ethanol (70%), has a dense texture, different colors, and a pungent taste, which is due to its richness in phenolic compounds, pigments, and acids, as described in this variety. The total yield is around 42.5%, which is within the world reference limits, up to 48%. These differences are directly related to growing conditions, model experience, and agricultural diversity in the variety (Zhang et al., 2023).

Assessment of the effect of Black pomegranate peel ethanolic extract (BPPEE), sulfasalazine and sirolimus on ulcerative colitis-Clinical observation: The clinical signs, that observed throughout the experiment period in the animals of various experimental groups are illustrated in the Table 1.

Table 1. Body weight changes in experimental groups

Group	Pretreatment (g)	4 weeks (g)	LSD	P-value
Negative control	205.50 ± 2.40 ^{bB}	231.00 ± 4.71 ^{aC}	12.92	<0.001
Positive control (DNBS)	202.30 ± 2.29 ^{aB}	168.30 ± 2.78 ^{bH}	8.81	<0.001
G1 (200 mg BPPEE)	203.30 ± 1.38 ^{bB}	232.80 ± 1.93 ^{aC}	5.81	<0.001
G2 (300 mg BPPEE)	184.80 ± 1.03 ^{bC}	190.30 ± 2.48 ^{aG}	3.27	<0.001
G3 (Sulfasalazine)	189.30 ± 2.18 ^{bC}	207.50 ± 1.85 ^{aE}	6.98	<0.002
G4 (200 mg BPPEE + sirolimus 30 µg/kg)	214.00 ± 2.04 ^{bA}	256.00 ± 2.94 ^{aB}	8.76	<0.001
G5 (300 mg BPPEE + sirolimus 30 µg/kg)	216.80 ± 1.65 ^{bA}	277.30 ± 2.29 ^{aA}	11.06	<0.001
G6 (Sirolimus 30 µg/kg)	211.80 ± 3.79 ^{bA}	224.50 ± 3.59 ^{aD}	12.78	<0.001
LSD	5.22	6.86		

Values are expressed as mean ± SEM (n = 10). Means within the same row that do not share a common lowercase letter are significantly different ($P < 0.05$). Means within the same column that do not share a common uppercase letter are significantly different ($P < 0.05$).

Results briefly show the clinical signs included diarrhea and bloody stools during the trial period. There was a difference in the disappearance of diarrhea between the groups, as the positive control group continued for approximately 4 weeks, while the therapeutic groups disappeared after a week of treatment, and the best group in the disappearance of diarrhea was group 5 (300 mg/kg of BPPEE).The appearance of stools changed from liquid to semiliquid pasty at 2 weeks of treatment and then to normal after 3 weeks of treatment. Other clinical signs including dehydration, abdominal pain, back arching, lethargy, anorexia, increased water consumption, rough hair coat, dullness, calming, hunching over and reduced movement observed in all experimental groups. In the control positive group, clinical signs continued about 2 weeks of the experiment while treated groups BPPEE 200 mg/kg BW, 300 mg/kg BW, Sulfasalazine, BPPEE 200 mg/kg BW +sirolums 30 µg/kg BW, BPPEE 300 mg/kg BW+ sirolums 30 µg /kg BW1 , and sirolums 30 µg/kg BW) showed gradual improvement. The recovery was evaluated by ceasing of diarrhea, hematochezia, weight stabilization, returning to normal status patterns. There were no differences in animal weight or feces consistency between the negative control and treated groups

(BPPEE 200 mg/kg BW, BPPEE300 mg/kg BW, Sulfasalazine, BPPEE 200 mg/kg BW +sirolums30µg /kg BW, BPPEE 300 mg/kg BW +sirolums 30µg /kg BW and sirolums 30 µg/kg BW alone) after 4 weeks of treatment.

Body weight Changes(g) of experimental groups: The pre-treatment body weights are clearly homogenous, with no significant differences among the groups. As expected, all groups had equal baseline weights. After four weeks of treatment, however, there were already significant differences between groups according to the type of therapeutic intervention. In the negative control, a significant increase in body weight was observed after the trial period compared to baseline values ($P < 0.05$), which reflects normal growth in healthy animals. In contrast, the positive control (DNBS) had a reduction in weight after induction showed statistical significance decrease, as shown by $P > 0.05$. The control positive group recorded the lowest final body weight values compared to the other treatment groups (G1-G6), indicating a detrimental effect of colitis induction on body weight indicators. Group G1 treated with 200 mg BPPEE showed a significant increase in weight compared with the pre-treatment value, and it was superior to the treatment groups; however, its improvement was less than that obtained in combined treatment groups. Group G2 received 300 mg BPPEE and showed a significant increase ($P<0.05$); the degree of improvement remained relatively lower than that with the lower dose, indicating a dose-response variability. In addition, group G3, Sulfasalazine showed significant weight gain after treatment with a P-value of less than 0.05. The overall improvement body weight was at a moderate level, as compared to the extract alone and combination treatment. Group G4 (200 mg BPPEE + Sirolimus) showed a clear and significant increase in weight, with $P < 0.05$. The improvement body weight was higher as compared to all single-dose groups, indicating a synergistic effect between the extract and sirolimus. In group G5 (300 mg BPPEE + sirolimus), the highest rate in weight gain was obtained when compared with all groups where $P < 0.05$ versus baseline and other groups after treatment. It was thereby the most effective of all groups. Group G6 on the other hand showed sirolimus only, revealing a significant increase in weight after treatment ($P < 0.05$), with its improvement being better than the infected group but remained less than the combination treatment (Table 2).

Table 2. Macroscopic scoring of colonic tissue of the experimental groups

Group	30 days post-treatment	Significance
Negative control	1.00 ± 0.00	E
Positive control (DNBS)	4.50 ± 0.28	A
G1 (200 mg BPPEE)	3.25 ± 0.25	B
G2 (300 mg BPPEE)	2.50 ± 0.28	CD
G3 (Sulfasalazine)	2.75 ± 0.25	C
G4 (200 mg BPPEE + sirolimus 30 µg/kg)	1.50 ± 0.28	E
G5 (300 mg BPPEE + sirolimus 30 µg/kg)	1.50 ± 0.28	E
G6 (Sirolimus 30 µg/kg)	2.25 ± 0.25	D
LSD	0.62	
P-value	<0.05	

Data are presented as mean ± SE (n = 10). Different superscript letters indicate statistically significant differences among groups based on LSD post-hoc test ($P < 0.05$).

Macroscopic scoring of colonic tissue of the experimental animals: Macroscopic inspection of the colon samples 4 weeks after DNBS-induced colitis. The gross injury scores observed for the 200 mg/kg of BPPEE and 300 mg/kg of BPPEE combination plus 30µgm/kg sirolimus treatment groups were lower compared to those of the other groups, with scores of 1.50 ± 0.28 indicating significant $P<0.05$ recovery. In comparison, the injury score of the positive control (DNBS only) (4.50 ± 0.28) was significantly $P<0.05$ higher than those of other experimental groups, indicating the most severe damage after inflammation without medication (Table 3).

Table 3. Concentration of Myeloperoxidase (MPO) in colonic tissue of experimental groups

Group	MPO (ng/g tissue)	Significance
Negative control	0.73 ± 0.06	G
Positive control (DNBS)	3.35 ± 0.04	A
G1 (200 mg/kg BPPEE)	2.33 ± 0.06	B
G2 (300 mg/kg BPPEE)	1.73 ± 0.06	C
G3 (Sulfasalazine)	1.33 ± 0.06	D
G4 (200 mg/kg BPPEE + sirolimus 30 µg/kg)	0.91 ± 0.06	F
G5 (300 mg/kg BPPEE + sirolimus 30 µg/kg)	0.62 ± 0.06	G
G6 (Sirolimus 30 µg/kg)	1.16 ± 0.02	E
LSD	0.14	
P-value	<0.05	

Data are presented as mean ± SE (n = 10). Different superscript letters indicate statistically significant differences among groups based on LSD post-hoc test ($P < 0.05$).

Transmission electron micrographs-Positive control group: The results of transmission electron micrographs of positive group of colons induced colitis by DNBS 4 weeks showing the architecture of colon epithelial cells. Images show the ultrastructure of epithelial cells and their content: nucleus, non-active mitochondria with epithelial cells, sloughed microvilli, cytoplasmic ultra vacuoles, necrotic structures and regular gap junction. Image identified also goblet cells, and some necrotic mucous with apoptotic cells (Figure 1).

Negative control group: The results of transmission electron micrographs of negative control group of rat colon for 4 weeks showing the architecture of colon epithelial cells. Images show the ultrastructure of epithelial cells and their content: nucleus, active mitochondria with epithelial cells, regenerated microvilli, cytoplasmic ultra vacuoles and regular gap junction. Image identified also goblet cells, and some active fibroblast (Figure 2).

G1 (200 mg BPPEE): The results of Transmission electron micrographs of G1 on the rat colon induced colitis by DNBS after dosing of BPPEE PO for 4 weeks showing the architecture of intestinal epithelial cells and several types of cells in the colon. Images (a, b, c, & d) show the ultrastructure of epithelial cells and their content actives mitochondria, organized microvilli and clearly gap junction (GJ). Images are identified some goblet cells, inflammatory cells, cytoplasmic vacuolation and fibroblasts (Figure 3).

G2 (300 mg BPPEE): The results of transmission electron micrographs on G2 of rat colon induced colitis by DNBS administered BPPEE PO for 4 weeks showing the architecture of colon epithelial cells. Images (a, b, c, & d) show the ultrastructure of epithelial cells and their content of clear nucleus, active mitochondria, cytoplasmic ultra vacuoles. Image identified active goblet cells, epithelial cells, newly capillary and inflammatory cells (Figure 4).

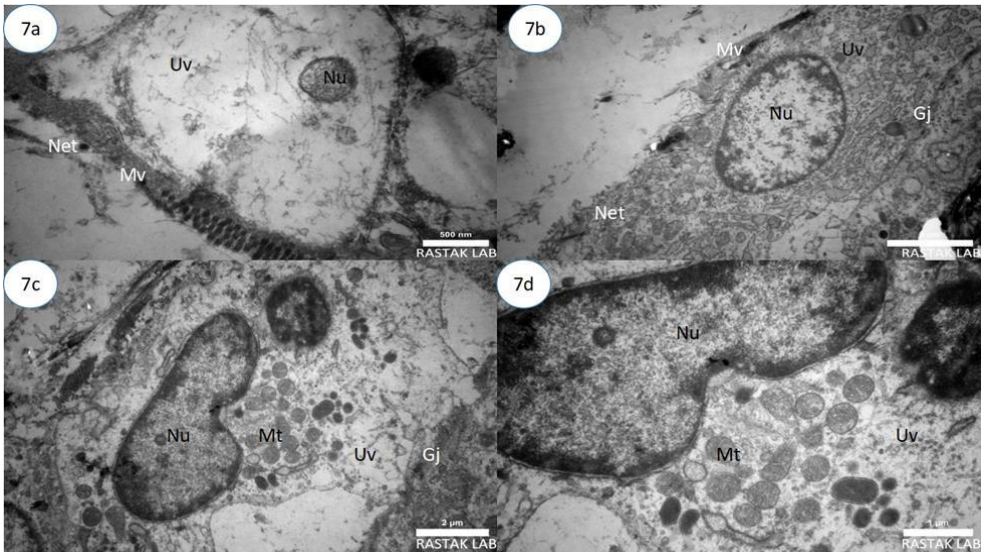

Figure 1. Transmission electron micrographs of the colon of rats with DNBS-induced colitis after 4 weeks, showing the ultrastructure of epithelial cells (Positive control). Images (a-d) show epithelial cell components including nucleus (Nu), mitochondria (Mt), microvilli (Mv), vacuoles (V), and gap junctions (Gj). Goblet cells (GC) and necrotic tissue (Net) are also observed. Scale bars: a = 5 µm, b = 1 µm, c = 2 µm, d = 1 µm

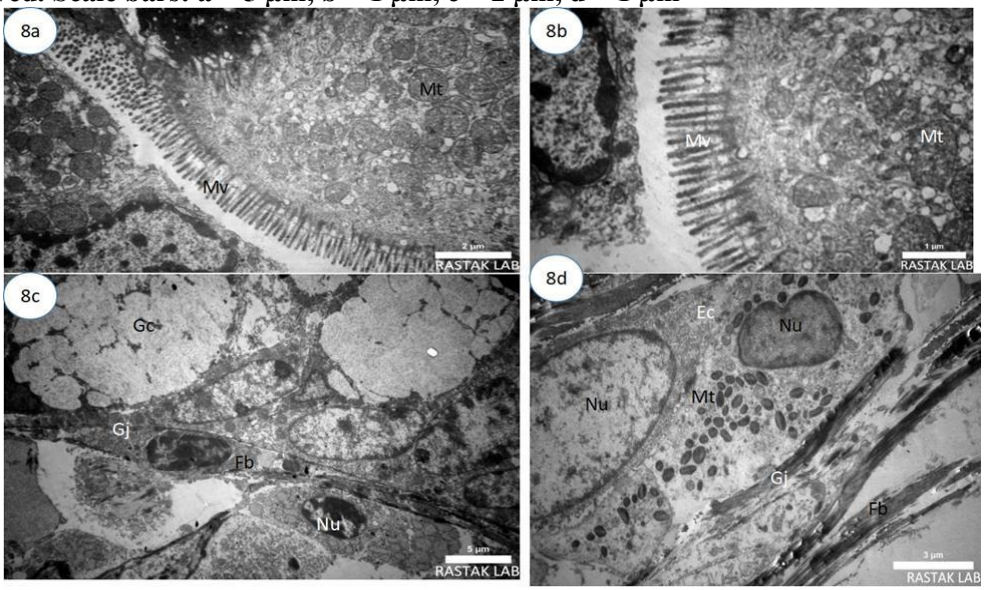

Figure 2. Transmission electron micrographs of normal rat colon after 4 weeks, showing preserved epithelial architecture (Negative control). Images (a-d) show nucleus (Nu), active mitochondria (Mt), well-developed microvilli (Mv), vacuoles (V), epithelial cells (Ec), fibroblasts (Fb), and gap junctions (Gj). Goblet cells (GC) are also present. Scale bars: a = 2 µm, b = 1 µm, c = 5 µm, d = 3 µm

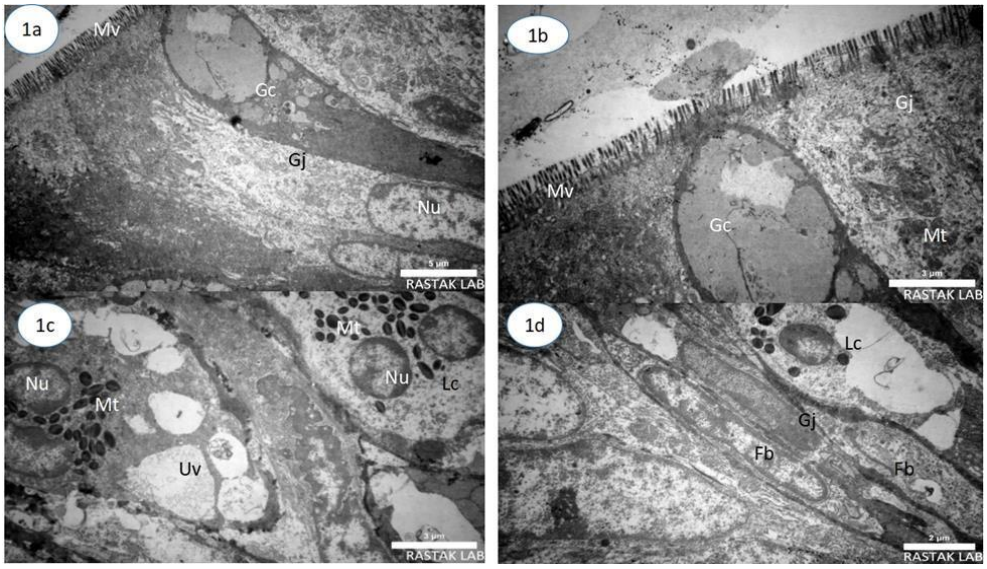

Figure 3. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with BPPEE (200 mg/kg) for 4 weeks (G1: BPPEE 200 mg/kg). Images (a-d) show nucleus (Nu), mitochondria (Mt), microvilli (Mv), and gap junctions (Gj). Leukocytes, cytoplasmic vacuolation, goblet cells, and fibroblasts are also observed. Scale bars: a = 5 µm, b-c = 3 µm, d = 2 µm

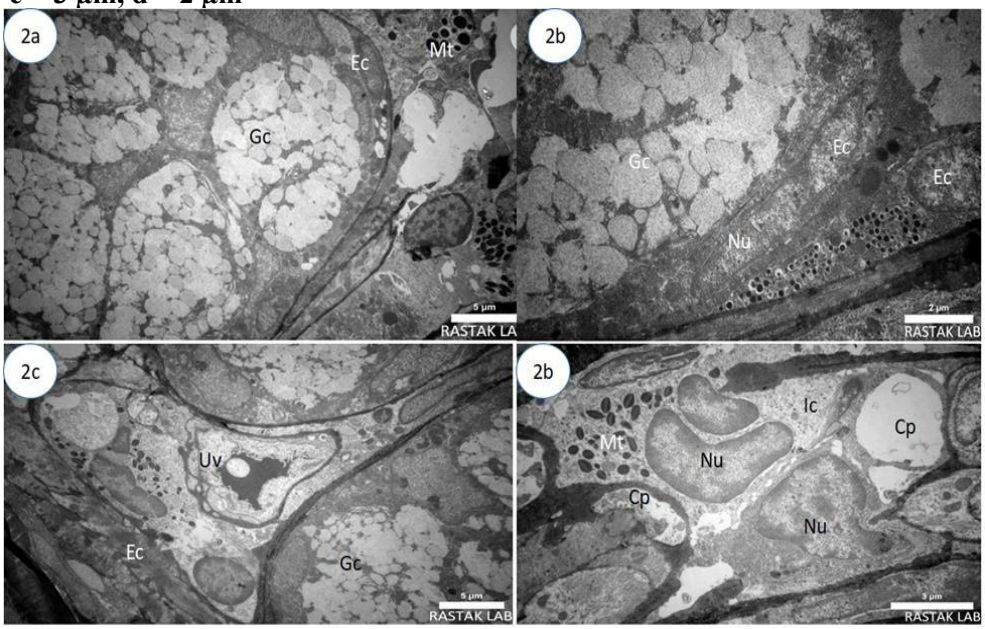

Figure 4. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with BPPEE (300 mg/kg) for 4 weeks (G2: BPPEE 300 mg/kg). Images (a-d) show nucleus (Nu), mitochondria (Mt), and vacuoles (V). Goblet cells (GC), epithelial cells (Ec), capillaries (Cp), and inflammatory cells (Ic) are also identified. Scale bars: a = 5 µm, b = 3 µm, c = 5 µm, d = 3 µm

G3 (sulfasalazine): The results of the transmission electron micrographs of G3 on rat colon induced colitis by DNBS after administered sulfasalazine 25 mg/kg PO for 4 weeks showing the architecture of colon epithelial cells. Images show the ultrastructure of epithelial cells and their content nucleus, active mitochondria (Mt), degeneration with cytoplasmic ultra vacuoles. Image identified also microvilli, gap junction and fibroblasts (Figure 5).

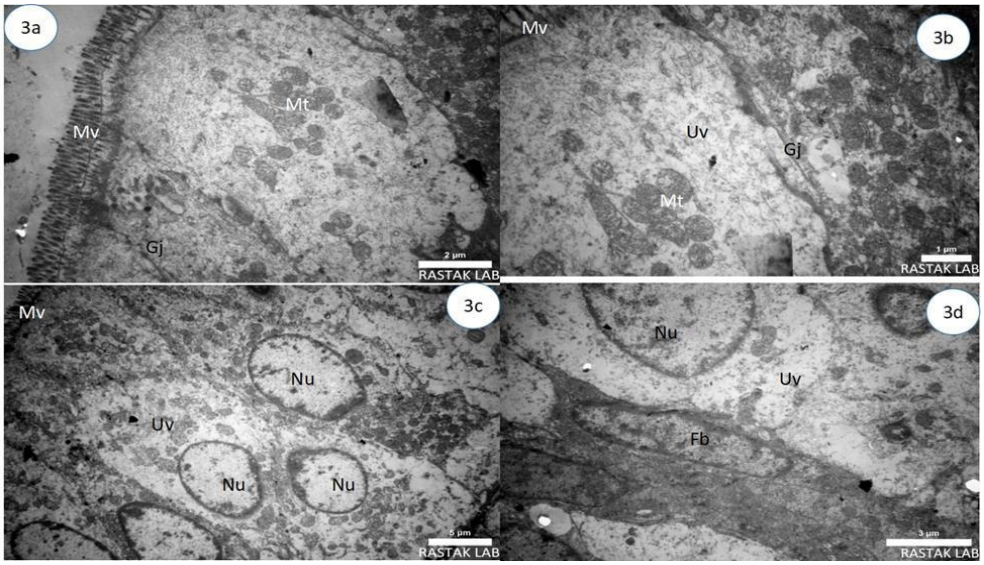

Figure 5. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with sulfasalazine (25 mg/kg, PO) for 4 weeks. Images (a-d) show nucleus (Nu), mitochondria (Mt), vacuoles (V), microvilli (Mv), and gap junctions (Gj). Scale bars: a = 2 µm, b = 1 µm, c = 5 µm, d = 3 µm

G4 (200mg BPPEE+ sirolimus 30 µg/kg BW): The results of transmission electron micrographs of G4 on the rat colon induced colitis by DNBS after administered the 200 mg/kg VW of BPPE PO plus sirolimus 30 µg/kg BW day for 4 weeks showing the architecture of colon epithelial cells. Images show the ultrastructure of epithelial cells and their content: nucleus, inactive mitochondria, degeneration with cytoplasmic ultra vacuoles. Image identified irregular goblet cells, degenerated epithelial cells with soughed microvilli, plenty of capillary and active gap junction (Figure 6).

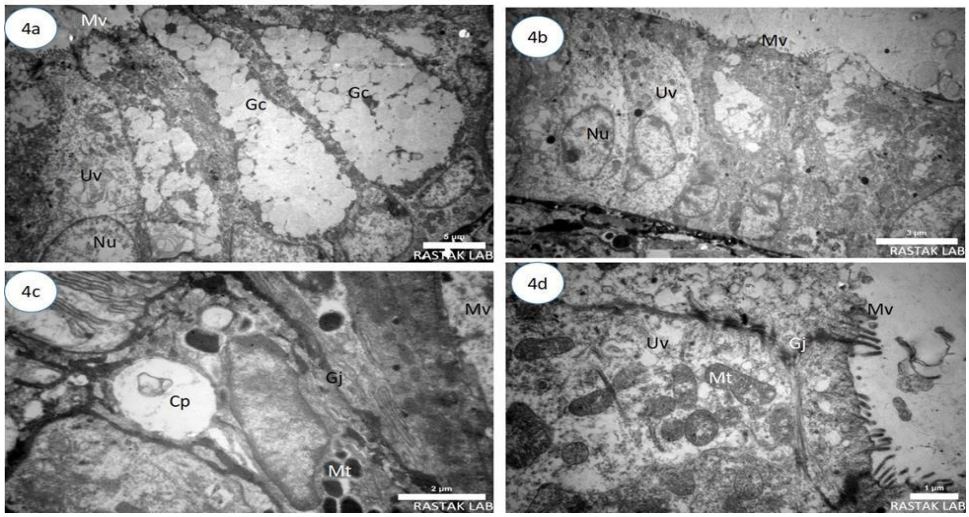

Figure 6. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with BPPEE (200 mg/kg) and sirolimus (30 µg/kg BW/day) for 4 weeks (G4: BPPEE 200 mg/kg + sirolimus). Images (a-d) show nucleus (Nu), mitochondria (Mt), vacuoles (V), microvilli (Mv), epithelial cells (Ec), capillaries (Cp), goblet cells (GC), and gap junctions (Gj). Scale bars: a = 5 µm, b = 3 µm, c = 5 µm, d = 3 µm

G5 (300mg BPPEE+ sirolimus30µg /kg BW): The results of transmission electron micrographs of G5 of rat colon induced colitis by DNBS after that induced colitis by DNBS after administered 300 mg/kg BW of BPPEE PO plus sirolimus 30 µg/kg BW day for 4 weeks showing the TEM analysis conducted for the G5 group revealed remarkable enhancement in suprastructural architecture of the colonic tissues, without any loss of nucleus organization and the arrangement of the cellular elements. The tissues seemed to be normal with reduced degenerative and necrotic properties in comparison with those in the positive control group. It is thus evident from these findings that the administration of BPPEE and sirolimus together was effective in reducing cell damage and regeneration of tissue. The small changes are merely indicative of any damage that may have been caused initially (Figure 7).

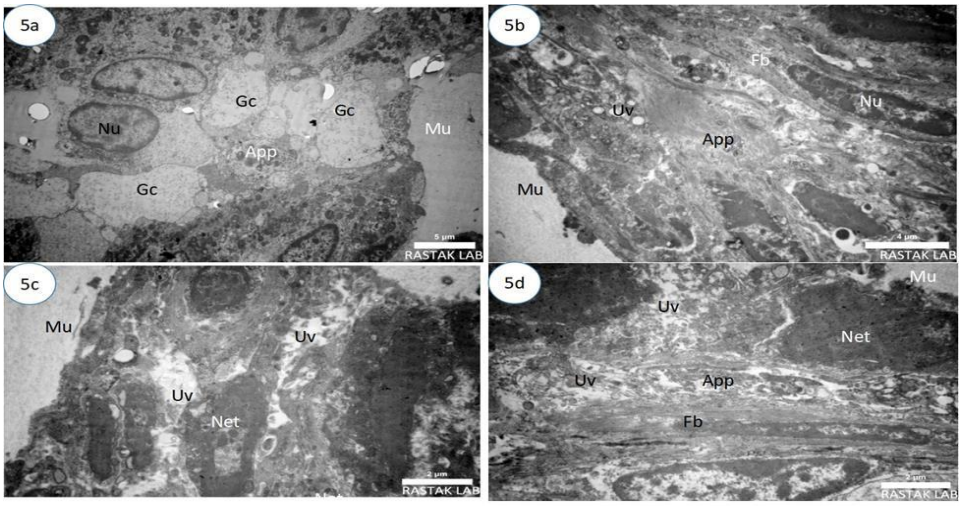

Figure 7. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with BPPEE (300 mg/kg) and sirolimus (30 µg/kg BW/day) for 4 weeks, showing marked improvement in epithelial ultrastructure (G5: BPPEE 300 mg/kg + sirolimus). Images (a-d) show nucleus (Nu), mitochondria (Mt), vacuoles (V), goblet cells (GC), mucin cells (Mu), and fibroblasts (Fb). Reduced necrosis (Net) and fewer apoptotic cells (App) are observed. Scale bars: a = 2 µm, b = 2 µm, c = 1 µm, d = 1 µm

G6 (only sirolimus 30 µg/kg BW): The results of transmission electron micrographs of G6 of rat colon induced colitis by DNBS after administered only sirolimus 30 µg/kg BW for 4 weeks showing the architecture of colon epithelial cells. Images show the ultrastructure of epithelial cells and their content: nucleus, active mitochondria with epithelial cells, regenerated microvilli, cytoplasmic ultra vacuoles and regular gap junction. Image identified also goblet cells, and some necrotic mucous with apoptotic cells (Figure 8).

The concentration of Myeloperoxidase (MPO) in the colonic tissue of the experimental animals: Myeloperoxidase enzyme activity measurements in colon tissue provided clear significant differences among the different study groups ($p<0.05$). The highest level of MPO and significantly higher than all other groups were found in the positive group (G^{+ve}) with untreated colitis. The lowest level of enzyme, the normal level in non-inflamed tissue, was found in the negative group (G^{-ve}). BPPEE treatment resulted in significant $P<0.05$lowering of MPO; activity

decreased in the 200 mg/kg treated group (G1) when compared with the G^{+ve} group, and further decrease was observed in the 300 mg/kg higher dose-treated group (G2) when compared to G1. Sulfasalazine (G3) treatment also caused substantial reduction in MPO activity, and its values were as low as were found in the high extract group (G2). Black pomegranate extract combination therapy with sirolimus (G4 and G5) caused the greatest reduction in MPO; The G5 group (300 mg extract + sirolimus) had the lowest level of enzyme, and this was not significantly $P<0.05$ different from the G^{-ve} healthy group. Sirolimus alone (G6) also suppressed MPO activity considerably compared to G^{+ve}, but less so than the combination treatments of the extract and the extract (Table 4).

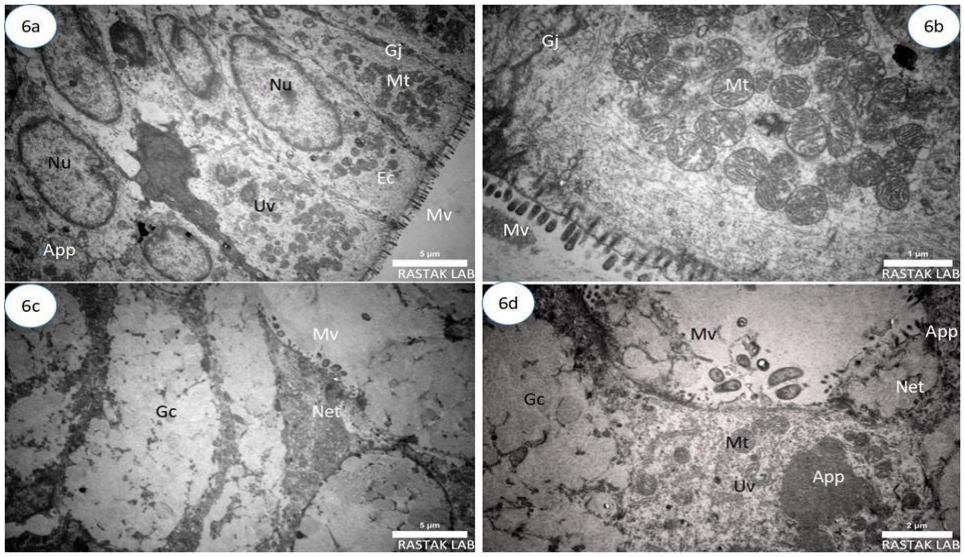

Figure 8. Transmission electron micrographs of colon tissue from DNBS-induced colitic rats treated with sirolimus (30 µg/kg BW/day) for 4 weeks (G6: sirolimus only). Images (a-d) show nucleus (Nu), mitochondria (Mt), microvilli (Mv), vacuoles (V), epithelial cells (Ec), and gap junctions (Gj). Goblet cells (GC), mucin (Mu), apoptotic cells (App), and necrotic tissue (Net) are also observed. Scale bars: a = 5 µm, b = 1 µm, c = 5 µm, d = 2 µm

Table 4. Effect of malondialdehyde (MDA) levels in the colonic tissue of experimental animals

Groups	MDA (ng/g tissue)
Negative control	59.8 ± 4.32 H
Positive control	269.7 ± 3.35 A
G1 (BPPEE, 200 mg/kg)	216.5 ± 3.05 B
G2 (BPPEE, 300 mg/kg)	160.1 ± 3.22 C
G3 (Sulfasalazine)	150.2 ± 3.20 D
G4 (BPPEE 200 mg/kg + sirolimus 30 µg/kg BW)	120.8 ± 3.27 E
G6 (sirolimus 30 µg/kg BW)	108.3 ± 3.09 F
G5 (BPPEE 300 mg/kg + sirolimus 30 µg/kg BW)	85.5 ± 3.15 G

Data are expressed as mean ± SE. LSD = 7.83; $P < 0.05$. Means with different letters indicate significant differences, while those sharing the same letter are not significantly different at $P < 0.05$ according to the LSD test

The concentration of Malondialdehyde (MDA) in the colonic tissue of the experimental groups: The results of the measurement of malondialdehyde concentration in colon tissue showed evident differences $P<0.05$ between the experimental groups (Table 4). The lowest values of MDA were recorded for the negative control group, which reflected integrity of the tissue and lack of oxidative stress. In contrast, the positive control group-wherein colitis was induced without any treatment, showed the highest MDA levels. This increase was significant ($P<0.05$) when compared to the negative control group and confirmed the occurrence of severe oxidative damage due to DNBS induction. Indeed, all the treatment groups (G1-G6) exhibited a significant decline, as compared to the positive control group, in the MDA level at $P<0.05$, though variations exist between the treatments. Group G1, which consisted of BPPEE 200 mg/kg, showed a significant decrease compared to the positive control group, suggesting an antioxidant activity at the dose of 200 mg/kg. Group G2 (BPPEE 300 mg/kg) had higher MDA level reductions than in Group G1, indicating that increasing the dose did indeed result in a more effective response and a greater reduction in oxidative stress. In addition, G3 (Sulfasalazine) treatment displayed a highly significant decrease in MDA levels compared to the positive group, being comparable in extent to the highest dose of the extract used. Group G6 (Sirolimus only) also demonstrated a significant decrease in MDA compared with the positive group, proving its ability to reduce oxidative damage in inflamed colon tissue. On the other hand, the two combined groups, G4 and G5, which received a mixture of black pomegranate peel ethanolic extract and sirolimus, respectively, had the greatest MDA reduction among all treatment groups. Group G5, which contained the highest dose of BPPEE plus sirolimus, resulted in the most significant reduction of all the treatment groups. This result points out the strong synergistic effect between the plant extract and sirolimus in lessening oxidative stress caused by colitis, which is supported by the significant P-value that confirmed clear differences in comparison to the rest of the groups. The findings of the present study validate the promising therapeutic potential of the ethanolic extract of black pomegranate peel (BPPEE) in the treatment of ulcerative colitis induced by DNBS in rat. This is clearly demonstrated when the combination of BPPEE and sirolimus is used; the best therapeutic response among all the clinical, histological, and biochemical parameters was shown by group G5, which received the high dose of the extract (300 mg/kg) along with sirolimus. The substantial clinical response in terms of the resolution of diarrhea and rectal bleeding in both treated groups following just one week of treatment, in comparison with the positive control group, was attributed to the greater capacity of phenolic compounds in BPPEE in promoting healing. The weight regained in both treated groups (G4, G5) also indicates the effectiveness of the treatment in addressing malabsorption/malassimilation and fluid loss associated with chronic inflammation, which is in line with other studies indicating that polyphenolic compounds have chemo preventive properties on the intestine (Bar-Ya'akov et al., 2019). Though there was presence of necrotic zone on TEM, it cannot refute the better therapeutic effects of the G5 group. The first necrotic zone is thought to be caused by late damage due to the chemically induced injury for about a month, rather than damage itself. Also, TEM observation involved tiny portions of tissue samples and could not reflect the general condition. On the contrary, the results in general showed that there was an improvement in macroscopical observation, histological study, and reduction in the level

of oxidative stress, MDA and MPO (significant MDA). Thus, the evaluation of therapeutic effects must involve thorough study, and not mere clinical observation. From the results, it was observed that there was a significant reduction in myeloid peroxidase (MPO) activity and myelondylaldehyde (MDA) levels in BPPEE-treated groups. The reduction in MPO activity directly reflects the reduction in neutrophil infiltration in colon tissue, thus limiting tissue damage due to the secretion of inflammatory mediators (Piechota-Polanczyk & Fichna, 2014). On the other hand, the significant reduction in MDA levels reflects the effectiveness of BPPEE in inhibiting lipid peroxidation and maintaining cell membrane integrity from oxidative stress. This is attributed to the richness of BPPEE in ellagitannins and anthocyanins, which serve as free radical scavengers (Hering et al., 2021). The use of BPPEE in combination with sirolimus is an innovative therapeutic approach. While sirolimus works by inhibiting the mTOR pathway to control the proliferation of immune cells, which in turn reduces the production of pro-inflammatory mediators, pomegranate extract works by improving the intestinal microenvironment, thus enhancing the mucosal barrier. This explains why the combined groups showed statistical significance in reducing the histological score of inflammation, as demonstrated by (Borichevsky et al., 2025). Transmission electron microscopy was used to demonstrate that cells in the treated groups showed microvilli regeneration, restoration of mitochondrial structures, and the formation of vacuole junctions, thus showing the restoration of the physiological function of the colonic epithelium, as demonstrated by (Zhang et al., 2025). The study concludes that BPPEE not only acts as an anti-inflammatory agent but also acts as an adjuvant that potentiates immunosuppressive drugs like sirolimus, which in turn provides new opportunities for reducing doses of chemical drugs with fewer side effects (Roychowdhury et al., 2025).

Novelty: While many studies have been conducted on the therapeutic potential of Punica granatum in the management of inflammatory bowel diseases, the novelty of the present study is its holistic and multidimensional approach. To the best of our knowledge, this is the first study to evaluate the potential effects of an ethanolic extract of the rare black pomegranate variety grown in Karbala, Iraq, in comparison with the immunotherapy drug Sirolimus and the traditional medication Sulfasalazine. In addition, the present study includes the comprehensive chemical analysis by GC-MS, which explains the role of the unique phenolic compounds in the therapeutic potential. The holistic approach includes the clinical, histological, and immunological aspects. The results demonstrate the potential value of the black pomegranate variety as an adjunct therapy or an alternative for the management of inflammatory bowel diseases.

Authors' Contribution

SSA and FMK designed the study. SSA conducted the experimental work and collected the samples. FMK performed the statistical analysis and interpretation of data. SSA wrote the manuscript. All authors read and approved the final version of the manuscript.

Data availability statement

The data of this research article are available from the authors upon reasonable request.

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Ethical considerations

The experimental procedures were approved by the Institutional Animal Ethics Committee and conducted at the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq (Bu180906 dated 15/7/2024). Ethical regulation of this work was assigned by the university research committee and adhered to the guidelines of the American Veterinary Medical.

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Conflict of interest

The authors declare that there is no conflict of interest related to this research.

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اثرات درمانی و تعدیل‌کننده ایمنی عصاره پوست انار سیاه (منشأ عراق) به‌همراه سی‌رولیموس در مدل آزمایشی کولیت اولسراتیو در موش صحرایی

سارا س. عبدالعباس

* نویسنده مسئول. گروه فیزیولوژی، بیوشیمی و فارماکولوژی، دانشکده دامپزشکی، دانشگاه بغداد، بغداد، عراق. ایمیل: Sara.sameer.ms12@gmail.com

فلاح م. ک. الرکابی

گروه فیزیولوژی، بیوشیمی و فارماکولوژی، دانشکده دامپزشکی، دانشگاه بغداد، بغداد، عراق. ایمیل: falah@covm.uobaghdad.edu.iq

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چکیده

هدف: کولیت اولسراتیو نوعی بیماری التهابی مزمن روده است که با آسیب مزمن مخاط کولون مشخص می‌شود. درمان‌های موجود عمدتاً فقط علائم بیماری را کنترل می‌کنند و درمان قطعی ارائه نمی‌دهند. هدف این مطالعه بررسی اثرات درمانی و آنتی‌اکسیدانی عصاره اتانولی پوست انار سیاه (BPPEE) به‌صورت تکی یا در ترکیب با سی‌رولیموس و مقایسه آن با درمان استاندارد سولفاسالازین در مدل کولیت القاشده با ۲،۴–دی‌نیتروبنزن‌سولفونیک اسید در موش صحرایی بود.

مواد و روش‌ها: موش‌ها به‌طور تصادفی به هشت گروه تقسیم شدند: گروه کنترل منفی، گروه کنترل مثبت، و گروه‌های درمانی شامل دوزهای مختلف BPPEE (۲۰۰ و ۳۰۰ میلی‌گرم بر کیلوگرم)، سولفاسالازین، سی‌رولیموس، و ترکیب BPPEE با سی‌رولیموس به مدت چهار هفته. میوه‌های انار سیاه در سپتامبر ۲۰۲۴ از باغ‌های کربلا در عراق جمع‌آوری شدند و عصاره با استفاده از اتانول ۷۰٪ همراه با ۱٪ اسید استیک تهیه گردید. عصاره دارای رنگ بنفش تیره، بافت غلیظ و چسبناک، طعم ترش و بوی معطر بود و بازده استخراج ۴۲.۵٪ گزارش شد. اثرات درمانی بر اساس مشاهدات بالینی، تغییرات وزن بدن، بررسی‌های بافت‌شناسی و میکروسکوپ الکترونی عبوری از بافت کولون، و اندازه‌گیری شاخص‌های استرس اکسیداتیو شامل مایلوپراکسیداز (MPO) و مالون‌دی‌آلدئید (MDA) ارزیابی شد.

نتایج: در تمامی گروه‌های تیمار شده در مقایسه با گروه کنترل مثبت، بهبود قابل‌توجهی در علائم بالینی و افزایش تدریجی وزن بدن مشاهده شد. بررسی‌های بافت‌شناسی و میکروسکوپ الکترونی نشان داد که یکپارچگی ساختار سلولی اپی‌تلیوم کولون به‌طور قابل‌توجهی بهبود یافته است، به‌ویژه در گروه‌هایی که درمان ترکیبی دریافت کردند. همچنین در این گروه‌ها کاهش معنی‌داری در سطوح MPO و MDA مشاهده شد.

نتیجه‌گیری: یافته‌های این مطالعه نشان می‌دهد که عصاره پوست انار سیاه دارای خواص ضدالتهابی و آنتی‌اکسیدانی است. همچنین استفاده همزمان از این عصاره با سی‌رولیموس اثر هم‌افزایی قابل‌توجهی در بهبود شاخص‌های بالینی، بافتی و بیوشیمیایی در مدل کولیت اولسراتیو دارد. بنابراین، این ترکیب می‌تواند به‌عنوان یک رویکرد امیدوارکننده در مدیریت کولیت اولسراتیو مطرح شود.

کلمات کلیدی: اثرات درمانی، بیماری التهابی روده، عصاره پوست انار سیاه، کولیت اولسراتیو، موش صحرایی

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