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Anti-colitic, nephroprotective, and acute toxicity profiles of *Punica granatum* peel aqueous extract in experimental models

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Summary

This investigation was conducted to determine the potential of *Punica granatum* as a protective treatment for ulcerative colitis and for nephroprotection. The aqueous extract of *Punica granatum* peel (APPE) was subjected to phytochemical examination by qualitative and quantitative techniques. Sulfasalazine at a dose of 600 mg/kg and APPE at doses of 200 and 400 mg/kg were orally administrated to male Wistar rats during 21 days. On day 17, the rats were transrectally given acetic acid to induce ulcerative colitis. A comparative analysis was conducted using a positive control group with colitis and a negative control group without colitis. Colon macroscopic damage, ulcer index, oxidative stress markers, histological investigation, and anti-inflammatory factors were evaluated. Nephroprotective activity was investigated in an animal model of sulfasalazine-induced kidney damage. The phytochemical analysis of APPE revealed the presence of various bioactive compounds, and the HPLC-UV profile of APPE revealed an important content of polyphenolic compounds, such as resveratrol (552.17 µg/ml), chlorogenic acid (376.15 µg/ml), and 3,4-dihydroxybenzoic acid (329.00 µg/ml). The results obtained show that APPE exhibited activity against ulcerative colitis by significantly modulating antioxidant defense mechanisms, namely superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), in the colon tissues of APPE-treated groups in comparison with the positive group. The histopathological assessment showed a notable decrease in microscopic damage in groups receiving sulfasalazine and APPE at 400 mg/kg, which facilitated mucosal healing and reduced inflammatory cell infiltration. The examination of the kidneys revealed histopathological changes confirmed by tubulointerstitial necrosis. Nevertheless, the biochemical parameters were substantially improved in rats pre-treated with graded oral doses of the extract in a dose-related manner. The extract provided the most effective nephroprotection at 400 mg/kg/day. It would be interesting to conduct further tests in the use of APPE extracts as a pre-treatment for ulcerative colitis and nephropathy.

Keywords: *Punica granatum*, oxidative stress, acute toxicity, ulcerative colitis, nephroprotection

Ulcerative colitis (UC) is frequently associated with problems outside of the intestines. Extra digestive manifestations can affect virtually any organ. The joints, skin, eyes, liver, and bile ducts are the most often affected, with spleen and liver damage occurring in 4-23% of cases (1, 2). At present, there is an increasing understanding of renal signs and problems in individuals with inflammatory bowel disease (IBD), primarily because of increased attention from healthcare professionals. The most frequent problems are renal lithiasis, glomerulonephritis, tubule interstitial nephritis, and type AA secondary amyloidosis (3). According to recent research, oxidative stress is implicated in the development of intestinal inflammation through a number of underlying processes, such as the generation of excessive reactive oxygen species (ROS), immune cell infiltration, and overexpression of inflammatory cytokines (4, 5). Furthermore, sulfasalazine, the first-line drug for treatment of ulcerative colitis, can cause renal injuries, such as kidney enlargement, interstitial nephritis, tubular atrophy, and renal necrosis (6, 7). Drug-induced nephrotoxicity continues to be a significant issue due to the inevitable usage of nephrotoxic medicines in clinical settings. Moreover, some studies have demonstrated a decline in kidney function following sulfasalazine treatment (8, 9), with a potentially irreversible damage (10, 11).

The specific mechanism underlying kidney damage caused by sulfasalazine remains unexplained. Several investigations have demonstrated the involvement of reactive oxygen species and oxidative stress in the organ damage caused by this medication (12, 13). There is currently no specific therapeutic treatment available for organ lesions caused by sulfasalazine. Medicinal plants have substantial value and effectiveness in treating ailments and are the main source of several recently discovered medications (14). Historically, pomegranate peel has been utilized in various cultures for medicinal purposes. The Romans employed it as an anthelmintic, while some ethnic groups in the Middle East and South America have used boiled pomegranate peel for treating dysentery (15). In traditional Chinese medicine, pomegranate peel is regarded as a potent astringent and anti-inflammatory agent used to treat traumatic bleeding, infections, and gastrointestinal disorders, such as diarrhea (16). The diversity of the medicinal functions of pomegranate peel has been attributed to its bioactive components (17). Prior studies have revealed many pharmacological activities of pomegranate (*Punica granatum* L.), including antioxidative, antimicrobial, and anticancer activities (18, 19). The objectives of this study were to analyze the qualitative composition of APPE by phytochemical screening and to determine its quantitative composition by HPLC-UV, to conduct an acute toxicity study at a dose of 5 g/kg, and to evaluate the cyto-protective effects of the aqueous extract of *Punica granatum* (APPE) in ulcerative colitis and in an experimental model of sulfasalazine-induced kidney injury.

Material and methods

Preparation of the samples

The aqueous extract of the basic plant product was prepared according to a method described by Guede-Guina et al. (20). In fact, 50 grammes of *Punica granatum* L. peel powder was dissolved in 500 milliliters of distilled water and homogenized with a blender. The homogenate was left to macerate for 48 hours and then filtered through cotton wool and Whatman paper. The filtrate was oven-dried at 40°C for 48 hours to obtain the aqueous extract (APPE).

Qualitative analysis of phenolic compounds by preliminary phytochemical screening

Qualitative analysis was performed to identify the main secondary metabolites in the APPE extract, such as steroids, alkaloids, glycosides, flavonoids, anthocyanins, tannins, coumarins, terpenoids, and sterols (21). For that purpose multiple chemical assays were used based on color and/or precipitation reactions.

Quantitative analysis of phenolic compounds by high-performance liquid chromatography Ultra-violet (HPLC-UV)

Preparation of samples for HPLC-UV analysis. APPE (2 mg/ml) was diluted in methanol. Before injecting sample solutions into an HPLC-UV device, they were filtered through a Millipore nylon filter disk with a Millipore 0.45 m.

Preparation of polyphenol standards. Analytical standards (all with purity ≥ 98%), dihydrocaffeic acid, benzoic acid, 3,4-dihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3-hydroxybenzoic acid, gallic acid, syringic acid, catechol, 4-methylcatechol, homovanillic acid, catechol, pyrogallol, phloroglucinol, hydrocinnamic acid, chlorogenic acid, 3-methoxyhydrocinnamic acid, trans-cinnamic acid, and resveratrol were purchased from Sigma-Aldrich. HPLC-grade methanol, acetonitrile, and HPLC-grade acetic acid were purchased from Sigma-Aldrich. Ultra-pure water was obtained from a Mega Purity water purification system (Billerica, MA, USA).

Stock standard solutions of each compound were prepared by dissolving 6 mg of analytical standard in methanol. To ensure complete dissolution, ultra-sonication was performed for 10 min. All solutions were stored at 4°C.

Chromatographic instruments and HPLC-UV analysis conditions. HPLC is the most common way to determine the various phytochemical components of APPE. The analysis was performed using HPLC-UV. At room temperature 25°C, 25 µl of the analyte solution was injected with a Terumo syringe into an Agilent Technologies 1200 Series HPLC valve. Phenolic compounds were separated on a Thermo Scientific (HPLC RP 18) ZORBAX column (150 mm*4,6 mm*1.8 µ) packed with C18 stationary phase at a mobile phase flow rate of 0.5 ml/min.

The mobile phase consisted of a binary solvent system comprising bi-distilled water (solvent A) and with 95% acetic acid (solvent B). In order to identify chromatographic

peaks, we compared the retention times of our analyses with those of reference chemicals (6). The phenolic composition was quantified by plotting a standard curve with respective standards.

Reagents

The chemicals and reagents used in this study were of high analytical quality and were purchased from Sigma (St. Louis, MO), Merk (Mannheim, Germany), Biochemical (Germany) and (Troikaa Pharmaceuticals, India). Methanol (MeOH) was an HPLC grade.

Animals and housing

Adult male and female Wistar rats were obtained from the animal breeding division of Pasteur Institute (IPA) of Kouba (Algiers, Algeria). The rats were housed in an animal facility (ENSV Oued Smar, Algiers) in separate stainless steel cages under controlled conditions of humidity, lighting, and temperature (50 ± 10% RH, 12-h light/dark, 23 ± 2°C). They were given water and standard diet *ad libitum* throughout the experiment. The feed consisted of 49.80% carbohydrate, 23.50% protein, 5% fat, and 5.7% mineral-vitamin complex.

All experiments were carried out in compliance with the institutional guidelines for animal care as well as the guidelines of the Algerian Association of Experimental Animal Sciences (AASEA) following approval by the local Ethical Committee of the Houari Boumediene University of Sciences and Technology (USTHB), Algeria (approval number 45/DGLPAG/DVA.SDA.14).

Acute toxicity study

The acute oral toxicity was evaluated in female rats following OECD Guideline No. 423 (22). The rats (n = 3) were administered a single oral APPE dose of 5000 mg/kg body weight, while the vehicle-treated control group (n = 3) received the same volume of water. Both groups were observed closely for any toxic effects within the first 6 hours and then at regular intervals for a total period of 14 days. Surviving rats were observed to determine the onset of toxic reactions. Special attention was given to the first three hours after the administration of the complex. Behavioral changes and other parameters, such as body weight, urination, food intake, water intake, respiration, convulsions, tremors, temperature, constipation, and changes in eye and skin color, were carefully monitored.

Colitis induction technique and experimental groups

Induction of colitis. Acetic acid-induced colitis is an animal model that replicates certain acute inflammatory reactions observed in ulcerative colitis. The induction of colitis in rats with acetic acid is a well-established technique employed to create an experimental model of human inflammatory bowel disease. On day 17, after one day of absolute fasting with only water, the rats were given anesthesia, and a medical-grade polyurethane tube for feeding (with an external diameter of 2 mm) was inserted into their anus (23). The tube was then advanced 7 cm towards the proximal end, away from the anus verge. Subsequently, a volume of 1 ml of 4% acetic acid was administered into the colon. The animals were euthanized on day 21. The distal colons were incised in an ice bath, carefully washed with normal saline, and thereafter evaluated for macroscopic scores. Afterwards, the colons were divided into two identical portions, one for histopathological examination (preserved in 5 ml of 10% formalin) and the other for the analysis of biochemical markers. The colons were quickly removed, rinsed with cold water followed by isotonic saline, and then blotted with filter paper. Next, the tissues were homogenized using Tris-HCl buffer (0.1 M) at pH 7.4.

Experimental groups. The control group consisted of rats with ulcerative colitis. These rats were given oral saline every day for 21days and received an intra-rectal injection of acetic acid on day 17. The APPE groups consisted of rats with induced ulcerative colitis that received the APPE extract orally at daily doses of 200 and 400 mg/kg body weight for 21 days. On day 17, these rats were also injected rectally with acetic acid (2 mL of 3% (v/v) in 0.9% NaCl). The reference group consisted of rats with ulcerative colitis that were given sulfasalazine (100 mg/kg/day) orally for 21 days as a reference drug. On day 17, they were injected intrarectally with acetic acid (2 mL of 3% (v/v) in 0.9% NaCl).

Determination of ulcer index

The macroscopic scoring was conducted using a magnifying glass according to the following criteria (24):

The scoring system used was: 0 = no damage, 1 = patch-type superficial hyperemia, 2 = generalized patch-type hyperemic regions with normal mucosa in between, 3 = generalized hyperemia and hemorrhage. After the macroscopic analysis, tissue samples of full thickness were taken from the distal parts of the colon adjacent to the rectum.

To assess the microscopic characteristics, the tissue was preserved in formaldehyde buffered with phosphate, embedded in paraffin, and sliced into 5-mm pieces. The tissue was stained with hematoxylin and eosin for the light microscopy analysis. A histological grading scale was used in which each of the specific parameters assessed, including inflammation intensity, inflammation extent, and crypt damage, was assigned a score ranging from 0 to 3. A score of 0 indicated no change, while scores of 1, 2, and 3 indicated mild, moderate, and severe changes, respectively (25). The parameters assessed were erosion, ulceration, mucosal necrosis, mucosal hemorrhage, edema of the lamina propria and submucosa, and infiltration of inflammatory cells. The magnitude of alterations was assessed subjectively and compared to those in the standard group.

Nephrotoxicity induction technique and experimental groups

The study was designed for 14 days, according to the procedure previously described by Nazari-Khanamiri et al. (29). Animals were randomly assigned to four groups of six rats each. Group I (n = 6) was the vehicle-treated control group. Group II (n = 6) consisted of rats that received sulfasalazine orally at 600 mg/kg for 14 consecutive days to induce renal impairment. Group III (n = 6) received sulfasalazine (600 mg/kg) and 200 mg/kg of APPE, both once daily by gavage for 14 days. Group IV (n = 6) received sulfasalazine (600 mg/kg) and 400 mg/kg of APPE, both once daily by gavage for 14 days. On day 15, the rats were intramuscularly euthanized with ketamine (100 mg/kg). The kidneys were quickly removed and preserved in 10% formalin.

Blood sampling

Blood samples were obtained after a 12 hour fasting period by puncture at the retro-orbital sinus, using a Pasteur pipette. Blood for biochemical and oxidative analysis was collected into EDTA-coated tubes and immediately centrifuged. Plasma was prepared by centrifugation at 3500 rpm for 15 minutes and stored at –20°C until analysis.

Biochemical analysis

Biochemical parameters and oxidative stress were measured by spectrophotometry. Common plasma biochemical parameters, i.e. urea, creatinine, total protein (PT), and albumin levels, were measured with standard and colorimetric commercial kits (SPINREACT, Sant Esteve De Bas (GI). SPAIN) following the manufacturers' protocols.

Catalase concentration

500 µl of 0.2% H_2O_2 solution was placed in a spectrophotometer cell; then 950 µl of 0.1 M PBS buffer and 50 µl of heart tissue supernatant were added. After mixing, the absorbance was measured at 240 nm, and CAT content was expressed as µg/100 mg per tissue (ε = 39.4 µmol/L/cm).

SOD Activity Assay

The activity of SOD was measured by a modified method of Heikkila and Cabbat. The method is based on the measurement of the inhibitory effect of SOD on the spontaneous autoxidation of 6-hydroxydopamine (6-OHDA). Stock solution of 6-OHDA was prepared daily in 1 mM KC1, at pH 2.0. Soluble oxygen was removed from the stock solution by passing pure N2 through pyrogallate solution. During the experiments, stock solution was preserved in N_2 media at +4°C. An autoxidation rate of 6-OHDA (0.4 mM) in 0.1 M phosphate buffer (pH 7.4) saturated by air O_2 (8.2 mg/l) at 20°C was determined by observing absorbance changes over time at 490 nm and 20°C. 1 IU of superoxide dismutase activity is the amount of superoxide dismutase required for 50% inhibition of the initial rate of 6-hydroxydopamine autoxidation.

Reduced Glutathione (GSH) Assay

Plasma glutathione content (nmol/mL) was evaluated by measuring the absorbance of free thiol (SH) groups at 412 nm. 150 mL of plasma was mixed with 300 mL of 0.25% sulfosalicylic acid and incubated at +4°C for 1 h. Afterward, all was centrifuged at 3000 g for 10 min at room temperature. 200 mL of the resulting supernatant containing free SH groups was mixed with 100 mL of 0.004% 5,5' dithiobis2-nitrobenzoic acid (DNTB) and 800 mL of 200 mM phosphate buffer, pH = 8. The values were expressed as mmol mL^{-1}, where ε = 13.6 mmol mL^{-1}.

Histological analysis

Colon and kidney were fixed in 10% formol and embedded in paraffin. Sections of 5 µm were stained with hematoxylin and eosin and examined under a light microscope. Microscopic observation was performed to verify changes in tissues. Digital images were obtained with a Leica optical microscope connected to an AmScop 3.7 camera.

Data and statistical analysis

All values are expressed as means ± standard error of mean in SPSS (version 19.0, Chicago, USA). One-way analysis of variance was employed for analyzing the data, followed by Tukey's test for multiple comparisons. Statistical significance was assigned when $p < 0.05$.

Abbreviations

Abbreviations used in the manuscript are explained in Table 1.

Tab. 1. List of abbreviations

APPE	Aqueous *Punica granatum* peel extract
HPLC-UV	High-performance liquid chromatography ultra violet
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
AA	Acetic acid
OECD	Organisation for Economic Co-operation and Development
LD50	Median lethal dose
SOD	Superoxide dismutase
CAT	Catalase
GSH	Reduced glutathione

Results and discussion

Identification and qualification of phenolic compounds in APPE

The phytochemical analysis of APPE revealed the presence of various bioactive compounds, including flavonoids, tannins, polyphenol, coumarins, flavanols, saponosides, triterpenoids, steroids, reducing compounds, alkaloids, sterols, and triterpenes. APPE did not contain anthocyanins (Tab. 2).

Tab. 2. Phytochemical screening results for APPE

Secondary metabolites	
Tannins	+
Flavonoids	+
Anthocyanins	–
Polyphenol	+
Saponosides	+
Triterpenoids	+
Steroids	+
Mucilages	–
Coumarins	+
Reducing compounds	+
Cardenolides derivatives	–
Alkaloids	–
Sterols and triterpenes	+

Explanations: (+) – positive; (–) – negative

Identification and quantification of phenolic compounds in APPE

The HPLC-UV conditions used to identify and quantify polyphenolic compounds in APPE were the same as those for standards. Phenolic acids and flavonoids present in the APPE samples were identified by comparing their retention times with those of 25 standards available in the laboratory, whereas their amounts were determined using the equations obtained from standard calibration curves after a necessary dilution of samples if needed. Among the standards tested, 24 were found in APPE. The most abundant phenolic acids (Fig. 1) were resveratrol (552.17 µg/mL), chlorogenic acid (376.15 µg/mL), 3,4-dihydroxybenzoic acid (329.00 µg/mL), hydrocinnamic acid (284.64 µg/mL), 3,5-dimethoxyphenol (168.79 µg/mL), and dihydrocaffeic acid (123.55 µg/mL). In addition to these major compounds, 3-methoxyhydrocinnamic acid and 3-methoxycinnamic acid were also present

Tab. 3. Retention times (min) and concentrations of phenolic compounds detected in APPE

Standards	Chemical formulas	RT (min)	[µg/mL]
Gallic acid	$C_7H_6O_5$	4.58	12.78
3,4-dihydroxybenzoic acid	$C_7H_6O_4$	8.18	329.00
2,3,4-trihydroxybenzoic acid	$C_7H_6O_5$	9.19	54.93
Catechol	$C_6H_6O_2$	12.82	37.79
Phloroglucinol	$C_6H_6O_3$	18.47	44.96
3,5-dihydroxybenzoic acid	$C_7H_6O_4$	18.98	0.54
Homovanillic acid	$C_9H_{10}O_4$	19.07	2.16
3-hydroxybenzoic acid	$C_7H_6O_3$	20.02	1.80
4-methylcatechol	$C_7H_8O_2$	20.76	1.92
Pyrogallol	$C_6H_6O_3$	21.81	1.69
Phenol	C_6H_6O	23.00	0.20
Syringic acid	$C_9H_{10}O_5$	25.91	34.85
Dihydrocaffeic acid	$C_9H_{10}O_4$	28.31	123.55
Chlorogenic acid	$C_{16}H_{18}O_9$	31.19	376.15
2-acetylresorcinol	C_8H_8O	32.48	7.00
Caffeic acid phenethyl ester	$C_{17}H_{16}O_4$	42.73	13.80
3-methoxyhydrocinnamic acid	$C_{10}H_{10}O_3$	44.71	93.16
Lawsone	$C_{10}H_6O_3$	47.85	43.80
Benzoic acid	$C_7H_6O_2$	48.31	17.00
3,5-dimethoxyphenol	$C_8H_{10}O_3$	50.73	168.79
Hydrocinnamic acid	$C_9H_{10}O_2$	51.08	284.64
Resveratrol	$C_9H_{10}O_2$	54.20	552.17
Tert-butylhydroquinone	$C_{10}H_{14}O_2$	54.81	55.88
Trans-cinnamic acid	$C_9H_8O_2$	55.83	38.43

in amounts not exceeding 100 mg/mL (Tab. 3). The structures of these major compounds are presented in Figure 2. The nature, number, and amount of phenolic compounds identified in the extracts depended on the solvent used for their extraction.

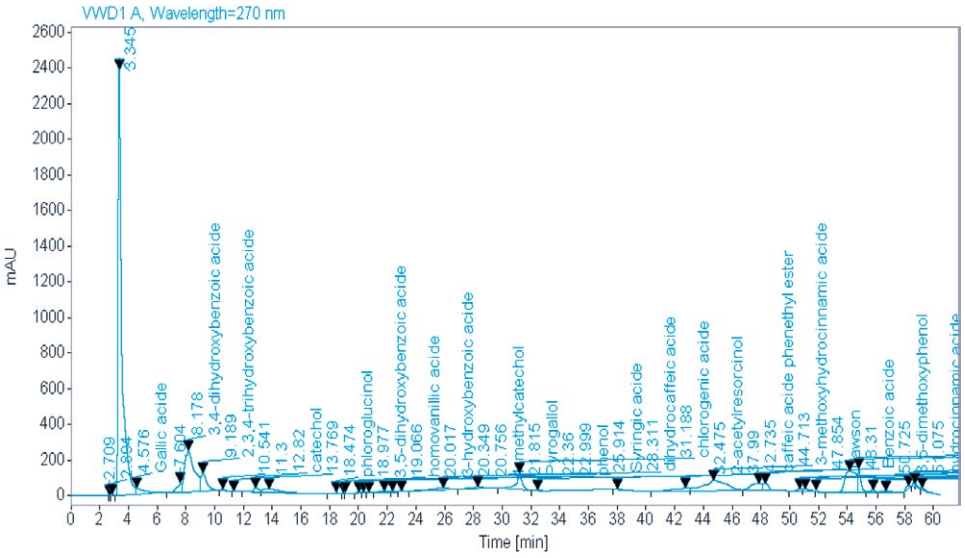

Fig. 1. Chromatographic profile of APPE

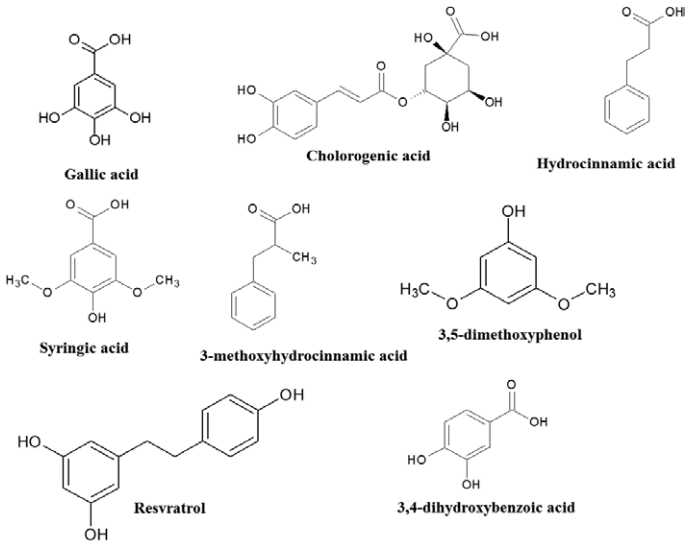

Fig. 2. The structure of major phenolic compounds detected in APPE

Acute toxicity study

In our study, all animals that received an APPE dose of 5000 mg/kg body weight were alive during the test period and did not exhibit any obvious pathological abnormalities, toxicity, or mortality.

Effect of APPE on colon macroscopic changes in rats with acetic acid-induced colitis

On day 4 of colitis induction, instillation of acetic acid into the colon of rats caused a strong inflammatory response. The distal colon displayed severe macroscopic edematous inflammation. The mucosa exhibited inflammation, ulceration, hyperemia, and hemorrhage, unlike the mucosa of the control group (Fig. 4 A). Nevertheless, the administration of APPE and sulfasalazine (100 mg/kg/day) directly into the colon reduced visible damage and improved the overall scores for the effects observed (Tab. 4). Sulfasalazine, administered at a dose of 100 mg/kg, effectively protected the colon of rats from harm caused by acetic acid. An APPE dose of 200 mg/kg resulted in an improvement in macroscopic damage scores compared to those in the AA group, reducing the score from 4 to 3.21. However, an APPE dose of 400 mg/kg caused a more significant decrease to 1.8, with no statistically significant difference compared to the sulfasalazine group (Tab. 4).

Effect of APPE on colon histopathology of rats with acetic acid-induced colitis

The histological analysis in the control group revealed a normal structure of colonic mucosa, characterized by intact epithelium, without any evidence of inflammation or necrosis

Tab. 4. Macroscopic and histological scoring under preventive APPE treatment

Group	Dose (Per Os)	Wet weight/length of the colon (g/8 cm)	Macroscopic damage score	Histological damage score
Control (distilled water)	10 ml/kg	0.49 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Acetic acid		1.19 ± 0.03^{d}	4.00 ± 0.03^{d}	3.80 ± 0.09^{d}
Sulfasalazine	100 mg/kg	0.53 ± 0.01^{ab}	1.70 ± 0.03^{b}	2.08 ± 0.03^{b}
APPE	200 mg/kg	0.88 ± 0.00^{c}	3.21 ± 0.10^{c}	3.12 ± 0.02^{c}
	400 mg/kg	0.69 ± 0.03^{bc}	1.86 ± 0.04^{b}	2.10 ± 0.05^{b}

Explanations: Each value is expressed as mean ± SEM (n = 6). Means with different letters within the same column are significantly different ($p < 0.05$)

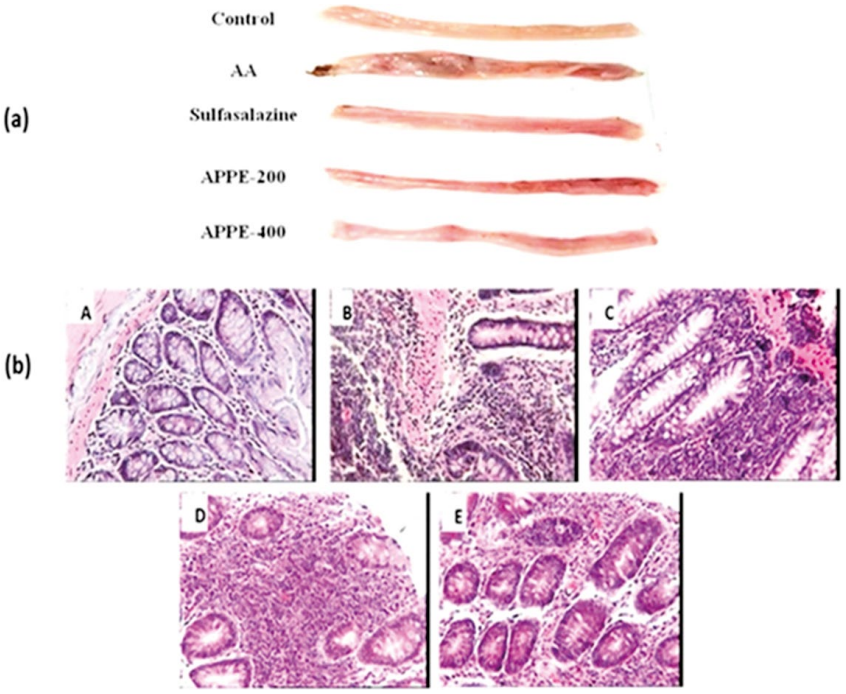

Fig. 3. Effect of APPE on the colon macroscopy (a) and histopathology (b) of rats with acetic acid-induced colitis. Panel (a): The control group exhibited an intact colon. Severe edematous inflammation was observed in the AA group. Groups treated with APPE-400 mg/kg and Sulfasalazine-100 mg/kg showed an apparent reduction in colon damage. A slight improvement was observed under the effect of APPE-200 mg/kg. Panel (b): The colon of the control group (A) maintained its structural integrity showing highly organized crypts (beehive cell appearance). The AA group (B) showed focal ulceration, severe necrosis, and moderate to severe crypt destruction with lymphocyte and plasma cell infiltration, indicating the initiation of inflammation. The group treated with APPE-200 mg/kg (C) exhibited a slight crypt damage and necrosis with mild inflammation. A notable decrease in microscopic damage was observed in the groups treated with APPE-400 mg/kg and sulfasalazine (D and E), which facilitated mucosal healing and reduced inflammatory cell infiltration

(Fig. 3A). The colon of the AA-treated group (Fig. 3B) showed multiple areas of tissue necrosis, hemorrhage, submucosal edema, and a significant infiltration of white blood cells in the mucosa. The inflammation propagated through the muscular layer and submucosa. The crypts had been deformed, and the surface layer of cells was extensively damaged.

In the groups treated with sulfasalazine and APPE at 400 mg/kg and 200 mg/kg, histological abnormalities were reduced, notably so for the first two groups, as evidenced by the healing of the mucosa, decrease in edema, and reduction in the number of inflammatory cells recruited (Fig. 3D and E). No significant difference was observed between these groups, which showed a significant decrease in the pathological scores to 2.02 and 2.1, respectively, compared to that of the AA group (Fig. 3; Tab. 4). The AA group exhibited focal breakdown of the epithelium and the presence of inflammatory cells in lamina propria.

Effect of APPE on colitis-induced colon oxidative stress

The colon tissues of all experimental groups were evaluated for their antioxidant activity. As indicated in Table 5, the group with AA-induced colitis showed a significant reduction in oxidative stress markers, namely superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), compared to the control group. The levels of oxidative stress markers were increased after treatment with APPE at a dose of 200 mg/kg and significantly increased for APPE at 400 mg/kg. The latter dose appears to be quite effective, as it resulted in levels close to those in the reference group (sulfasalazine). Higher doses could potentially restore these levels to normal.

Tab. 5. Effect of APPE on SOD, CAT, and GSH levels in colitis-induced colon oxidative stress

Treatment	Dose (mg/kg)	SOD (U/g protein)	CAT (U/g protein)	GSH (nmol/g protein)
Control		56.71 ± 1.09^{a}	54.72 ± 0.49^{a}	72.69 ± 0.07^{a}
AA		32.73 ± 0.08^{d}	26.64 ± 0.17^{b}	31.32 ± 0.03^{d}
Sulfasalazine	100	52.60 ± 0.09^{ab}	53.85 ± 1.20^{a}	67.49 ± 1.12^{ab}
APPE	200	41.20 ± 0.66^{c}	32.91 ± 0.48^{b}	41.92 ± 0.03^{c}
APPE	400	48.17 ± 0.75^{b}	52.23 ± 0.31^{a}	61.41 ± 1.02^{b}

Explanations: Each value is expressed as mean ± SEM (n = 6). Means with different letters within the same column are significantly different ($p < 0.05$)

Tab. 6. Effect of the plant extract on urea, creatinine, total protein, and albumin levels in rats with sulfasalazine-induced nephrotoxicity

Treatment	Dose (mg/kg)	Plasma urea (mmol/L)	Plasma creatinine (mmol/L)	Plasma total protein (g/dl)	Plasma albumin (g/dl)
Control		2.67 ± 0.11	0.064 ± 0.004	11.22 ± 0.78	3.43 ± 0.17
Sulfasalazine	600	6.47 ± 0.11	0.165 ± 0.009	6.82 ± 0.19	1.22 ± 0.13
Sulfasalazine + APPE	600 + 200	3.41 ± 0.11$^{\#\#\#}$ ***	0.079 ± 0.003***	9.05 ± 0.26$^{\#}$ *	3.02 ± 0.25***
Sulfasalazine + APPE	600 + 400	3.00 ± 0.04***	0.067 ± 0.002***	10.07 ± 0.51***	3.24 ± 0.11***

Explanations: Data are mean ± SEM; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to sulfasalazine group

Nephroprotective activities

Serum biochemical analysis

The administration of sulfasalazine at a dose of 600 mg/kg/day for 14 days resulted in a significant impairment of kidney function. This was evidenced by significantly elevated levels of serum urea and creatinine and a notable reduction ($p < 0.05$) in total protein and albumin, compared to the control group ($p < 0.05$). However, rats treated with APPE at doses of 200 mg/kg and 400 mg/kg had significantly lower levels of these parameters, compared to the group receiving sulfasalazine alone (Tab. 6). In terms of the levels of total protein and albumin, sulfasalazine caused a statistically significant reduction compared to the control group. These parameters appeared to be significantly increased in the groups that received APPE at doses of 200 mg/kg and 400 mg/kg (Tab. 6).

It is interesting to note that there was no significant difference in the levels of the four parameters (Tab. 6) between the control group and the groups treated with APPE extract at doses of 200 mg/kg and 400 mg/kg. In the case of the higher dose of APPE, the values were almost identical to those in the control group.

Effect of APPE in histopathological evaluation

Examination of the control group (Fig. 4 A) showed normal histoarchitecture of the kidney tissues, while in the sulfasalazine group it revealed a severe inflammatory cell infiltration, irregularly dilated lumina, interstitial hemorrhage, vacuolated cytoplasm, and tubular epithelial injury with widened Bowman's space along with congestion in capillaries of the glomerulus. The groups treated with APPE at doses of 200 mg/kg and 400 mg/kg showed improvement in the renal tissue architecture, which appeared more pronounced for the higher dose (Fig. 4 D).

Major adverse effects, treatment failure, and high costs of current medications justify exploring plant-based remedies with fewer side effects and multi-target actions. In this context, we evaluated the anti-inflammatory effect of aqueous pomegranate peel extract (APPE) in an animal model of acetic acid-induced ulcerative colitis. As sulfasalazine is the treatment of choice for ulcerative colitis, we evaluated the nephroprotective effect of APPE in an animal model of kidney injury in Wistar rats. An acute toxicity study was conducted beforehand.

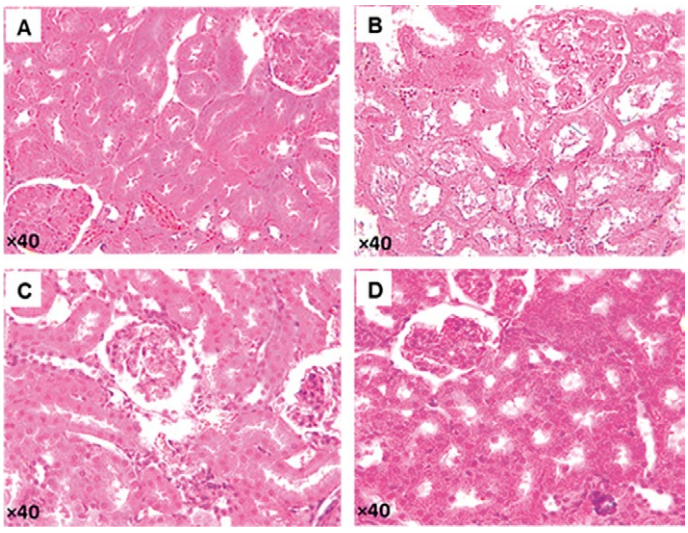

Fig. 4. Histopathological changes in rat kidney after sulfasalazine-induced nephrotoxicity and APPE treatment (hematoxylin-eosin staining). (A) Control group: the normal appearance of glomeruli. (B) Group of rats treated with sulfasalazine: degeneration and necrosis in renal tubules and infiltration of inflammatory cells (neutrophils). (C): Group of rats treated with APPE 200 mg/kg: a clear nephroprotective effect, with moderate degenerative changes in the glomeruli. (D): Group of rats treated with APPE 400 mg/kg: an almost normal appearance of glomeruli

Phytochemical screening confirmed the presence of tannins and terpenoids in the aqueous *Punica granatum* peel extract (APPE). HPLC profiling further identified abundant phenolic acids and flavonoids – especially resveratrol, chlorogenic acid, 3,4-dihydroxybenzoic acid, hydrocinnamic acid, 3,5-dimethoxyphenol, and dihydrocaffeic acid – in line with prior reports on APPE. In addition, 3-methoxyhydrocinnamic acid and 3-methoxyhydrocinnamic acid were also detected.

The results presented in this study are similar to those of other authors (50), who reported that *Punica granatum* peel extract is a good source of phenolic and flavonoid compounds, which are abundantly present in APPE. These compounds have anti-inflammatory properties, which can be linked to their ability to prevent ulcers (17-19).

Phenolic acids and flavonoids, the major compounds with a total quantity of 2296.99 µg/mL, represent 80% of the compounds identified in the APPE sample. These results are consistent with those obtained by other authors (33-37).

Toxicological evaluation of medicinal plants is essential to assess the potential toxicity of aqueous pomegranate peel extract (APPE) and ensure its safety as a therapeutic candidate. An acute toxicity study at the dose of 5000 mg/kg indicates that APPE can be considered non-toxic to the animals tested.

The acetic acid-induced UC model is a reproducible model that recreates pathological pathways resembling human UC histologically and biochemically. The acetic acid-induced UC model is widely acknowledged as a reliable model for experimental investigation of inflammatory bowel disease (38).

Changes indicating inflammation of the intestinal tract were significantly correlated with a notable rise in the wet weight/length of the colon specimens. The oral administration of sulfasalazine and APPE significantly improved the inflammatory indices after ulcer induction. These findings are consistent with those of previous research (39). Macroscopic examination of the colon demonstrated a significant increase in the weight-to-length ratio of the colon. This phenomenon can be attributed to substantial tissue oedema, goblet cell hyperplasia, and infiltration of inflammatory cells. The results obtained are consistent with those of previous research (40).

The intestinal content of rats treated with APPE and sulfasalazine consisted of well-formed faecal pellets with no visible traces of blood or mucus. This can be attributed to the role of the mucus layer in facilitating the restoration of chemically damaged epithelium (41). This observation indicates the extract's potential therapeutic efficacy in reducing colonic ulceration and inflammatory scores.

The intra-rectal administration of AA resulted in notable histological changes, including the thickening of the colon, excessive growth of goblet cells, and the presence of inflammatory infiltrations (42, 43). The results of histopathological evaluation demonstrated that the administration of APPE to rats effectively maintained the structural integrity of the colonic mucosa while also suppressing the infiltration of inflammatory cells, congestion, ulceration, erosion, necrosis, and hyperplasia induced by acetic acid. This also demonstrates the extract's capacity to safeguard the animals and hinder the progress of the disease.

Experimental studies indicate that oxidative stress results from a shift in equilibrium between the pro-oxidant and anti-oxidant systems in favor of the pro-oxidant system as a result of excessive production of free oxygen radicals (44).

The importance of enzymatic and non-enzymatic antioxidant systems in safeguarding tissues against pro-oxidants is widely recognized. The equilibrium between these systems plays a significant role in the pathogenesis of diverse disorders (45). SOD and CAT are naturally occurring enzymatic antioxidants, while GSH is a non-enzymatic antioxidant (46). These molecules serve as a defense mechanism for cells and organisms against harmful free oxygen radicals. The AA treatment reduced the levels of SOD and CAT in the tissue.

The glutathione (GSH) cycle is an important intracellular mechanism for antioxidant defense. The substance serves as a substrate for the enzymatic activity of several antioxidants. Reduced GSH activity heightens oxidative stress and results in the buildup of harmful substances (47).

In this study, we demonstrated that the *in vivo* administration of APPE enhances SOD, CAT, and GSH activity in colonic tissue of rats compared with those in the AA-induced colitis group. The antioxidant capacity of APPE could be attributed to the presence of several phytochemical components.

Sulfasalazine is widely used as the first-line drug for the treatment of rheumatoid arthritis and other inflammatory-mediated disorders, such as Crohn's disease in humans, but exposure to it is usually associated with nephrotoxicity caused by stress, inflammation, and apoptosis due to the generation of reactive oxygen species (ROS). The treatment with 600 mg/kg of sulfasalazine resulted in kidney damage, as evidenced by a noticeable increase in urea and creatinine levels and a decrease in total protein and albumin levels. The literature documents many cases of sulfasalazine-induced renal injury (48, 49). Kidneys of sulfasalazine-treated rats showed the presence of inflammatory collections and cell necrosis. The APPE-treated group showed no necrosis and a minimal inflammatory environment with normal renal architecture, which demonstrates a high degree of nephroprotective action.

This study demonstrates that APPE exhibited a protective effect, as indicated by its ability to cause serum creatinine and urea to decrease and total proteins and albumin to increase.

In conclusion, our study demonstrates that *Punica granatum* peel extracts (APPE) exhibit notable antioxidant and anti-inflammatory properties, which suggests their potential use as protective agents in renal and inflammatory disorders. More research is needed to move toward a clinical application, which should include testing standardized methods of extraction, pharmacokinetics studies, and review studies of long-term toxicology.

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