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Anti-angiogenic and Anti-Inflammatory Activity of *Punica granatum* Peel on Experimentally -Induced Gastric Ulcer in Rats

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ABSTRACT

Pomegranate (*Punica granatum*) has been used in folk medicine for the treatment of gastric ulcer. Thus, the current study aimed to evaluate the reparative and anti-inflammatory activity of methanolic extracts of pomegranate peel (mesocarp and epicarp). This study was conducted on male Wistar rats divided into 9 groups: Group 1; normal controls, Group 2; received 80% ethanol (positive control), Groups 3, 4, 5, 6, 7, 8 and 9; gastric ulcer treated with 250mg/kg, 500mg/kg (mesocarp extract), 250mg/kg, 500mg/kg (epicarp extract), Ranitidine, Omeprazole and Sucralfate, respectively. Serum pro-angiogenic [vascular endothelial and platelet derived growth factors (VEGF and PDGF) and inflammatory mediators [Interleukin (IL-1β), IL-6, tumor necrosis factor (TNF-α) and cyclooxygenase (COX-2)] were performed by enzyme-linked immunosorbent assay (ELISA). Pomegranate peel methanolic extracts are found to have antiulcer property. They have been proved as anti-inflammatory agents by reducing gastric mucosal COX-2 and plasma TNF-α level. Healing effect was approved by elevation of PDGF level. Maximum ulcer healing effect was elicited in epicarp extract at 500mg/kg. Taken together, pomegranate peel methanolic extracts could be considered as one of the antiulcer therapeutic strategies based on its anti-inflammatory and reparative activities. Further pharmacokinetic analysis of these extracts will be taken in consideration in future study.

Keywords: *Punica granatum*, Gastric ulcer, Angiogenesis, Inflammation.

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INTRODUCTION

Peptic ulcer disease (PUD) is the most common gastrointestinal disorder in clinical practice. It is a chronic, multifactorial disease distinguished by different degrees of denudation in gastric or duodenal mucosa [1,2] . Ulcer pathogenesis is resultant of an imbalance between aggressive factors [acid, pepsin, bacterial infection (*Helicobacter pylori*) and non-steroidal anti-inflammatory drugs (NSAIDs)] and defensive factors (mucus bicarbonate, blood flow, gastric mucin and prostaglandins) that are needed for the maintenance of integrity of gastro-duodenal mucosa [3] .

Gastric ulcer therapy faces a major drawback in modern days due to the undesirable side effects of the long term uses of commercially available drugs [4]. The conventional drugs used in the treatment of gastric ulcer include histamine (H_2) receptor antagonist, proton pump inhibitors, antacids and anti-chollinergics [5,6] . As it affects approximately 15-20% of the global population [7] , the treatment of this disease has become one of the challenging problems today. Thus, the search is still on to discover a drug possessing antiulcer activity, which will serve as a powerful therapeutic agent for gastric ulcer.

Due to lack of side effects compared to synthetic drugs, new trends in treatment have relied on plants for medication. From ancient times, plants have been proved to be powerful therapeutic agent for the treatment of various human diseases. One of these plants is *Punica granatum* (Punicaceae), a commonly called pomegranate, is an edible fruit cultivated in Mediterranean countries, Asian countries and some parts of the United States. *P. granatum* has been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases [8,9,10] . Pomegranate fruit is a rich source of two types of polyphenolic compounds: anthocyanins (such as delphinidin, cyanidin and pelargonidin) and hydrolyzable tannins (such as punicalin, pedunculagin, punicalagin and ellagic acid esters of glucose). A number of biological activities such as anti-tumour [11] , anti-bacterial [12] , anti-diarrhoeal [13] , and anti-fungal [14] activities have been reported with various extracts/constituents of different parts of this plant. Pomegranate is now gaining importance because of its potent antioxidant activity. As some potent antioxidants have been isolated from the fruit juice and have been found to be bioavailable, effective and safe [15]. Moreover, it has been used previously in folk medicine for the treatment of ulcer [16].

Inflammation can stimulate angiogenesis, and angiogenesis can facilitate inflammation [17]. Inflammatory mediators can, either directly or indirectly, promote angiogenesis. Angiogenesis, in turn, contributes to inflammatory pathology. New blood vessels can maintain the chronic inflammatory state by transporting inflammatory cells to the site of inflammation and supplying nutrients and oxygen to the proliferating inflamed tissue. The increased endothelial surface area also creates an enormous capacity for the production of cytokines, adhesion molecules, and other inflammatory stimuli [18].

Based on these facts, the present work aimed to evaluate in *vivo* antiulcer, anti-angiogenic and anti-inflammatory activity of different methanolic extracts from *P. granatum* peel (mesocarp and epicarp) and comparing the healing activity of these extracts with three well documented anti-ulcer drugs act by different pharmacokinetics; Omeprazole (Omz) (proton pump inhibitor), Ranitidine (Ran) (histamine H2-receptor antagonist) and Sucralfate (Suc) (cytoprotective agent).

MATERIALS AND METHODS

Animals

Ninty male Wistar rats (body weight 150–200g) were purchased from VACSERA, Egypt. The animals were maintained under standardized environmental conditions (22-28°C, 60-70% relative humidity, 12 h dark/light cycle). They were allowed to access to food and water *ad libitum*. All experimental protocols described in this study were in accordance with the rules and regulation of the Animal Ethics rules, Menofiya University, Egypt.

Plant material

The fruits of *P. granatum* were collected from the local market in Cairo, Egypt in October, 2010 and identified by experts in National Herbarium reference.

Extraction

The peels of *P. granatum* were manually separated from the whole fruits to epicarp and mesocarp, dried in hot-air woven, powdered and extracted with a mixture of methanol: water (7:3, v/v) by a Soxhlet apparatus at 65 ºC. The solvent was completely removed and the dried crude extract thus obtained was used for investigation.

Phytochemical analysis

The aqueous methanol extract of the *P. granatum* epicarp and mesocarp was subjected to qualitative chemical screening for the identification of the tannins, and flavonoids using standard procedures [19].

Test for tannins

The aqueous extract (1 ml) was mixed with 10 ml of distilled water and filtered. Ferric chloride reagent (3 drops) was added to the filtrate. A blue-black or green precipitate confirmed the presence of gallic tannins or catechol tannins, respectively.

Test for flavonoids

A portion of the aqueous extract (2 ml) was heated, and metallic magnesium and concentrated hydrochloric acid (5 drops) were added. A red or orange coloration indicated the presence of flavonoids.

Ethanol (80%) induction of gastric ulcer in rats and role of *P. granatum* methanolic extracts

The rats were randomly divided into 9 groups (10 animals each). Gastric ulcer was induced as previously described by Paiva et al. [20].

Group (1) was kept as normal controls without any treatment. All other groups were fasted from 24 to 36h before 80% ethanol administration. All treatments were administered orally, 1h prior to the ethanol administration. All groups were sacrificed 4hr after ethanol administration. Effect of *P. granatum* extracts were compared with different synthetic drugs such as Ranitidine (MUP, Egypt), Omeprazole (MUP, Egypt) and Sucralfate (EIPICO, Egypt).
Group (2) (gastric ulcer, untreated group) was received 1 ml of 80% ethanol.
Group (3) and (4) were treated with *P. granatum* extract (mesocarp part) (250 mg/kg and 500 mg/kg body weight in water; respectively).
Group (5) and (6) were treated with *P. granatum* extract (epicarp part) (250 mg/kg and 500 mg/kg body weight in water; respectively).
Groups (7), (8) and (9) were treated with synthetic drugs Ranitidine (50 mg/kg body weight in water), Omeprazole (20 mg/kg body weight in water) and Sucralfate (360 mg/kg body weight suspended in 2% gum acacia) [21]; respectively.

Sample collection

At the end of experiments, animals were subjected to light ether anesthesia, blood samples were withdrawn by retro-orbital bleeding and collected in EDTA-centrifuged tube (BD Biosciences, CA, USA). The plasma were separated by centrifugation at 2000 rpm for 15 min at 4°C, aliquotted, and stored at -80°C for further investigations. All rats were sacrificed after plasma collection. Gastrectomy specimens were removed from the control, ulcerated and treated groups, opened along the greater curvature, and thoroughly rinsed with normal saline, fixed and processed for haematoxylin and eosin (H&E) staining.

Histopathological examination

The gastrectomy specimens for each rat were fixed in 10% neutral buffered formalin solution. Each specimen was assessed grossly for dissection and detection of ulcer. Each specimen dehydrated in a graded alcohol series. After xylene treatment, the specimens were embedded in paraffin blocks. Five-micron thick

sections were cut and stained with H&E staining and the mucosal and submucosal changes were examined by light microscopy. Damaging effect of ethanol-induced ulcer in both mucosa and submucosa; ulceration, erosion, congestion, inflammatory infiltrates type and intensity, epitheliotropism, edema, and reactive atypia were investigated in addition to reparative changes; angiogenesis and restoration of epithelium. Unintentional bias was prevented by coding rat's tissue samples.

Immunohistochemical (IHC) analysis of COX-2

IHC staining was performed on formalin fixed, paraffin embedded material that were sectioned at 5 µm thickness and placed onto positive charged slides. COX-2 mouse monoclonal antibody (Santa Cruz Biotechnology, INC., U.S.A.) (200 µg/ml) was diluted 1:100 by phosphate buffered saline (PBS). IHC staining was performed using the labeled streptavidin biotin (LSAB)+System-HRP (Dakocytomation, Glostrup Denmark). All slides were de-paraffinized using xylene then dehydrated in decreasing concentrations of ethanol. After inhibition of endogenous peroxidase activity using 0.3% hydrogen peroxidase for 15 min, antigen retrieval using microwave heating (20 min; 10 mM Citrate buffer, pH 6.0) was performed. The slides were incubated overnight with anti-COX-2 primary antibody at room temperature, and washed by PBS then incubated with biotinylated secondary antibody for 15 min. After washing with PBS, the detection of bound antibody was accomplished using a modified labeled avidin–biotin reagent for 20 min. A 0.1% diaminobenzidine (DAB) (Sigma Chemical Company, St. Louis, MO) was used for 5 min as a chromogen. Slides were counterstained with Mayer's hematoxylin for 5–10 min.

Measurement of plasma angiogenesis markers [vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF)]

Levels of VEGF and PDGF were quantified by enzyme linked immunosorbent assay (ELISA) as previously described by Talaat et al. [22]. Absorbencies were measured at 450nm using ELISA plate reader (Sunrise, Tecan Group Ltd.). The ELISA reader-controlling software (Softmax) processes the digital data of raw absorbance value into a standard curve from which cytokine concentrations of unknown samples can be derived directly. Results were expressed as pictogram of cytokine per milliliter (pg/ml).

Measurement of pro-inflammatory cytokines [interleukin -1 (IL-1β), IL-6 and Tumor necrosis factor-alpha (TNF-α)]

Total concentrations of IL-1β, IL-6 and TNF-α in plasma samples were measured using a commercial ELISA kit (RayBiotech, Inc. Norcross GA) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 13 (SPSS, Inc., Chicago). Data are presented as means with corresponding SE. Comparisons between different groups were performed by one-way analysis of variance (ANOVA). Tukey's test was used as a post-hoc test. Pathology data was calculated using MedCalc statistical software for biomedical research. Correlation among variables was determined using Pearson's correlation test. A *P* value of $p<0.05$ was deemed to indicate statistical significance.

RESULTS

Histopathological assessment

Different histopathological changes in ulcer-induced rats treated either with different pomegranate peel extracts or pharmaceutical drugs were summarized in Table (1). Histopathological examination of stomach of ulcerogenic untreated (Figure 1), extract-treated (Figure 2) and drug-treated groups (Figure 3) were performed. Ethanol administration induced gastric lesions mainly in the body and fundus when compared to normal control group (Figure 1). All extracts and the 3 selected drugs (Ranitidine, Omeprazole and Sucralfate) induced a significant dose dependent reduction in the gastric mucosal damage induced by ethanol when compared to untreated rats ($p<0.05$) (Figure 2) (Table 1).

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Table 1: Different histopathological changes in ulcer-induced rats treated either with different pomegranate peel extracts or pharmaceutical drugs.

Variable	Control	Epi-250	Epi-500	Meso-250	Meso-500	Ranitidine	Sucralfate	Omeprazole
Mucosal changes								
Epithelial changes								
Ulcer	3 (30)	3 (30)	1 (10) **	1 (10)	5 (50)	2 (20)*	3 (30)*	3 (30)
Erosion	7(70)	5 (50)	2 (20)	7 (70)	5 (50)	3 (30)	2 (20)	5 (50)
intact	0 (0)	2 (20)	7 (70)	2 (20)	0 (0)	5 (50)	5 (50)	2 (20)
Degenerative changes								
Present	4 (40)	0 (0)	0 (0)	0 (0)	3(30)	0 (0)	0 (0)	5 (50)
Absent	6 (60)	10 (100)	10 (100)	10 (100)	7 (70)	10 (100)	10 (100)	5 (50)
Congestion								
Mild	2 (20)	6 (60)	10 (100) ***	8 (80)*	3 (30)	8 (80)*	8 (80)*	5 (50)
Moderate	6 (60)	4 (40)	0 (0)	2 (20)	7 (70)	2 (20)	2 (20)	5 (50)
Severe	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Inflammatory cells								
Chronic	5 (50)	6 (60)	9 (90)	9 (90)	6 (60)	5 (50)	5 (50)	3 (30)
Mixed	5 (50)	4 (40)	1 (10)	1 (10)	4 (40)	5 (50)	5 (50)	7 (70)
Epitheliotropism								
Present	7 (70)	2 (20)**	0 (0)**	3 (30)	0 (0)**	0 (0) **	0 (0) **	0 (0)**
Absent	3 (30)	8 (80)	10 (100)	7 (70)	10 (100)	10 (100)	10 (100)	10 (100)
Odema								
Mild	0 (0)	8 (80)***	10 (100)***	9 (90)***	10 (100)***	9 (90)***	10 (100) ***	6 (60)**
Moderate	7 (70)	2 (20)	0 (0)	1 (10)	0 (0)	1 (10)	0 (0)	4 (40)
Severe	3 (30)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Reactive atypia								
Mild	3 (30)	2 (20) **	0 (0) ***	4 (40) **	3 (30) **	0 (0)***	0 (0)***	4 (40) ***
Moderate	6 (60)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Severe	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Absent	0 (0)	8 (80)	10 (100)	6 (60)	7 (70)	10 (100)	10 (100)	6 (60)
Angiogenesis								
Present	10 (100)	7 (70)	6 (80)	7 (70)	8 (80)	6 (60)	7 (70)	6 (60)
Absent	0 (0)	3 (30)	4 (20)	3 (30)	2 (20)	4 (40)	3 (30)	4 (40)
Restoration of epithelium								
Absent	10 (100)	4 (40)**	1 (10)***	1 (10)***	7 (70)	2 (20) ***	3 (30) **	4 (40)**
Incomplete	0 (0)	4 (40)	2 (20)	7 (70)	3 (30)	3 (30)	2 (20)	4 (50)
Complete	0 (0)	2 (20)	7 (70)	2 (20)	0 (0)	5 (50)	5 (50)	2 (10)
Inflammatory degree								
Mild	0 (0)	6 (60) **	10 (100) ***	10 (100)***	6 (60) **	7 (70) **	5 (50)*	5(50) *
Moderate	10 (100)	4 (40)	0(0)	0 (0)	4 (40)	3 (30)	5 (50)	5(50)
			Submucosal	**Changes**				
Congestion								
Mild	0 (0)	7 (70) **	10 (100)***	10 (100)***	7 (70) ***	10 (100) ***	5 (50)*	7(70) ***
Moderate	10 (100)	3 (30)	0 (0)	0 (0)	3 (30)	0 (0)	5 (50)	3(30)
Odema								
Mild	0 (0)	10 (100)*** 0 (0)	10 (100)***	10 (100)***	10 (100)***	10 (100) ***	10 (100) ***	8(80)***
Moderate	10 (100)		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2(20)

< 0.01and ***P< 0.05,**P<*Significance between treated groups and ulcerated-untreated group. (Meso, Mesocarp; Epi, Epicarp).).*P 0.001.

Epi-Ex 500 and Ranitidine displayed near similar healing process indicators as regarding to the histopathological parameters with statistically insignificant differences between them. On the other hand, Epi-Ex 500 had a statistically significant ability to enhance healing and reducing the damaging effect as compared with Omeprazole and Sucralfate (p<0.05) (Figure 3). The promising efficacy of Epi-Ex 250 was appeared in its significant improvement of degenerative epithelial effect in relation to Sucralfate (p<0.05) (Figure 2&3) and simultaneously with insignificant changes in all histopathological parameters as compared to the selected 3

drugs. Ranitidine and Omeprazole has a statistically significant ($p<0.05$) ability in enhancing healing capacity than Meso-Ex 500 ($p<0.05$). In contrast, Meso-Ex. 250 is more effective than Omeprazole and Sucralfate in enhance healing and reduce the cellular damage ($p<0.05$) (Table 1).

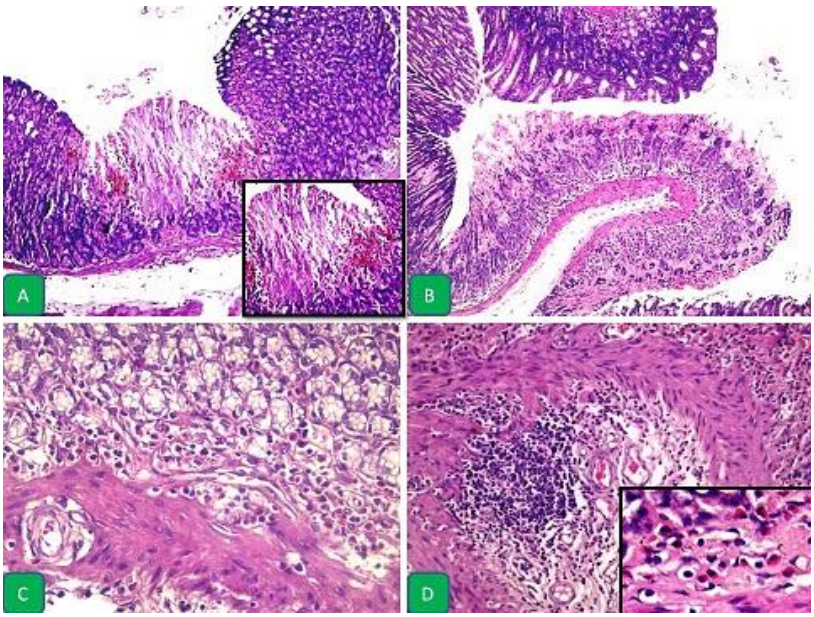

Figure 1: Section in gastric mucosa of untreated rats. A: Solitary ulceration at the body resting on muscularis mucosa and covered by necrosis and hemorrhage. Submucosa showed moderate degree of oedema (H&E X100). Inset; high power view showing necrosis and haemorrhage (H&E X400). B: Superficial erosion with reactive atypia in adjacent mucosa (H&E X200). C: Base of gastric mucosa showed epitheliotropism and scattered eosinophilic infiltrates (H&E X400). D: Submucosa showed congestion, lymphoid aggregates and scattered eosinophils (H&E X200). Inset showing prominent eosinophilic infiltrates (H&E X400).

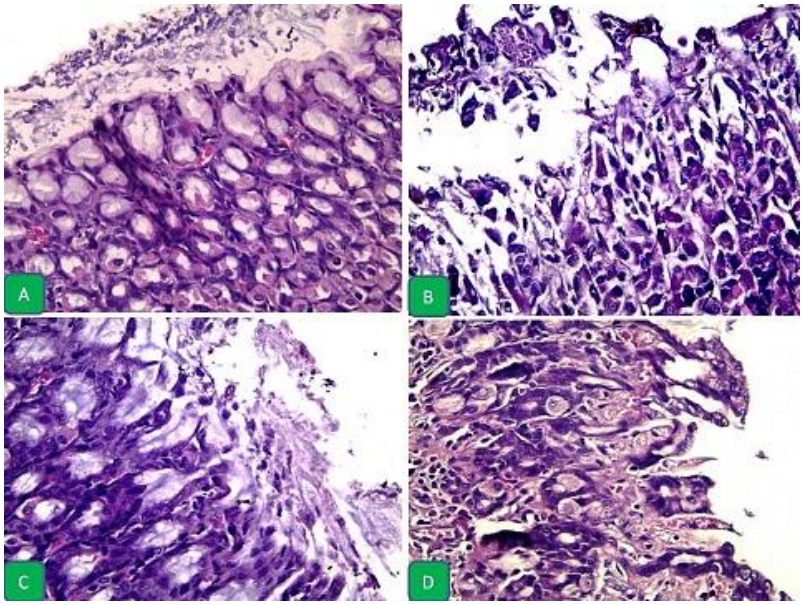

Figure 2: A: Section in gastric mucosa of a rat treated with Epi-Ex 500 showed great reduction of damage, complete restoration of epithelium and effective healing. B: Section in gastric mucosa of a rat treated with Meso-Ex 500 showed gastric mucosa with erosion of mucosa, minimal healing in form of focal incomplete restoration of epithelium. C: Section in gastric mucosa of a rat treated with Epi-Ex 250 showed superficial erosion of mucosa with partial restoration of normal epithelium and few angiogenic blood vessels. D: Section in gastric mucosa of a rat treated with Meso-Ex 250 showed superficial erosion of mucosa, partial restoration of normal epithelium, prominent reactive atypia, chronic inflammatory infiltrates and angiogenesis (H&E X400). (Meso-Ex, Mesocarp Extract; Epi-Ex, Epicarp Extract).

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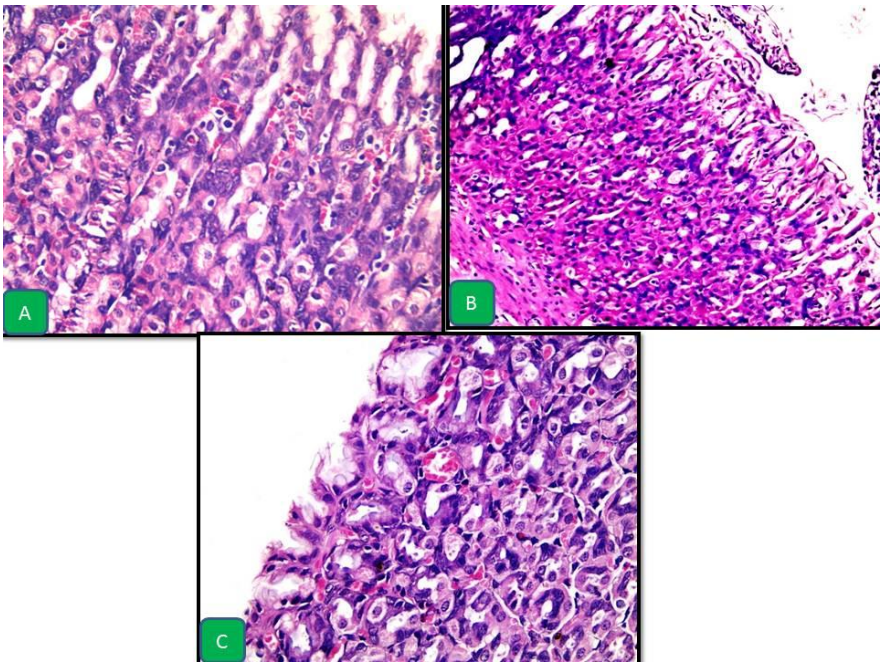

Figure 3: A: Section in gastric mucosa of a rat treated with Ranitidine showed complete restoration of epithelium with few angiogenic blood vessels. B: Section in gastric mucosa of a rat treated with Sucralfate showed incomplete restoration of epithelium with few angiogenic blood vessels. C: Section in gastric mucosa of a rat treated with Omeprazole showed partial complete restoration of epithelium with few angiogenic blood vessels.

COX-2 expression in rat stomach

Table 2: COX-2 H score in ulcer-induced rats treated either with different pomegranate peel extracts or pharmaceutical drugs.

Groups	COX-2 H score Mean ± SD	P value
Control	149.7±5.23	-
Meso Ex 250	135±3.7	*P*<0.001
Meso Ex 500	144.8±3.15	*P*<0.05
Epi Ex 250	107.4±5.9	*P*<0.001
Epi Ex 500	85.3 ±3.33	*P*<0.001
Ranitidine	254.7±3.33	*P*<0.001
Omeprazole	288.95±5.61	*P*<0.001
Sucralfate	195±10.21	*P*<0.001

*Significance between treated groups and ulcerated- untreated group (Meso Ex, Mesocarp Extract; Epi Ex, Epicarp Extract)

COX-2 IHC expression was assessed by using 2 objective methods; localization topography and Histo-score (H score). Both intensity and percent of distribution were used to calculate H score for each case. Table (2) showed COX-2 concentration in ulcerated and all treated groups. In normal gastric mucosa, COX-2 was present in the lower half only, while the upper half showed complete negativity (Figure 4A). In untreated ulcer (positive control group), COX-2 expression showed positively in all thickness of mucosa with differential distribution of stain as moderate to intense in the lower half while mild to moderate in the upper half of mucosa (Figure 4 B&C). The most powerful anti- COX-2 effect on gastric mucosa is exerted by Epi-Ex 500 (Figure 5A). As COX-2 expression in gastric mucosal with erosion that either associated with complete or incomplete healing showed restoration of the normal COX-2 pattern localization topography in normal gastric mucosa with minor different extent. Gastric mucosa with administration of Epi-Ex 500 showed reduction of COX-2 expression that simulating the expression in normal gastric mucosa but with focal residual superficial COX-2 positively in recently healed with complete restoration of the epithelium. COX-2 H score in gastric mucosa with administration of Epi-Ex 500 showed the lowest values in comparison with other groups. The powerful anti-inflammatory (anti COX-2) effect is arranged in descending orders as follows; Epi-Ex 500, Epi-Ex

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250, Meso-Ex 250 and Meso-Ex 500. In Figure (6) Omeprazole and Ranitidine treated group showed moderate intensity of COX-2 expression while Sucralfate group express mild to moderate intensity of COX-2 expression in all layers.

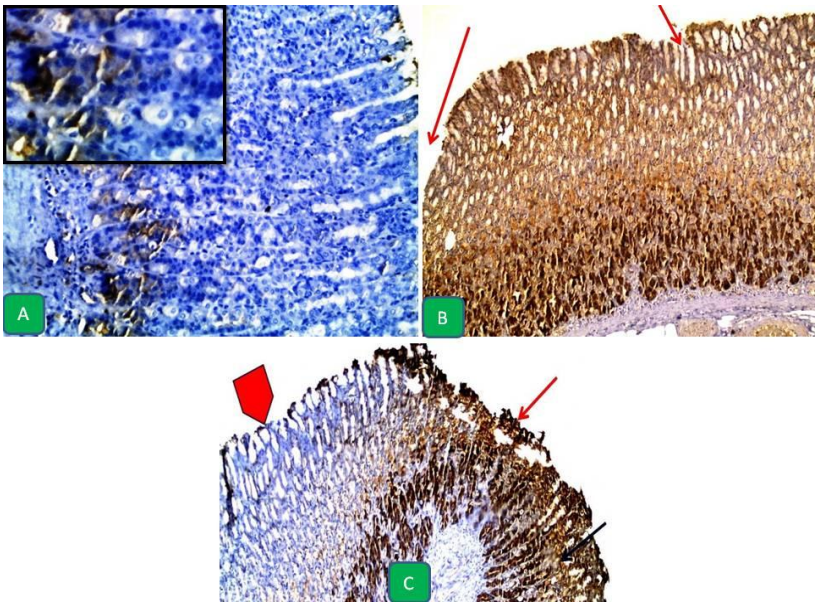

Figure 4: A: Section in normal gastric mucosa showed intense positive COX-2 expression in the lower half of gastric glands and negative expression in the upper half of glands (IHC X100), Inset high power view (IHC X400). B: Section in gastric mucosa of untreated ulcer rats showed full thickness COX-2 expression with intense stain in the lower half. C: Section in gastric mucosa of untreated ulcer rat showed area of deep erosion (red arrow) and area of healing with incomplete restoration of epithelium (black arrow) exhibiting full thickness COX-2 expression in contrast with normal mucosa (head arrow) (IHC X100).

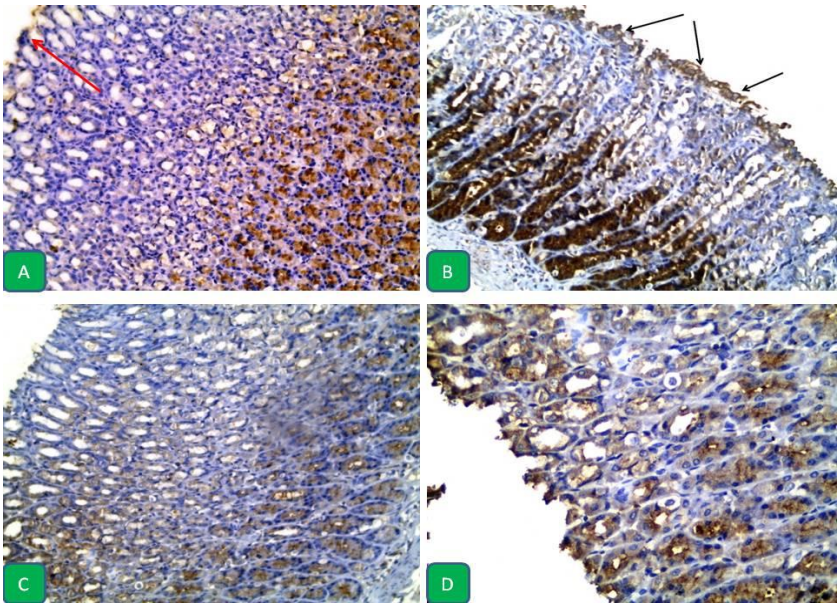

Figure 5: A: Section in gastric mucosa of a rat with administration of Epi-Ex 500 showed reduction of COX-2 expression that simulating the expression in normal gastric mucosa but with residual superficial COX-2 positively (Red arrow) in recently healed with complete restoration of the epithelium. B: Section in gastric mucosa of a rat with administration of Epi-Ex 250 showed reduction of COX-2 expression that near simulating the expression in normal gastric mucosa but with more residual superficial COX-2 positively (Black arrows) in healing mucosa with incomplete restoration of epithelium. C: Section in gastric mucosa of a rat with administration of Meso-Ex 500 showed intense COX-2 expression in the whole thickness of ulcerated poorly healing mucosa. D: Section in gastric mucosa of a rat with administration of Meso-Ex 250 showed moderate COX-2 expression in the whole thickness of eroded healing mucosa (IHC X100 for A, B and C X200 for D). (Meso-Ex, Mesocarp Extract; Epi-Ex, Epicarp Extract).

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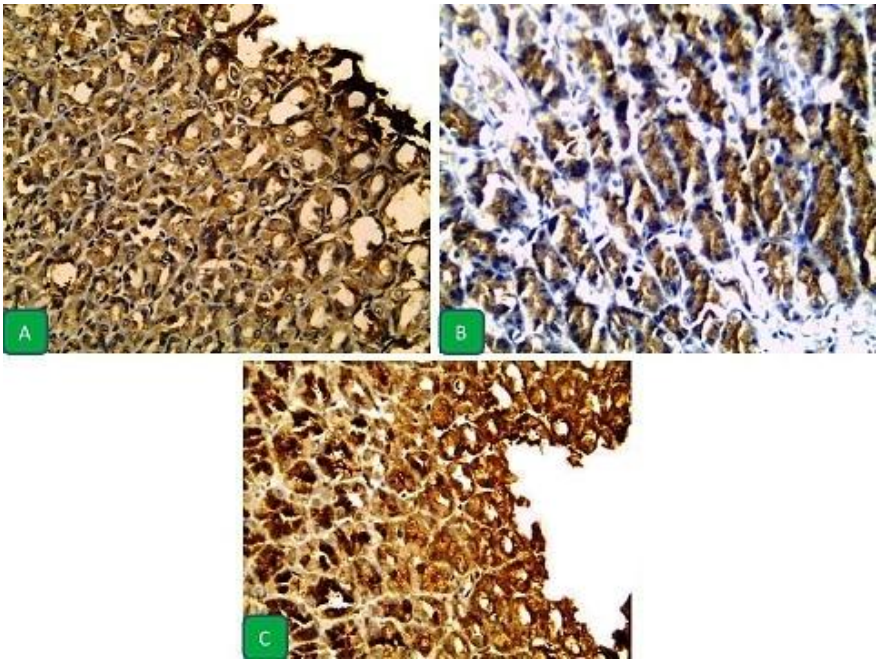

Figure 6: A: Section in gastric mucosa of a rat treated with Omeprazole showed moderate intensity of COX-2 expression in all layers B: Section in gastric mucosa of a rat treated with Sucralfate showed mild to moderate intensity of COX-2 expression in all layers. C: Section in gastric mucosa of a rat treated with Ranitidine showed moderate intensity of COX-2 expression in all layers (IHC X400 for all).

Effect of peel extracts on angiogenesis

VEGF: The result in Figure (7a) showed a significant increase of VEGF level in untreated gastric ulcer group as compared to control group (p<0.01). A positive correlation was reported between ulcer formation and elevation in VEGF level (r= 0.635, p<0.01). A slight reduction in VEGF secretion levels was observed in all treated groups; however, it is statistically insignificant. The maximum reduction was observed in Sucralfate-treated group as compared to untreated ulcer group (p<0.001).

PDGF: Figure (7b) showed a remarkable decrease in PDGF level in ulcerated group as compared to their normal control counterpart. A negative correlation was reported between ulcer formation and reduction in PDGF level (r= -0.874, p<0.001). A statistically significant (p<0.001) increase of PDGF levels was observed in all treated groups as compared to untreated ulcer group. Dose 250 mg/kg of both extracts produced more PDGF than 500 mg/kg (p<0.001 and p<0.05; respectively).

Effect of peel extracts on pro-inflammatory cytokines

IL-1β: Compared with control group, IL-1β level was increased by untreated ulcer group, although it was statistically insignificant. Elevation in IL-1β was directly correlated with ulcer formation (r= 0.740, p<0.001). A pronounced elevation (p<0.001) in IL-1β was demonstrated in all ulcer treated groups in relation to normal controls (Figure 8a). Although IL-1β production was increased by treatment, the significant elevation was reported in Meso-Ex 500, Ranitidine and Omeprazole (p<0.001).

IL-6: As shown in the (Figure 8b), a slight insignificant increase of IL-6 level was observed in untreated ulcer group as compared to control group. Animal group received 250 mg/kg from Meso-Ex has a statistically elevated IL-6 level in relation to normal, ulcer and 500 mg/kg Meso-Ex groups (p<0.001). Only Sucralfate displayed a significant increase of IL-6 level in relation to negative (p<0.01) and untreated ulcer (p<0.05) groups. IL-6 level remain higher in all treated groups except for Ranitidine.

TNF-α level: The results in (Figure 8c) demonstrated a significant increase (p<0.001) in TNF-α in untreated group in relation to control group. Elevation in TNF-α was positively correlated with ulceration (r= 0.676, p<0.001).A statistically significant reduction in TNF-α levels was observed in extract-treated groups (p<0.001,

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p<0.05, *p*<0.001, *p*<0.01 for Meso-Ex 250, Meso-Ex 500, Epi-Ex 250, Epi-Ex 500; respectively) and drug-treated groups (*p*<0.001); except Ranitidine, in relation to untreated ulcer group.

Figure (7a)

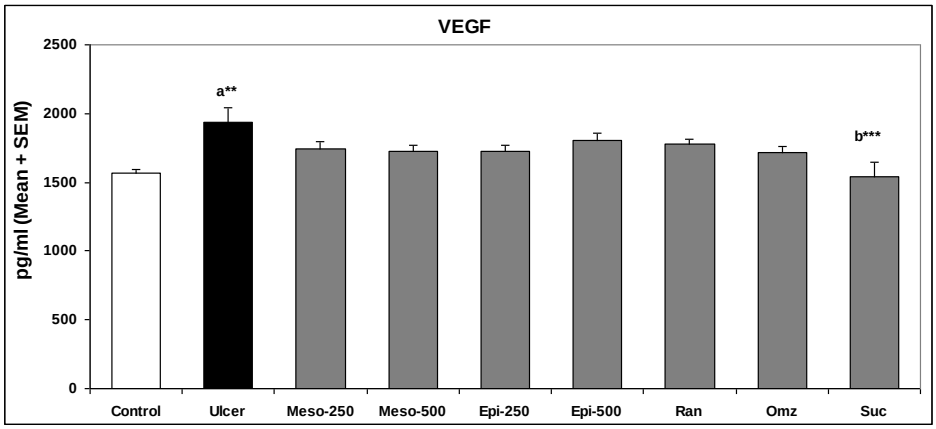

Figure (7b)

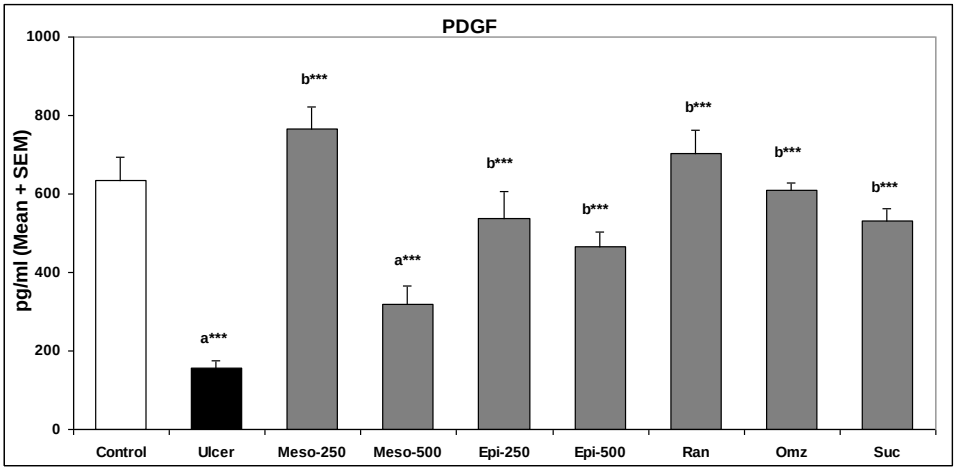

Figure 7a & 7b: Plasma level of Vascular Endothelial Growth factor (VEGF) (a) and Platelet Derived Growth factor (PDGF) (b) in Ethanol-induced gastric ulcer. Results are expressed as mean ± standard error (Meso, Mesocarp; Epi, Epicarp; Ran, Ranitidine; Omz, Omeprazole; Suc, Sucralfate). a: denotes groups statistically significantly different from control (normal group); b: groups statistically significantly different from ulcer (ethanol-untreated group). **p*<0.05, ***p*<0.01 and ****p*<0.001.

Figure (8a)

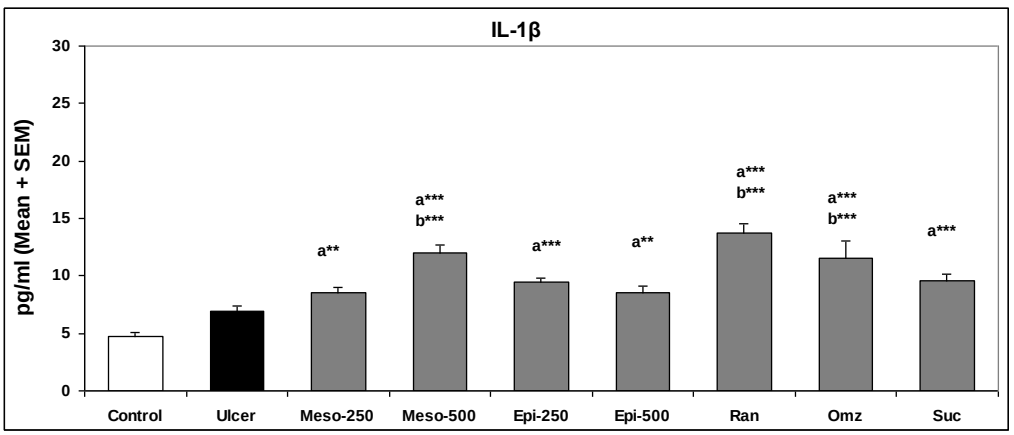

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Figure (8b)

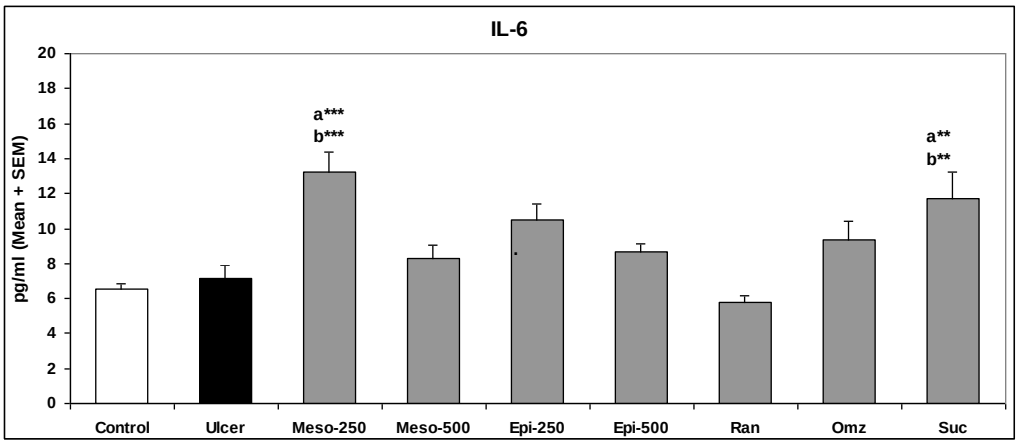

Figure (8c)

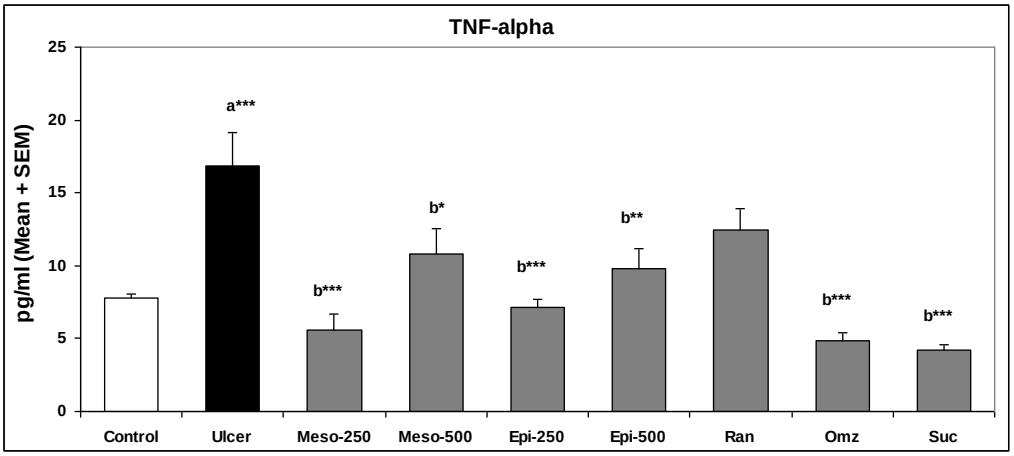

Figure 8a, 8b & 8c: Plasma level of Interleukin-1β (Il-1β) (8a), IL-6 (8b) and (TNF-α) (8c) in Ethanol-induced gastric ulcer. Results are expressed as mean ± standard error (Meso, Mesocarp; Epi, Epicarp; Ran, Ranitidine; Omz, Omeprazole; Suc, Sucralfate). a: denotes groups statistically significantly different from control (normal group); b: groups statistically significantly different from ulcer (ethanol-untreated group). *p<0.05, **p<0.01 and ***p<0.001.

DISCUSSION

Peptic ulcer is one of the most common gastrointestinal diseases. It is more common among male adults and in low socio-economic groups of people [23]. The etiology of the peptic ulcer may be due to overproduction of gastric acid or decrease in gastric mucosal production [24]. Gastric tissues upon exposure to ulcerogenic agents such as ethanol, NSAIDs, bile acids, and ischemia shows characteristic morphologic and functional changes leading to gastric injury [25].

Ethanol-induced ulcer model is useful to measure the cytoprotection activity of a substance or element, as previously described by Robert et al. [26]. Its ulcerogenic activity is due to its ability to disturb gastric secretory activity, alter cell permeability and deplete gastric mucus [27]. The necrotizing effect of ethanol is caused due to the production of free radicals and is associated with severe hemorrhage [28].

Gastric ulcer process generically involves cell migration, proliferation, re-epithelization, angiogenesis and deposition of extracellular matrix [29]. Many factors regulate ulcer formation, such as cytokines, prostaglandins, nitric oxide, growth factors and sensorial neurons [30]. Gastric ulcer formation could be divided into two stages. The first stage presents quick cicatrisation and depends on contraction of the ulcer basis; and the one present's slow cicatrisation and depends additionally on mucosa regeneration. The

epithelial reconstitution is performed by proliferation of undifferentiated epithelial precursors, which migrate from the ulcer border to the granulation tissue thus covering the base of the ulcer [31,32].

To recover all of the above mentioned harmful changes, various therapeutic agents including plant extracts were used such as *Nigella sativa Linn* [33], *Aloe barbadensis Mill* [34] and *P. granatum*. Therefore the proposed anti-ulcerogenic, anti-angiogenic and anti-inflammatory potential of the pomegranate peel extracts were evaluated in the current study on ethanol-induced gastric ulcer.

In the work presented herein, the anti-ulcerogenic efficacy of methanolic extract of pomegranate peel was evident from significant reduction of the gastric ulcer with pomegranate peel treatment. The most effective treatment was Epi-Ex at 500mg/kg. Phytochemical studies of pomegranate peel revealed the presence of flavonoids and tannins [35]. Previous studies suggested that intake of flavonoids, a group of polyphenolic compounds found in vegetables and fruits, is beneficial for prevention of cardiovascular [36], inflammatory and other diseases [37,38]. Several reports have shown that flavonoids (such as flavones, flavanones, aurones and chalcones) exert protective effects against experimentally induced lesions of the stomach [39]. Polyphenols (phenolic acids, flavonols, flavones and tannins) are able to inhibit the proton pump present on the parietal cell [40,41] and by this means participate in protecting the stomach against damaging agents [42]. Also, polyphenols present free-radical-scavenging properties, a stimulatory effect on prostaglandine E2 [43]and therefore of mucus secretion [44], the three main components of the gastric mucosal barrier [45]. Tannins represent a unique group of phenolic metabolites in numerous woody and some herbaceous higher plants species [46]. Their capacity to prevent ulcer development may be due to protein precipitating and vasoconstricting effects [47]; their astringent action helps in precipitating microproteins on the ulcer site, thereby forming an impervious layer over the lining that hinders gut secretions and protects the underlying mucosa from toxins and other irritants [48]. Since tannins and flavonoids have been demonstrated in pomegranate peel extract in the current study, it is possible that these components may be involved in its antiulcer activity.

COX-2 is an inducible enzyme expressed in injures for inflammatory processes or facing mutagenic agents [49]. It is an important enzyme for the gastric mucosa because it, in cicatrisation process, induces angiogenesis, cell proliferation and cytoprotection, related to the production of prostaglandins that stimulates mucus and bicarbonate production [50]. The present study was in agreement with Sun et al. [51]who demonstrated that normal gastric mucosa express a lower level of COX-2, whereas gastric mucosal epithelium expresses this isoform after various stimuli. In the current study, pomegranate peel extract administration showed a significant dose-dependent down regulation of COX-2 IHC expression. This finding confirmed that pomegranate extract had anti-inflammatory effect by inhibition of COX-2. Our results comply with previous reports, where pomegranate and the selected chemical constituents isolated from peel have been found to have a large range of effects such as inhibition of COX-2 expression [52].

In view of the antioxidant and anti-inflammatory properties of pomegranate, phenolics and/or its derived metabolites have a beneficial effect on inflammation. Larrosa et al. [53] evaluated the effects of pomegranate intake and its main microbiota-derived metabolite urolithin-A (UROA) on colon inflammation. They reported a reduction in COX-2 in colonic mucosa. Also, pomegranate, widely used as an antipyretic analgesic in Chinese culture as reported by Lee et al. [54].

In addition to its local anti-inflammatory effect (as proposed by reduction of COX-2 in gastric mucosa), pomegranate may have systemic anti-inflammatory and anti-angiogenic effect. To confirm this idea, serum VEGF and PDGF (pro-angiogenic) and TNF-α, IL-1β and IL-6 (pro-inflammatory) were examined.

A previous study of detailed histological examination of ethanol-induced gastric mucosal injury revealed areas of focal hyperemia and hemorrhage in the damaged portions of the stomach, which suggests a role for impaired blood flow in the genesis of ethanol-induced gastric lesions [55]. In the current study, gastric ulcer induction by ethanol increased VEGF level that was reduced when the rats were treated with pomegranate Peel extract. The anti-angiogenic potential of pomegranate was evaluated by Toi et al. [56] where VEGF was measured in estrogen sensitive MCF-7, estrogen resistant MDA-MB-231 human breast cancer cells and MCF-10A immortalized human breast epithelial cells. Pericarp polyphenols were shown to strongly down regulated VEGF in MCF-10A and MCF-7 representing a marked potential for down regulation of angiogenesis by pomegranate fractions. Interestingly, in a test using the chicken chorioallantoic membrane

model, pomegranate fermented juice polyphenols were found to inhibit angiogenesis, but the pomegranate pericarp polyphenols were not active [56]. In the present study, VEGF production after the induction of gastric ulcer in rats by ethanol was in agreement with Jones et al. [57] who reported a 4-6 fold increase in VEGF mRNA and protein in the mucosa-bordering necrosis after 24 hour from the induction of ulcer by ethanol.

In the current study, ethanol administration reduced PDGF level, which increased in pomegranate peel extract treated animals. Reduced PDGF concur with reports that PDGF is a critical regulator of cell proliferation. Such inhibitory effects of ethanol may result from interference with mitogenic growth factors, specifically with the PDGF. This implies that ethanol acts on the signal transduction system mediating growth factor-stimulated cell proliferation [58]. Although pomegranate peel extract has anti-angiogenic effect, elevated PDGF levels demonstrated in our work complies with previous studies reporting that ulcer healing process include reconstruction process of mucosa through formation of granulation tissue. Granulation tissue consists of proliferation of connective tissue cells, such as macrophage, fibroblast and proliferative endothelial cell which form microvessels through angiogenesis process [59]. The migration from fibroblast to granulation tissue and its proliferation process are induced by PDGF [60]. To our knowledge, no previous work has been carried out on the effect of pomegranate peel extract on PDGF induction in ulcer healing.

Cytokines play a central role in the regulation of the mucosal immune system and therefore are extremely important in mucosal defense. Several pro-inflammatory cytokines are implicated in the pathogenesis of peptic ulcer, such as IL-1β, IL-2, IL-6, IL-8 and TNF-α [61]. Gastric mucosa inflammation by ethanol is accompanied by increased production of TNF-α which augments neutrophil-derived superoxide generation and stimulates the production of IL-1β, leading to neutrophil accumulation [62]. However; IL-1β, IL-6 and TNF-α can drive inflammation in pathological contexts. They also have important beneficial roles in the control of infections [63]. In view of this, IL-1β, IL-6 and TNF-α were studied in ulcer-untreated and pomegranate peel extract-treated groups. Our results showed that ulcerated-untreated rats had elevated pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) levels. However, pomegranate peel extract treatment modulated TNF-α in all doses, it simultaneously induced production of IL-1β and IL-6.

Previous studies have reported the anti-inflammatory properties and immunomodulatory effects of polyphenolic compounds, where many studies showed a decreasing effect of polyphenols on a previously increased biomarker for inflammation, such as IL-1β, IL-6, and TNF-α [64,65]. Reduced TNF-α observed in our results was simultaneous with Kowalski et al. [66] who found that pomegranate peel flavonols (e.g., kaempferol) suppress the expression of TNF-α. In treated animals, an increase of IL-1β and IL-6 could be explained depending on the previous reports that have determined polyphenols to influence immune-relevant biomarkers[67-] [67,68,69,70].

Also reported is a powerful anti-ulcer, anti-secretory, and prostaglandin-stimulating effect of IL-1β [71,72]. It has been found that IL-1β reduces injury through a paradoxical inhibitory action on leukocyte adherence. Furthermore, IL-1β has played a role in the inhibition of gastric acid secretion [73,74]. It stimulates the release of prostaglandin and nitric oxide possibly by inducing inducible nitric oxide synthase (iNOS) expression and COX-2 expression, thus provide a protective role to gastroduodenal mucosa [75]. In contrary, Amandeep et al. [76] demonstrated the role of immune system in the pathogenesis (not in protection) of ulcers, mainly T-lymphocytes and cytokines produced by them.

CONCLUSION

In conclusion, methanolic extract of *P. granatum* fruit peel, either mesocarp or epicarp, is protective against ethanol-induced stomach injury. Pomegranate peel extract acts on inflammation via decreasing plasma TNF-α level and down regulating IHC COX-2 expression in gastric mucosa, however, its effect on IL-1β and IL-6 elevation should be examined. Pomegranate peel extract treatment reversed the effect on VEGF production, although, they induce PDGF secretion that important for the process of granulation tissue proliferation. Hence, it might be suggested that pomegranate peel extracts could be used as a therapeutic agent for gastric ulcer patients. Further pharmacokinetic analyses of these extracts are required to identify the most powerful antiulcerogenic component(s) in the studied extracts.

Conflict of interest: Authors declares that no conflict of interest.

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Glossary: COX-2, Cyclo-oxygenase; Epi Ex, Epicarp Extract; iNOS, inducible Nitric Oxide Synthase; IL, Interleukin; Meso Ex, Mesocarp Extract; NO, Nitric Oxide; NFκ-B, Nuclear Factor Kappa; Omz, Omeprazole; PBS, Phosphate Buffered Saline; PDGF, Platelet Derived Growth Factor; *P. granatum, Punica granatum;* Ran, Ranitidine; Suc, Sucralfate; TNF-α, Tumor Necrosis Factor-alpha; UROA, Urolithin-A; VEGF, Vascular Endothelial Growth Factor.

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