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RESEARCH ARTICLE

Potential Health Benefits of a Pomegranate Extract, Rich in Phenolic Compounds, in Intestinal Inflammation

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Abstract: ***Background*:** Pomegranate is a fruit rich in bioactive compounds such as punicalagins, gallic acid, and ellagic acid derivatives. It has been widely used since ancient times in traditional medicine for a wide variety of diseases. It has been reported that bioactive compounds, such as polyphenols, are able to induce the expression of cytoprotective enzymes, including HO-1. The contribution of HO-1 activity to the prevention of intestinal inflammation has been shown in different models of Inflammatory bowel diseases (IBD).

***Objective*:** Aim of the present research was to investigate the molecular mechanisms involved in the beneficial effects of a pomegranate extract (PE), rich in bioactive compounds in intestinal inflammation.

***Methods*:** Caco-2 cells exposed to LPS and DSS induced colitis were chosen as convenient experimental models of intestinal inflammation.

***Results*:** Results obtained in our experimental conditions showed that PE *in vitro* was able to induce HO-1 and to reduce cellular damage and oxidative stress through an increase of GSH levels. Moreover, PE was able to decrease the pro-inflammatory marker IL-8 levels and activate TIGAR pathway. The results obtained *in vivo*, in agreement with the data obtained *in vitro*, highlighted the ability of PE to reduce intestinal inflammation, preserve the colon length and histological features and reduce IL-6 levels compared to the DSS treated group.

***Conclusion*:** PE, rich in bioactive compounds, could contribute, as a supportive therapy, to enhance the effects of the conventional therapeutic strategies on the management of IBD.

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1. INTRODUCTION

Pomegranate is a fruit rich in bioactive compounds such as punicalagins, gallic acid, and ellagic acid derivatives [1]. It has been widely used since ancient times in traditional medicine for a wide variety of diseases, including upper respiratory tract infections and flu [2]. Many investigators have reported that pomegranate extracts are rich sources of bioactive compounds with free radical scavengers and potent antioxidant capacity [3-6]. These extracts were also reported to possess anticancer, antiviral, anti-inflammatory and hypolipidemic bioactivities [7]. The potential health benefits of such extracts are attributed to the polyphenolic compounds contained in the pomegranate fruits [8-12]. Current evidence strongly supports the role of polyphenols in the prevention of various diseases, including cardiovascular diseases, cancers, neurodegenerative diseases, diabetes and Inflammatory bowel diseases (IBDs) [9, 13-15]. IBDs include Crohn's Disease and Ulcerative Colitis which are correlated with strong inflammatory conditions of the colon and small intestine [16, 17]. Several hypotheses have been proposed to determine the possible causes of IBDs: one hypothesis suggests that deregulation of the mucosal immune system leads to ex-

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cessive immunologic responses against the normal gut microflora, while another one proposes that changes in the composition of the gut microflora associated with a disrupted epithelial barrier lead to an abnormal inflammatory response from the intestinal mucosa [18, 19]. An imbalance of the cytokine profile in favour of pro-inflammatory cytokine (such as IL-6 or IL-8) overproduction leads to inflammatory conditions, as observed in IBD [20]. Nowadays, the treatment of IBD is based on drugs able to reduce symptoms and inflammation, such as 5-aminosalicylic acid, corticosteroids and immunomodulators [21]. However, these chronic treatments are not curative, but the only palliative and can have significant side effects. For this reason, there is considerable interest in the identification of new therapeutic strategies. Anti-inflammatory properties of polyphenols could contribute, as a supportive therapy, to enhance the effects of the conventional therapeutic strategies on the management of IBD. It has been reported that plant-derived polyphenols are able to activate the transcription factor called nuclear factor erythroid-derived 2 (Nrf2), which translocates into the nucleus to bind to antioxidant response element (ARE) and, as a consequence, modulates the expression of hundreds of genes, including the cytoprotective enzyme heme oxygenase-1 (HO-1).

This cytoprotective enzyme catalyzes the rate-limiting step in heme degradation, leading to the generation of equimolar amounts of iron ions, CO and biliverdin. This last one, together with bilirubin, formed to the action of biliverdin reductase (BVR), are potent antioxidants, but also the other products of HO-1 activity regulate important biological processes, including inflammation [22]. Sebastian *et al.* reported that the contribution of HO-1 activity to the prevention or reduction of intestinal inflammation had been shown in different models of IBD [23]. Evidence from intestinal *in vitro* and *in vivo* studies, indicates that anti-inflammatory effects related to pomegranate consumption may be due to its derived products, which may have a role in IBD prevention [24]. The aim of the present research was to investigate, in an *in vitro* model of IBD, the intestinal beneficial effects of phenolic compounds contained in high percentages in a pomegranate extract and their ability to upregulate the cytoprotective enzyme HO-1. Moreover, the effects of the pomegranate extract in an *in vivo* model of DSS induced colitis were also evaluated.

2. MATERIALS AND METHODS

2.1. Chemicals

The dry powdered pomegranate extract (PE) employed in this study, (Dermogranate®) manufactured by Medinutrex (Catania, Italy), was prepared from the residue of industrial processing of pomegranate fruits. Pomegranate fruits cv. "Wonderful", were processed at commercial maturity, and the residue of the fruits (exhausted peels, membranes and arils) was dried in an oven until a loss on drying of about 5.0%. The dried residue of pomegranate fruits was extracted with hydroalcoholic solutions (food grade). After distillation and recovery of alcohol (ethanol), the concentrated aqueous extract was subjected to a spray drying process to obtain the dry powdered extract.

The extract was standardized to contain ≥ 20.0% total polyphenols, ≥ 10.0% ellagic acid derivatives and ≥ 7.0% punicalagins. In particular, the extract had the following chemical composition: total polyphenols (22.4%), ellagic acid derivatives (15.6%) and punicalagins (8.3%). The total polyphenols content was determined using Folin-Ciocalteu spectrophotometric method as previously reported [1]. Separation and quantification of phenolic compounds were performed by HPLC-PDA-ESI/MS^n analysis as previously described [1]. Antioxidant activity of PE was tested by their ability to reduce the stable DPPH radical and to inhibit superoxide anion formation as previously reported [1].

2.2. Cell Culture and Cell Viability Assay

Caco-2 cells were used as a stable *in vitro* model for the intestinal epithelium. Caco-2 cells (heterogeneous human epithelial colorectal adenocarcinoma cells) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and grown in DMEM supplemented with 10% fetal bovin serum (FBS), 0,1% streptomycin-penicillin, 1% L-glutamine and 1% non-essential amino acids. Caco-2 cells were seeded at a concentration of 2×10^4 cells per well of a 96-well, flat-bottomed 200-µl microplate. Dose-response experiments were performed in cells incubated at 37°C in a 5% CO_2 humidified atmosphere and cultured for 24 h in the presence and absence of different concentrations (3.4, 1.7, 0.85, 0.56, 0.22, 0.11, 0.055, 0.028, 0.014 mg/ml) of PE. Cell viability was measured by MTT assay as previously reported [25].

2.3. Western Blotting

Caco-2 cells were cultured in T75 flasks and treated with different non- toxic concentrations of PE (0.56, 0.056, 0.028 mg/ml) corresponding to total polyphenols ranging from 134 µg/ml to 6.7 µg/ml, ellagic acid and derivatives ranging from 70.9 µg/ml to 3.5 µg/ml and Punicalagins A+B ranging from 63.5 to 3.2 µg/ml) for 24h. Electrophoresis, transfer blot and protein detection were carried out as previously described [10].

2.4. ELISA HO-1 Measurements

The commercially available ELISA kit (ADI-EKS-800, Enzo Life Sci) was used to measure HO-1 protein concentration in Caco-2 cellular lysates. The test was performed in agreement with the protocol reported by the manufacturer. Absorbance at λ=450 nm was measured and the concentration of HO-1 was calculated from a standard curve previously created using purified HO-1 protein. Results were expressed as pg HO-1/mg proteins. Individual measurement was performed in triplicate and data are reported as average.

2.5. Treatments to Induce *In Vitro* Intestinal Inflammation

Caco-2 cells ($2x\ 10^4$) were seeded in six-well plates and cultured to reach the confluence. An experimental inflamma-

tory condition in Caco-2 cell monolayers was induced by the exposure for 12 h to lipopolysaccharide (LPS) (100 μg/ml). Twenty-four hours pre-treatment with the chosen concentration (0.56 mg/ml) of freshly prepared PE dissolved in PBS, in presence and absence of the inhibitor of heme oxygenase activity, stannous meso-porphyrin (SnMP) (5 μM), was applied before inflammatory stimuli.

2.6. RNA Extraction and qRT-PCR

Quantitative Real-Time PCR was performed in 7900HT Fast Real-Time PCR System Applied Biosystems using the SYBR Green PCR MasterMix (Life Technologies) as previously described [26].

2.7. GSH Content Determination and Oxidative Stress Assay

Determination of glutathione (GSH) was performed by the spectrophotometric assay as previously described [25]. The quantitative measurement of cellular populations undergoing oxidative stress was performed using the Muse Oxidative Stress Kit (Merck Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The kit determines the percentage of cells that are negative [ROS (−)] and positive [ROS (+)] for reactive oxygen species (ROS). The count and percentage of cells undergoing oxidative stress were quantified using the Muse Cell Analyzer and Muse analysis software (Merck Millipore, USA) [26].

2.8. Treatments to Induce *In Vivo* Intestinal Inflammation: Effect of PE in DSS-Induced Colitis in Mice

The DSS model is a well-established model to induce *in vivo* intestinal inflammation [27]. C3H female mice (5-7 weeks) were obtained from The Laboratory Animal Care Facility (LACF) of the AGU, Bahrain (Arabian Gulf University Ethical Approval No: E022-PI-1). All mice were housed in pathogen-free conditions and maintained in the same room on a 12:12-hour light/dark cycle. The temperature was maintained at 22 ± 2°C for the duration of the study. Prior to commencing the study, animals were provided with tap water and food ad libitum. On Day 0 of the study, mice were randomly separated into three groups (n=five per group): 1) Control mice group (CTRL); 2) DSS mice group (DSS); 3) DSS+PE mice group (DSS+PE). Control mice group (CTRL) received tap water without DSS. Two random groups (DSS, DSS+PE) were given 6% DSS in drinking water for 7 days and then switched to drinking water without DSS for 21 days to reproduce the inflammatory events in IBD. At day 14, the pomegranate group (DSS+PE) was treated with pomegranate extract (PE) dosed at 150 mg/kg (15mg of extract was weighed and dissolved in 1.5 ml of PBS) 0.3 ml was injected daily per animal (intraperitoneal) for 2 weeks. The dose of PE was chosen to base on the analyses described in the literature [15, 28-30]. It should be noted that the concentrations of polyphenols contained in PE used in the present study are close to the human plasma concentrations found in physiological conditions following the intake of pomegranate extract [31]. Mice weight and clinical conditions (stool consistency) were monitored daily and at the end of the treatments, weight change was expressed as a ratio of day 28 weights to day 14 weights of the same mouse and compared to the control group. After 4 weeks, mice were sacrificed with CO_2 .

Immediately after euthanasia, a midline incision was made, the inferior vena cava was exposed, and through a 24G needle syringe 0.3 to 0.4 ml was collected. The blood was kept in Eppendorf heparinized tubes. The whole length of the colon was excised from the cecum to the rectum. Collected blood was centrifuged at 2500 RPM for 15 minutes to separate the plasma.

Animal weight and colons length were measured and compared. Colon tissue samples were fixed in 10% formalin and underwent histological scoring by H&E staining to detect and compare cellular infiltration, depletion and damage between the different mouse groups. The Research and Ethics Committee of Arabian Gulf University (AGU), Bahrain approved all experimental procedures.

2.9. ELISA IL-6, TNF-alpha, CXCL1 Measurements

Plasma samples were used to quantitatively determine levels of IL-6 cytokine, TNF-alpha and CXCL1 by the sandwich enzyme immunoassay technique using Quantikine ELISA kits (Quantikine ELISA, Mouse IL-6, mouse TNF-alpha and mouse CXCL1-R&D Systems, Inc., Minneapolis, USA) and following the recommendation of the manufacturer.

Briefly, the samples, standards and controls were added to the assay diluent in duplicates and incubated for 2 hours at room temperature. After incubation, the wells were washed 4 times with wash buffer. The mouse-appropriate conjugate was added to all wells and incubated for another 2 hours at room temperature. The wells were washed 4 times, and the substrate solution was added and incubated for 30 minutes at room temperature in the dark. The reaction was stopped by a stop reagent, and the OD is detected at 450 nm and 540 nm. The readings at 540 nm were subtracted from the readings at 540 nm. The protein concentration was determined by plotting the OD corrected result against the standard curve. Results were expressed as pg/ml plasma. Individual measurement was performed in triplicate, and data are reported as average.

2.10. Statistical Analysis

Statistical analyses of multiple comparisons were performed by the Fisher method. P-values lower than 0.05 were accepted as significant. Data were analyzed using either single-factor analysis of variance (ANOVA) for multiple groups or the unpaired t-test for two groups, and the results are presented as mean ± SD.

3. RESULTS

3.1. Effect of PE in *In Vitro* Model of IBD

3.1.1. Effect on Cell Viability

The highest concentration of PE significantly reduced cell viability (3.4 mg/ml), whereas the other (1.7, 0.85, 0.56,

0.22, 0.11, 0.055, 0.028, 0.014 mg/ml) concentrations were not toxic as they had no significant inhibitory effect (Fig. **1**).

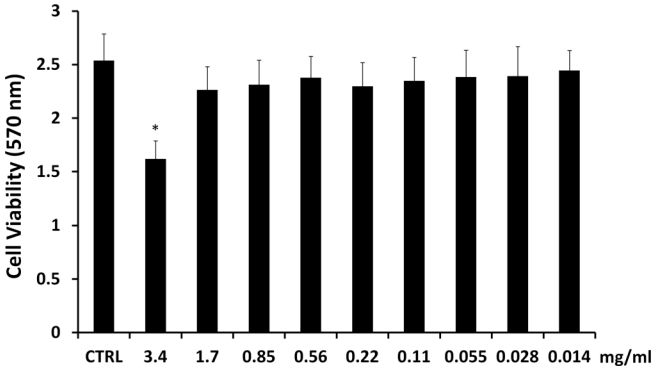

Fig. (1). Cell viability of Caco-2 in absence or presence of PE at different concentrations (3.4-1.7-0.85-0.56-0.22-0.11-0.055-0.028-0.014 mg/ml). Results are expressed as the means ± SD of 4 experiments performed in triplicate. Significant *vs.* untreated controls: *p<0.005.

3.1.2. Effect on HO-1 Eexpression

Western blotting was performed to evaluate levels of HO-1 expression in Caco-2 cells treated with different non-toxic concentrations of PE (0.56, 0.056, 0.028 mg/ml) for 24h. A significant HO-1 induction was observed in cells treated with 0.56 mg/ml. This concentration was chosen for further experiments (Fig. **2**).

3.1.3. Effect of LPS and PE on OH-1 Expression

ELISA was performed to evaluate levels of HO-1 expression in Caco-2 cells treated with LPS in the presence or absence of PE. In Caco-2 cells treated only with LPS, a significant increase of HO-1 protein was detected. PE pre-treatment resulted in HO-1 levels reduction. However, Caco-2 cells pre-treated with PE, showed higher HO-1 protein levels with respect to the control group (Fig. **3**).

3.1.4. Quantitative RT-PCR

Quantitative Real-Time PCR was performed to measure HO-1, TIGAR and IL-8 mRNA levels. In Caco-2 cells treated only with LPS, a significant increase of HO-1, TIGAR and IL-8 mRNA levels were detected. PE pre-treatment resulted in HO-1, TIGAR and IL-8 mRNA levels reduction. However, Caco-2 cells pre-treated with PE, showed higher mRNA levels of HO-1 and TIGAR with respect to the control group. Group of cells pre-treated with PE in the presence of SnMp, showed HO-1, TIGAR and IL-8 mRNA levels higher than LPS group (Fig. **4** Panels A, B, C).

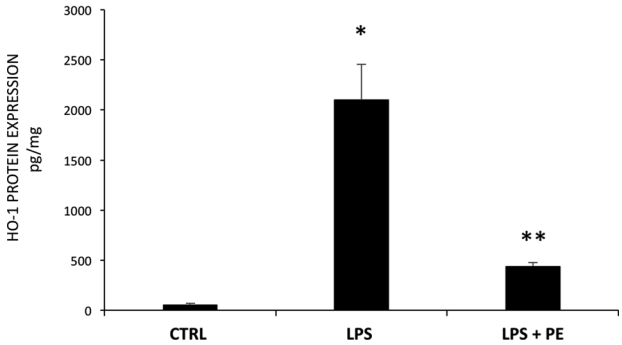

Fig. (3). HO-1 amount determined by ELISA in Caco-2 cells treated with LPS in presence or absence of PE. Results were expressed as the means ± SD of 4 experiments performed in triplicate. Significant *vs*. untreated controls: *p<0.005; **p < 0.005 *versus* LPS.

3.1.5. GSH and ROS Levels

According to data obtained with RT-PCR, in LPS group, GSH level was significantly reduced and ROS levels significantly increased with respect to the control group. In cells pre-treated with PE, GSH levels and ROS levels were similar to the control group; in cells pre-treated with PE and SnMP, GSH levels resulted significantly decreased and ROS levels moderately but significantly increased in respect to a positive control group (Fig. **4** Panel D and Fig. **5**).

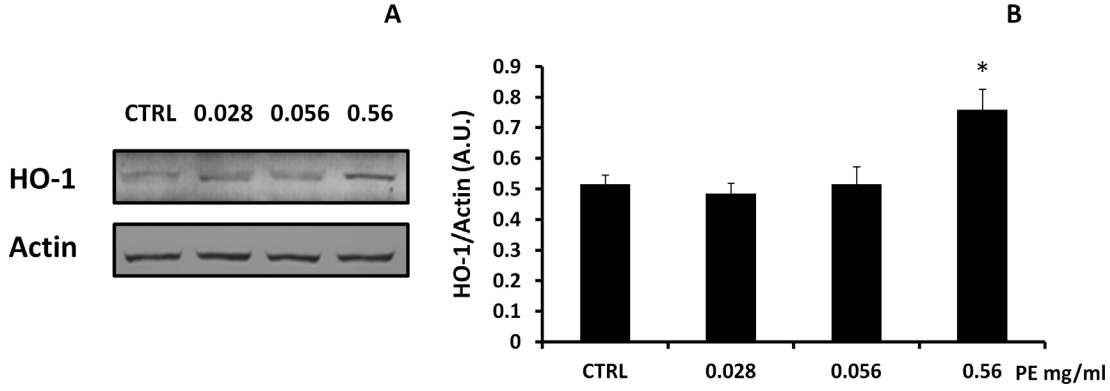

Fig. (2). Effect "*in vitro*" of different concentrations (0.028, 0.056, 0.56 mg/ml) on expression of HO-1 protein in Caco-2 cells (Panel **A**). Representative western blotting (Panel **B**). Results are expressed as the means ± SD of 4 experiments performed in triplicate. Significant *vs*. untreated controls: *p<0.005.

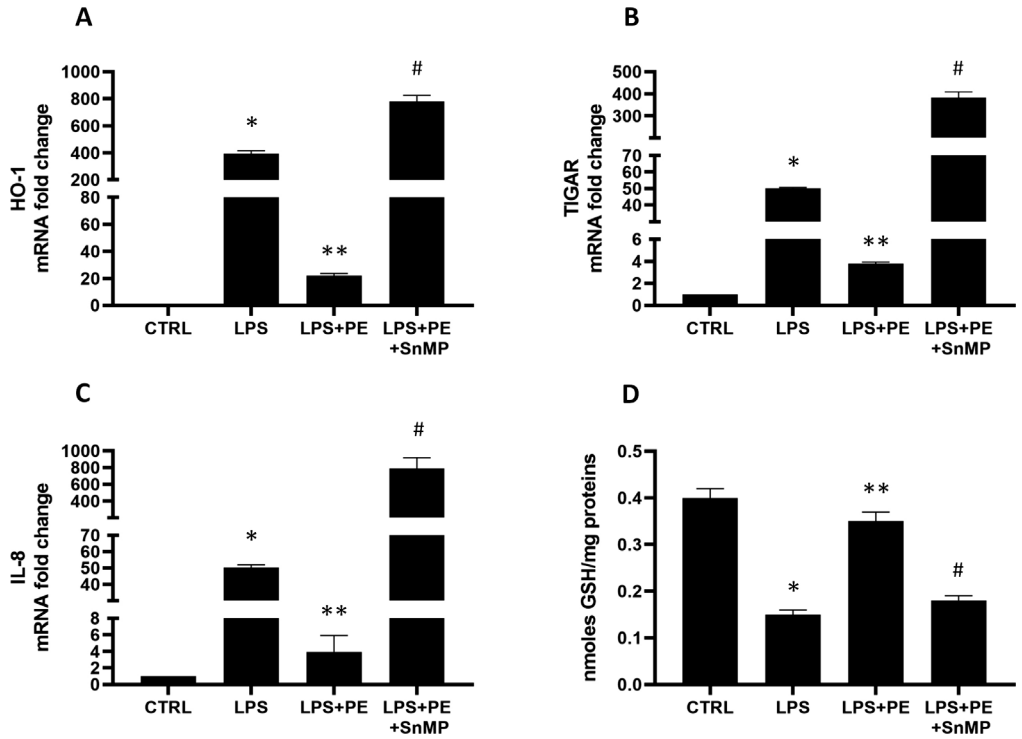

Fig. (4). Panels (**A**-**C**): "*in vitro*" analysis of gene expression by RT-PCR of HO-1, TIGAR, IL-8 mRNA in untreated and LPS (100 μg/ml), LPS (100 μg/ml)+PE (0.56 mg/ml), LPS (100 μg/ml)+PE(0.56 mg/ml)+SnMP (5 μM), treated cells. Panel **D**: Effects of LPS (100 μg/ml), LPS (100 μg/ml)+PE (0.56 mg/ml), LPS (100 μg/ml)+PE(0.56 mg/ml)+SnMP (5 μM), on GSH levels. Results are expressed as the means ± SD of 4 experiments performed in triplicate (*p < 0.005 *versus* control; **p < 0.005 *versus* LPS; #p < 0.005 *versus* LPS+PE).

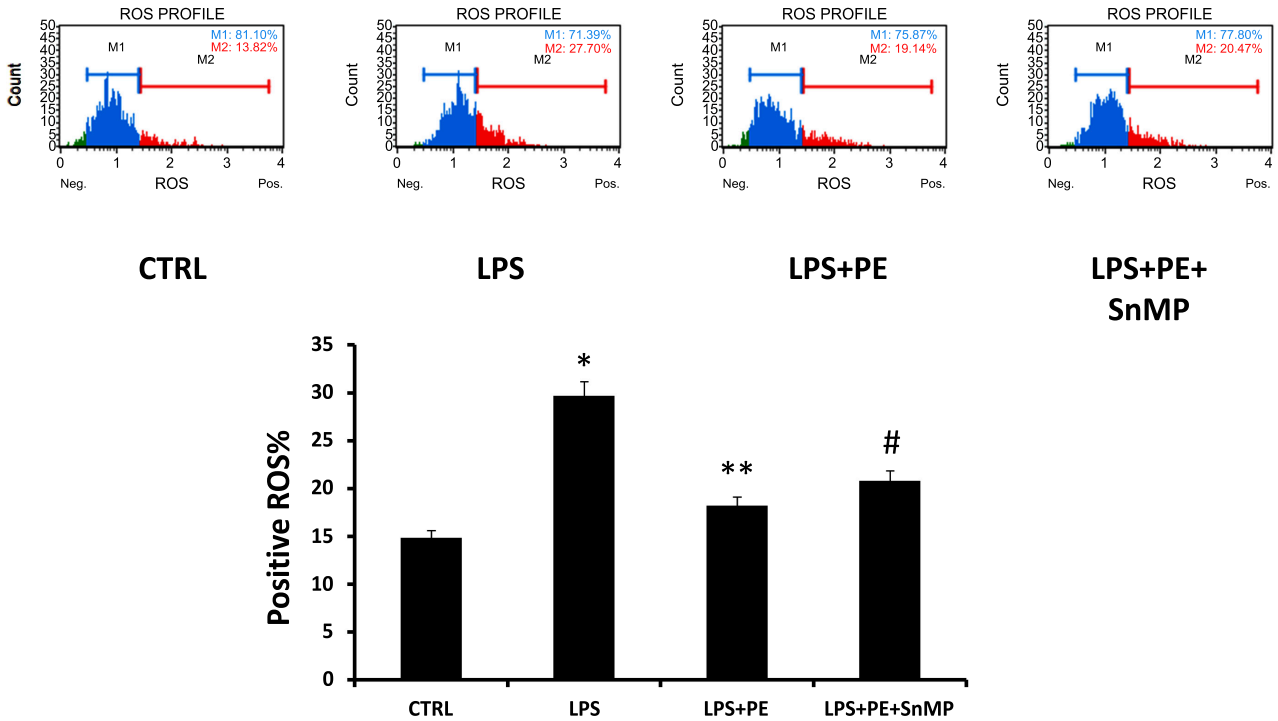

Fig. (5). "*In vitro*" ROS production in untreated and LPS (100 μg/ml), LPS (100 μg/ml)+PE (0.56 mg/ml), LPS (100 μg/ml)+PE(0.56 mg/ml)+SnMP (5μM), treated cells. The quantitative measurement of cells undergoing oxidative stress was evaluated by cytometry, using the Muse Oxidative stress kit. The graph showed the positive ROS percentage in the different groups. Results are expressed as the means ± SD of 4 experiments performed in triplicate (*p < 0.005 *versus* control; **p < 0.005 *versus* LPS; #p < 0.05 *versus* control).

3.2. Effect of PE against DSS Induced Colitis *In Vivo*

3.2.1. Effect on Animal Weight

Animal weight is a faithful and general measure of the DSS-induced toxicity [32]. Animal weight at day 14 was considered as 100% and compared to that at day 28. For the control group, slight change of 1.8 gm, which represented 7% weight increase, was observed. DSS only treated mice showed a significant 11% weight reduction (2.24 gm), while PE treated group on DSS drinking water, demonstrated stable weight 99% (0.2 gm). The difference between DSS group and PE group was statistically significant (T. test p-value = 0.03) (Fig. **6** Panel A).

3.2.2. Effect on Colon Length

As shown in Fig. (**6**) Panel B, colon length was significantly reduced in the DSS group (p-value = 0.002) compared to the control group. While the colon length in the PE group was comparable to that of the control.

3.2.3. Effect on Colon Histology

Fig. (**7**) illustrates the main histological findings in experimental groups. DSS treated group showed intense localized inflammation of the mucosa with atrophied epithelial glands, shortening of the crypts, and widened lumen. Longitudinal smooth muscle thickness is reduced, indicating atrophic changes compared to the control and PE treated group. This late change can be reflective of the effect of DDS after 28 days. However, the changes noticed in DDS group were not found in the DDS+PE group, which was histologically closest to the control group. DDS+PE group showed both preserved mucosal and smooth muscle thickness. Further inflammatory cell infiltration of the mucosa was absent or minimal.

3.2.4. Effect of DSS on IL-6, TNF-alpha and CXCL1

As shown in Table **1**, a significant increase of IL-6 levels was measured in DSS treated mice with respect to control mice. However, we found no significant difference of TNF alpha and CXCL1 levels between DDS and control group.

3.2.5. Effect of PE on IL-6

As shown in Fig. (**6**) (Panel C), PE significantly reduced IL-6 levels associated with DSS treatment. IL-6 levels were significantly higher in the DSS-treated group than in the control group (26.42 pg/ml compared to 9.68 pg/ml). However, IL-6 levels in mice treated with PE were lower than those in the DSS-treated group (17.5 pg/ml). Although the reduction in IL-6 levels did not reach the baseline of the control group, the reduction indicates the beneficial effect of PE.

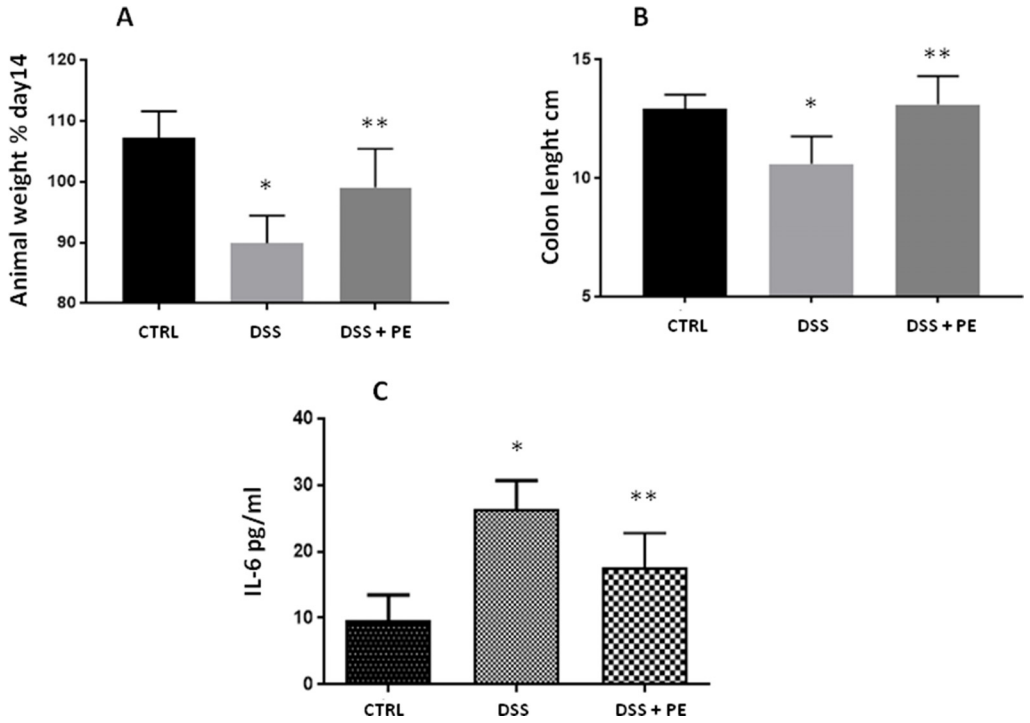

Fig. (6). Panels **A**-**B**: "*in vivo*" effects of PE (150 mg/kg) on animal weight and colon length in DSS treated mice. Results are expressed as the means ± SD, n=5, *p < 0.05 *vs*. control; **p < 0.05 *vs*. DSS; Panel **C**: "*in vivo*" plasmatic IL-6 levels in untreated, DSS and DSS+PE (150 mg/kg) treated mice. N=5, *p < 0.005 *vs*. control; **p < 0.05 *vs*. DSS.

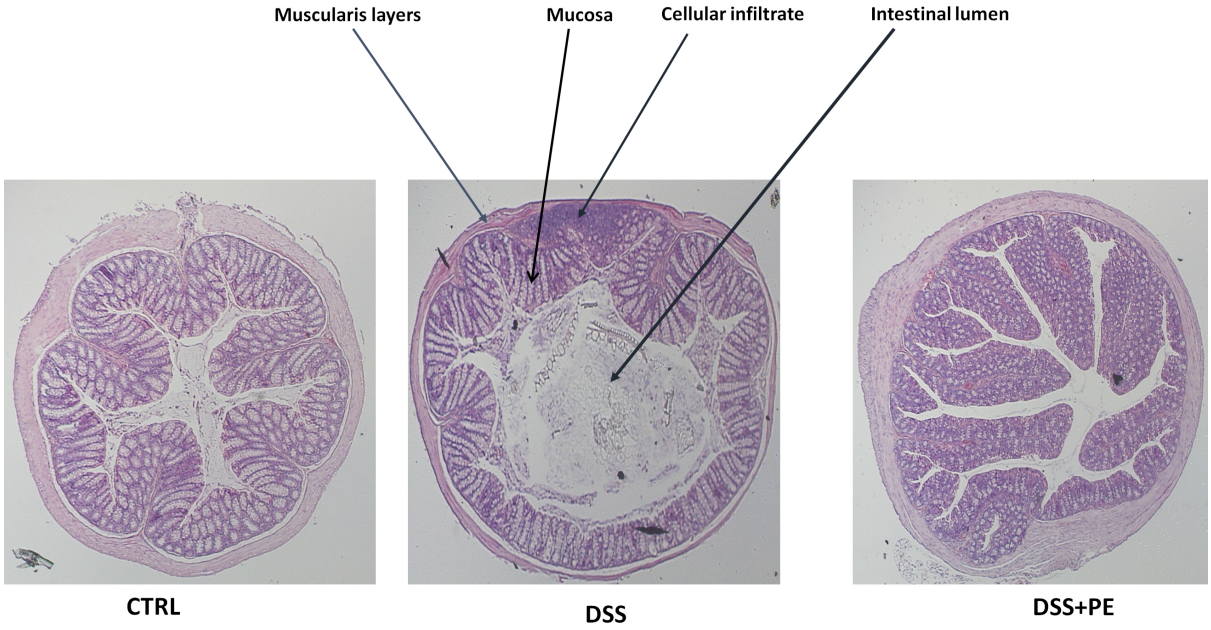

Fig. (7). Colon changes in DSS treated mice under PE (150 mg/kg) treatment: histological images of colon tissues stained with hematoxylin and eosin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. "*In vivo*" plasmatic IL-6, TNF-α and CXCL1 levels in untreated and 6% DSS treated mice. Results are expressed as the means ± SD of 4 experiments performed in triplicate *p < 0.005 *vs*. control.

-	IL-6	TNF-α	CXCL1
	(pg/ml)	(pg/ml)	(pg/ml)
DSS	26.42± 3.78*	13.74± 2.32	170.12± 4.81
Control	9.68± 2.41	11.94± 1.51	162.64± 5.9

4. DISCUSSION

Inflammatory bowel diseases are characterized by inflammation that compromises the integrity of the epithelial barrier. Caco-2 cells exposed to LPS have been chosen as a convenient experimental model of intestinal inflammation, as this human intestinal cell line has been widely used as an experimental model of the intestinal barrier [33, 34]. Moreover, this cell line spontaneously differentiates, leading to the expression of various morphological and functional characteristics of the mature enterocyte. Finally, Caco-2 cells are considered a well-recognized model to study the pharmacological modulation of epithelial barrier and integrity of Tight Junctions (TJs) [34, 35]. It has been reported that prolonged oxidative stress plays a key role in the initiation and development of inflammatory bowel disease (IBD) [36, 37]. Many studies have reported that IBD is associated with ROS overproduction or decreased antioxidant activity with consequent oxidative stress as a result of an imbalance between ROS and antioxidant activity [38, 39]. ROS production can specifically modify the expression of Tight Junction proteins and induce increased cell permeability, which may ultimately cause small intestinal mucosal injury [34, 40, 41]. In view of the increasing evidence supporting the importance of carbon monoxide and bilirubin (products of heme degradation catabolized by HO-1) in reducing stress conditions, molecules with HO-1-inducing activity could also represent pharmacological strategies to increase cytoprotection [22, 42-47]. It has been reported that HO-1 played a central role in protection against intestinal injury. High expression of HO-1 significantly improves intestinal epithelial barrier function. Therefore HO-1 has potential clinical applications for use in the treatment of intestinal dysfunction [23]. Pomegranate extract, used in the present study, was soluble in water and characterized through HPLC-PDA-ESI/MSn for its phenolic and anthocyanin content. PE contains high percentages of phenolic compounds, particularly ellagic acid, ellagic acid derivatives and the ellagitannins named punicalagins. PE showed antioxidant activities in a concentration-dependent manner, as shown in our previous study [13] for both the DPPH and superoxide anion scavenging assay. Results obtained in our *in vitro* experimental conditions evidenced that beneficial effects of pomegranate phenolic compounds, contained in PE, may be due to its ability to activate HO-1These data are in agreement with Xu *et al.* and Hseu *et al.* results which show that the cell treatment with the most abundant phenolic compounds contained in the pomegranate extract, Punicalagin and Ellagic acid, increased Nrf2 nuclear translocation and induced the expression of HO-1 [48, 49]. Moreover, TIGAR (TP53-inducible glycolysis and apoptosis regulator) represents another cytoprotective enzyme. It has been reported that TIGAR protein is required for colon regeneration in a model of ulcerative colitis [50]. The glycolysis modulator TIGAR, a fructose-2,6-bisphosphatase (Fru-2,6-BP), plays a key role in the modulation of

metabolism in response to oxidative stress. TIGAR protein controls fructose-2, 6-bisphosphate (Fru-2, 6-P2) levels during glucose metabolism and helps to maintain nicotinamide adenine dinucleotide phosphate (NADPH) levels to recycle the intracellular antioxidant GSH and to limit ROS [51]. TIGAR activates the pentose phosphate pathway (PPP), which produces NADPH required both for GSH restoration and HO-1 enzymatic antioxidant activity. Norden and Heiss reported that microbial gut metabolites of polyphenols are able to induce TIGAR protein [52].

HO-1 protein induction observed in our experimental conditions, both in LPS treated cells and LPS+PE treated cells, evidenced the cytoprotective role of NrF2 dependent/HO-1 protein, which can be stimulated at transcriptional levels by a plethora of pro-oxidant stimuli or by food polyphenols. Moreover, HO-1 and TIGAR mRNA increase, measured in the LPS group, represents an attempt by cells to defend themselves from oxidative damage. We could speculate a correlation between these two important antioxidant systems exerting their activity simultaneously in order for the cell to replenish its reductants and glutathione sources and provide for an appropriate response to oxidative stress conditions, both in the physiological and pathological state. In Caco-2 cells pre-treated with PE, the moderate but significant increase of TIGAR mRNA and HO-1, both mRNA and protein levels, compared to the control group, and the significant reduction of IL-8 mRNA (pro-inflammatory marker) levels with respect to LPS group, indicate that PE mediated HO-1 induction may be able to buffer the harmful effects of oxidative stress and to reduce damage. The harmful effect of pre-treatment with PE in the presence of the inhibitor of heme oxygenase activity SnMP evidenced a significative cytoprotective effect of PE/HO-1 induction in IBD model with consequent reduction of oxidative damage. According to data obtained with RT-PCR, in LPS group, GSH levels resulted significantly reduced and ROS levels significantly increased with respect to the control group. In cells pre-treated with PE, GSH levels and ROS levels were similar to the control group; in cells pre-treated with PE and SnMP, GSH levels resulted significantly reduced and ROS levels significantly increased with respect to the control group. In our experimental conditions, the HO-1-inducing activity of LPS may be attributable to its GSH-decreasing and ROS-increasing effect. A correlation between HO and glutathione has, in fact, been documented, where HO-1 levels are increased in conditions of glutathione depletion [53]. The potential beneficial effects of PE, demonstrated by *in vitro* results, were supported by the results obtained *in vivo*. It has been reported that DSS induces intestinal inflammation in mice by acting as a direct chemical toxin. The induced epithelial cell damage initiates the inflammation cascade characterized by immune cells infiltration. The ensuing inflammation leads to disruption of the intestinal mucosal integrity allowing access to intestinal bacteria, further propagating the immune reaction in the intestine. Tissue disruption and regeneration overlap and, eventually, culminate in atrophy and fibrosis. This outcome can be assessed by measuring the colon length upon DSS exposure [54]. In our *in vivo* model, DSS treated group showed extensive inflammation, whereas PE group showed both preserved mucosal and smooth muscle thickness. Further inflammatory cell infiltration was minimal. Our data, according to Wei *et al.*, showed that the chosen concentration of PE (150 mg/Kg b.w.) was able to induce protective effects, evidenced by marked improvement in the histopathological examination [28]. The colon length in the PE group was comparable to that of the control group. The effect denotes that in spite of the ensuing colon inflammation in response to DSS, this inflammation, in the presence of PE, did not result in significant morphological changes in the colon over a relatively long duration (28 days from starting DDS treatment). We measured, as inflammatory mediators, IL-6, TNF Alpha as well as CXCL1 [55-59].

However, we found no difference in TNF alpha and CXCL1 levels between DDS and control group. The lack of high levels of CXCL1 and TNF alpha in DDS treated mice, may be attributed to different factors such as the long duration since the last treatment of DDS, the different plasma half-life's of different mediators, the temporal production of different mediators at different stages of inflammation, and the specificity of the animal strain (C3H mice). However, under the same experimental conditions, a significant increase of IL-6 levels was measured in DSS-treated mice compared to the control group. Hence, to evaluate the ability of PE to reduce, not only *in vitro* but also *in vivo*, pro-inflammatory cytokines, we used IL-6 as inflammatory marker. On the other hand, the role of IL-6 as a proinflammatory marker is well recognized in the pathophysiology of IBD [60].

In clinical cases of IBD, such as Ulcerative Colitis or Crohn's disease, the cytokine IL 6 overproduction is induced in CD4+ T cells and macrophages. IL-6 binds to its receptor, sIL 6R, the IL 6-sIL 6R complex activates antigen-presenting cells and T cells [20]. Furthermore, the IL 6-sIL 6R complex reduces apoptosis of mucosal T cells and activates pro-inflammatory cytokine production by these cells. IL-6 pathway proved to be an attractive therapeutic target for the management of IBD. Inhibition of IL-6 signaling with monoclonal antibodies was effective in suppressing chronic intestinal inflammation in mouse models [61]. The ability of PE to reduce, not only *in vitro* but also *in vivo,* pro-inflammatory cytokine levels confirms that anti-inflammatory effects related to bioactive metabolites extracted from pomegranate may have a role in IBD prevention.

CONCLUSION

We can conclude that the potential health benefits of a pomegranate can be mediated by different molecular mechanisms. HO-1 protein increase, induced by a pomegranate extract, rich in bioactive compounds, in *in vitro* model of intestinal inflammation, may contribute to the prevention or reduction of intestinal inflammation. Moreover, PE is able to increase also TIGAR protein, which helps to maintain NADPH levels to recycle glutathione (GSH), a key intracellular antioxidant, with consequent reduction of oxidative stress. Overall, our data allow us to affirm that protective and anti-inflammatory properties of high concentrations of

pomegranate polyphenols could contribute, as supportive therapy to enhance the effects of the conventional therapeutic strategies on the management of IBD.

AUTHORS' CONTRIBUTIONS

Conceptualization, Valeria Sorrenti, Luca Vanella, Greish Khaled; *Funding acquisition*, Valeria Sorrenti, Loredana Salerno, Valeria Pittalà; *in vitro Investigation and Formal analysis*, Marco Raffaele, Giuseppe Carota; *in vivo Investigation and Formal analysis*, Fatemah Bahman, Khalid M Bindayna, Hicham Ezzat; *HPLC-PDA-ESI/MSn analysis,* Gabriele Ballistreri, Margherita Amenta; *Project administration*, Valeria Sorrenti, *Supervision*, Valeria Sorrenti; *Writing - original draft*, Valeria Sorrenti, Luca Vanella, Greish Khaled; *Writing - review & editing*, Valeria Sorrenti. All authors have read and agreed to the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Research and Ethics Committee of Arabian Gulf University (AGU), Bahrain approved all experimental procedures, (Approval No: E022-PI-1).

HUMAN AND ANIMAL RIGHTS

All animal research procedures were followed in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (published by the National Academy of Sciences, The National Academies Press, Washington, DC.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

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