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Antioxidant, anti-inflammatory, and anti-apoptotic efficacy of pomegranate molasses versus peel extract against sodium nitrate hepatotoxicity in rats

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ABSTRACT

The current research was performed to compare the efficacy of pomegranate molasses (PM) versus pomegranate peel aqueous extract (PPAE) in ameliorating the hepatotoxicity of sodium nitrate. The phytochemical screenings of PM and PPAE were analyzed using GC/MS. Sixty male rats were randomly assigned to six equal groups and treated for 10 successive weeks. The control group received distilled water orally, the PM and PPAE groups were orally administered PM (0.5 ml/rat) and PPAE (100 mg/kg), respectively, the nitrate group received sodium nitrate (500 ppm) in drinking water, the Nitrate+PM and Nitrate+PPAE groups received PM and PPAE, respectively with nitrate. Sodium nitrate-intoxicated rats showed significant elevations in the activities of serum alanine aminotransferase and aspartate aminotransferase, significant increases in malondialdehyde, nitric oxide, and hydrogen peroxide levels, as well as significant decreases in reduced glutathione content and catalase activity in hepatic tissues. Moreover, sodium nitrate caused histopathological alterations in the liver, along with significant increases in the expressions of caspase-3, Bax, Bax/Bcl-2 ratio, tumor necrosis factor α, and glial fibrillary acidic protein and decrease in B-cell lymphoma-2 expression. Conversely, the concomitant administration of either PM or PPAE with sodium nitrate mitigated the biochemical, histopathological, and histochemical toxic effects induced by sodium nitrate intoxication. Accordingly, pomegranate molasses and peel extract exhibited similar protective effects against sodium nitrate-provoked hepatotoxicity, mainly via their antioxidant, anti-apoptotic, and anti-inflammatory activities.

Introduction

Inorganic nitrate is one of the most ubiquitous nitrogenous environmental pollutants. Although nitrate is naturally found in soil, water, air, vegetables, and human body (Ogur *et al*., 2005; Silalahi *et al*., 2016), exposure to nitrate occurs also from anthropogenic sources through its use as inorganic fertilizers, explosives, pharmacological applications, food additives, and oxidizing agents in the chemical industries (Pokorny *et al*., 2006; Lidder and Webb, 2013). Moreover, nitrate and nitrite are widely used as food preservatives in the form of sodium and potassium salts to inhibit microbial growth in fish products, and processed meat (Silalahi *et al*., 2018). Beside the endogenous production of nitrate and its essential role in the nitric oxide pathway, there is ample evidence on its beneficial roles for human health (Lidder and Webb, 2013; Bryan and Loscalzo, 2011; Carlström *et al*., 2011).

Despite the low toxicity of nitrate salts, numerous *in vitro* and *in vivo* studies assured that nitrate is endogenously reduced to the more toxic nitrite in saliva and gastrointestinal tract by either bacterial or mammalian metabolic pathways (Gangolli *et al*., 1994; Sobko *et al*., 2005; Hord *et al*., 2009; Tiso and Schechter, 2015)

The main toxic effects of nitrites are the ability to induce methemoglobinemia and to generate carcinogenic nitrosamines (Silalahi *et al*., 2018). Further, nitrates and nitrites exert hepatotoxic, nephrotoxic, cardiotoxic, and reproductive toxic effects (Bouaziz-ketata *et al*., 2014; Fadda *et al*., 2018; El-Nabarawy *et al*.; 2020, Eissa *et al*., 2020; Soliman *et al*., 2021)

Oxidative stress has been reported to be the key mediator of nitrates- and nitrites- induced tissue damage, possibly via the excessive production of reactive oxygen and nitrogen species and the inhibition of antioxidant activities (Eissa *et al*., 2020; Soliman *et al*., 2021; Eissa *et al*., 2021). Additionally, it is now recognized that nitrate is a precursor of nitric oxide, which in turn interacts with reactive oxygen species forming a powerful oxidant and cytotoxic agent that results in cellular damage (Bouaziz-ketata *et al*., 2014).

Dietary natural antioxidants intake has a substantial role in the prevention of oxidative stress and protection against a variety of diseases and toxicities (Rani, 2017; Abd Eldaim *et al*., 2020; Orabi *et al*., 2020). Various epidemiological, *in vivo*, and *in vitro* studies confirmed an inverse association between oxidative stress-mediated disorders and consumption of vegetable concentrates and fresh fruits, including pomegranate (Esfahani *et al*., 2011; McMartin *et al*., 2013; Sun *et al*., 2017).

Pomegranate (*Punica granatum* L.), belonging to Punicaceae family, is an ancient fruit that is rich in various bioactive polyphenolic compounds which have nutritional and medicinal values (Teixeira da Silva *et al*., 2013). Nowadays, pomegranate has gained popularity due to the prospective health-promoting benefits from the consumption of fresh fruit or its by-products such as juice, molasses, jelly, wine, jam, oil, and other dietary supplements (Akpinar-Bayizit *et al*., 2016).

Pomegranate peel is an agricultural biomass waste product obtained during the processing of pomegranate juice and possesses a higher antioxidant efficacy than the edible part because of its high content of bioactive phytochemicals, complex polysaccharides, minerals, and vitamins (Sreekumar *et al*., 2014; Russo *et al*., 2018). The most predominant phytochemicals in pomegranate peel are anthocyanins, flavonoids, phenolic acids, and hydrolyzable tannins (Russo *et al*., 2018). These components exert antioxidant, anti-inflammatory, anti-mutagenic, anticarcinogenic, hepatoprotective, neuroprotective, cardioprotective, and cytoprotective potentials (Puneeth and Chandra, 2020).

Pomegranate molasses (PM), also known as "pomegranate sauce" or "debis", is a concentrated pomegranate juice that is simply prepared by boiling pomegranate juice with or without the addition of sugar or other additives. It is a traditional tasty condiment in salads and many dishes (Akpinar-Bayizit *et al*., 2016). Mounting evidence suggested the nutritive value of PM because of its high mineral contents. Further, PM has higher

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polyphenols than juice and may be valuable in the treatment of various disorders (Chalfoun-Mounayar *et al*., 2012).

Great interest has been paid recently to the medicinal value, not only of the edible parts of pomegranate fruit but also to the fruit processing by-products such as peels, which are considered an essential source of antioxidants of natural origin. To our knowledge, there are scanty investigations regarding the bioactive health effects of other pomegranate processing products, such as pomegranate molasses. Therefore, this study was proposed to assess the efficacy of pomegranate molasses versus pomegranate peel aqueous extract against sodium nitrate-induced hepatotoxicity, focusing on their antioxidant, anti-apoptotic, and anti-inflammatory activities.

Materials and methods

Chemicals

Sodium nitrate, NaNO3 (CAS No: 7631-99-4; purity of ≥ 99.0%) was obtained from Sigma–Aldrich Company, Germany. Pomegranate fruits and pomegranate molasses (Chtoura Garden Company) were commercially available at local markets at Sadat City, Egypt. The diagnostic kits used for measuring of serum and tissue biochemical biomarkers were obtained from Biodiagnostic Company, Dokki, Giza, Egypt. All other chemicals and reagents were of analytical grade and were commercially available.

Preparation of Pomegranate Peels aqueous extract

Pomegranate (*Punica granatum* L.) fruits were manually peeled. The peels were dried, ground, sieved and kept in a glass jar at room temperature until used. The pomegranate aqueous extract was prepared from the ground pomegranate peels following the method adopted by Salah-ud-Din *et al*. (2015) with slight modifications. The aqueous extract was freshly prepared by soaking 10g of the ground pomegranate peels in 100 ml of distilled water at 60°C for two hours in a shaking incubator at 120 rpm. This was followed by filtration through Wattman filter paper and then heating at 45°C in a hot air oven for drying. The dried extract was re-dissolved in distilled water just before administration.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis

The phytochemical compositions of PM and PPAE were analyzed using a Trace GC1300-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m×0.25 mm×0.25 µm film thickness). The column oven temperature was set to 50°C and then progressively increased to 250°C (5°C/min) and maintained for 2 minutes. The temperature was then continuously elevated to 300°C (25°C/min) and kept for 2 minutes. The temperatures of the injector and MS transfer line were set at 250 and 260°C respectively. Helium was used as a carrier gas and was transferred at a regular flow rate of 1 ml/min with injection of 1 µl of the diluted sample at 3 min solvent delay using Autosampler (AS1300) linked with GC at the split mode. Electron ionization mass spectra were collected at 70 eV over the range of 50–650 m/z at a full scan mode. The ion source was adjusted at 250°C. The phytochemical constituents of PM and PPAE were identified using the NIST 11 database and WILEY 09 mass spectral libraries.

Animals and experimental design

Sixty adults male Wistar albino rats (100-120g) were obtained from Al-Zyade Experimental Animals Production Center, Giza, Egypt. Rats were caged at a well-ventilated laboratory animal room (natural daily dark/light cycle; 23±2°C; 40-50% relative humidity) and provided with a standard commercial pellet and tap water ad libitum throughout the experimental period. The experimental design was ethically approved (Approval No. VUSC-010-1-17) by the International Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

Rats were indiscriminately assigned to six equal groups, 10 rats each, as follows:

Control group: Rats orally received distilled water, daily for 10 successive weeks.

Pomegranate molasses (PM) group: Rats orally received pomegranate molasses (0.5 ml/rat) (Hussein *et al*., 2018), daily for 10 successive weeks.

Pomegranate peels aqueous extract (PPAE) group: Rats orally received pomegranate peels aqueous extract (100 mg/kg b.w.) (Adiga *et al*., 2010), daily for 10 successive weeks.

Sodium nitrate (Nitrate) group: Rats received sodium nitrate (500 ppm) in drinking water (Anwar and Mohamed, 2015) for 10 successive weeks.

Sodium nitrate and pomegranate molasses (Nitrate+PM) group: Rats received sodium nitrate (500 ppm) in drinking water, simultaneously with daily oral administration of PM (0.5 ml/rat) for 10 successive weeks.

Sodium nitrate and pomegranate peels aqueous extract (Nitrate+PPAE) group: Rats received sodium nitrate (500 ppm) in drinking water, simultaneously with daily oral administration of PPAE (100 mg/kg b.w.) for 10 successive weeks.

Samples collection

At the completion of the experiment and 24 h following the last treatment, rats were fasted overnight and then anesthetized by inhalation of isoflurane for samples collection. Blood samples were collected from the retro-orbital venous plexus, centrifuged at 3000 rpm for 15 minutes. The separated sera were preserved at −20°C for serum biochemical analyses. The liver of each rat was instantly excised and assigned into two sets; one set was immersed in physiological saline and kept at −80°C for tissue biochemical analyses, while the other set was fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry investigations.

Estimation of serum liver enzymes

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were estimated following the methods adopted by Reitman and Frankel (1957), using commercial kits and following the manufacturer's instructions.

Estimation of hepatic oxidant/antioxidant biomarkers

Hepatic malondialdehyde (MDA), nitric oxide (NO), and reduced glutathione (GSH) contents were measured utilizing commercial kits and following the manufacturer's instructions according to the methods approved by Ohkawa *et al*. (1979); Montgomery and Dymock (1961) and Beutler (1963), respectively, and according to the method of Aebi (1984) for measuring hydrogen peroxide (H_2O_2) content and catalase (CAT) activity.

Histopathological examination

The formalin-fixed liver specimens were trimmed, processed, embedded in paraffin, sectioned using a microtome (LEICA RM 2135) at 4 µm thickness, and then routinely stained with hematoxylin and eosin stain (H&E) according to Bancroft and Layton (2013). Histopathological examination and photographing were done using a digital Leica photomicroscope (LEICA DMLB, Germany). Semi-quantitation of the hepatic alterations was scored according to Tahoun *et al*. (2021) with some modifications as follows: (-) normal 0%, (+) mild (<25% of sections), (++) moderate (26–50% of sections), (+++) severe (51–75% of sections), and (++++) very severe (>75% of sections).

Immunohistochemical Investigation

Immune-staining for localization of caspase-3, Bax (Bcl-2-associated X), Bcl-2 (B-cell lymphoma 2) was performed according to El-Maksoud *et al*. (2020). The formalin-fixed paraffin-embedded liver sections were deparaffinized, rehydrated in alcohol solutions, incubated in 3% H_2O_2 and then, incubated with the primary antibodies of anti-caspase-3 (diluted 1:200, Abcam, USA), anti-Bax (diluted 1:100, Santa Cruz Biotechnology Santa Cruz, CA, USA), anti-Bcl-2 (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TNF-α (diluted 1:500, Abcam, USA), or anti GFAP (diluted 1:300, Dako, Carpinteria, USA) antibodies overnight at 4◦C. The sections were rinsed with phosphate-buffered saline then incubated for 30 min at room temperature with the appropriate biotinylated secondary antibodies (Vecta stain Elite ABC Kit). The immune reactions were visualized using diaminobenzidine (DAB, Sigma Chemical Co, USA) under light microscopy then counterstained with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA). This was followed by routine dehydration in alcohol, clearing in xylene, and mounting using Aquatex fluid (Merk KGaA, Germany) under a coverslip. Semi-quantification analysis of caspase-3, Bax, Bcl-2, TNF-α, and GFAP expressions were represented by the percentage of positively immune stained cells by counting at least 1000 cells per slide in 10 random fields. AI% = (number of positive cells/ total number of calculated cells) × 100, HPF (40X), as previously described by Rahman *et al*. (2001).

Statistical Analysis

Data were analyzed using analysis of variance statistical test (ANOVA) followed by Duncan's Multiple Range test for post hoc analysis using SPSS software, version 16 (released in 2007). Data are presented as means±S.E. Statistical significance was set at $p < 0.05$.

Results

Phytochemical constituents of pomegranate molasses

The phytochemical screening of PM using GC–MS was displayed in Table 1, which shows the presence of eight phytochemical compounds at retention time, 4.98, 6.21, 7.77, 9.28, 21.49, 23.11, 24.19 and 24.48 min. The constituents with the highest peak were 9-Octadecenoic acid (27.29%) followed by, 2-Furancarboxaldehyde,5- hydroxymethyl (21.65%); hexadecanoic acid (13.44%); 5-Methyl-1,3-benzenediol (11.26%), benzoic acid (9.32%); octadecenoic acid (5.20%), and hexadecanoic acid, methyl ester (5.08%).

Phytochemical constituents of pomegranate peels aqueous extract

The result of GC–MS analysis of PPAE was displayed in Table 2, which shows the presence of nine phytochemical compounds at retention time,

Table 1. The Phytochemical Compositions of Pomegranate Molasses.

	Chemical compound	Phytochemical compound	RT/min	Area %	MW	Chemical formula	**Activity
1	5-Methyl-1,3-benzenediol	Orcinol	4.98	11.26	124	C7H8O2	Antioxidant, anti-proliferative, anticancer
2	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6- methyl	1,5-Anhydro-6-deoxyhexo-2,3-diulose (Charbohydrate, sugar- amino acid derivatives, flavonoid)	6.21	2.27	144	C6H8O4	Antimicrobial, anti-inflammatory, anti-proliferative, antioxidant
3	Benzoic acid	-	7.77	9.32	122	C7H6O2	Antimicrobial
4	2-Furancarboxaldehyde,5-hydroxymethyl	5-Hydroxy methyl furfural	9.28	21.65	126	C6H6O3	Antioxidative, anti-inflammatory
5	Hexadecanoic acid	Palmitic acid (Fatty acid)	21.49	13.44	256	C16H32O2	Anti-inflammatory, antioxidant
6	9,12-Octadecadienoic acid, methyl ester, (E,E)	Sterculic acid (Fatty acid)	23.11	1.4	294	C19H34O2	Anticancer
7	9-Octadecenoic acid	Oleic acid (Omega-9)	24.19	27.29	282	C18H34O2	Antibacterial,
8	Octadecanoic acid	Stearic acid (fatty acid)	24.48	5.2	284	C18H36O2	Antimicrobial

RT: Retention time; MW: Molecular weight; MF: Molecular formula. ** Sources: Mujeeb *et al*. (2014); Abubakar and Majinda (2016); Shapla *et al*. (2018); Suresh and Nadumane (2021)

Table 2. The Phytochemical Compositions of Pomegranate Peel Aqueous Extract

	Chemical compound	Phytochemical compound	RT/min	Area %	MW	Chemical formula	**Activity
1	5-Methyl-1,3-benzenediol	Orcinol	4.98	11.27	124	C7H8O2	Antioxidant, anti-proliferative, anticancer
2	4H-pyran-4-one, 3-hydroxy-2-methyl	Maltol	5.55	5.27	126	C6H6O3	Antioxidant
3	4H-Pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl	1,5-Anhydro-6-deoxyhexo-2,3-diulose (Charbohydrate, sugar- amino acid derivatives, flavonoid)	6.24	12.85	144	C6H8O4	Antimicrobial, anti-inflammatory, anti-proliferative, antioxidant
4	2-Furancarboxaldehyde,5- hydroxymethyl	5-Hydroxy methyl furfural	9.26	25.73	126	C6H6O3	Antioxidant, anti-inflammatory
5	2-Amino-5-guanidino-pentanoic acid	Arginine	10.93	2.03	174	C6H14N4O2	Antioxidant
6	Hexadecanoic acid	Palmitic acid (Fatty acid)	21.48	11.52	256	C16H32O2	Anti-inflammatory, antioxidant
7	9,12-Octadecadienoic acid, methyl ester, (E, E)	Sterculic acid (Fatty acid)	23.1	2.3	294	C19H34O2	Anticancer
8	Octadecanoic acid	Stearic acid (fatty acid)	23.61	1.93	284	C18H36O2	Antimicrobial
9	9-Octadecenoic acid	Oleic acid (Omega-9)	24.52	26.76	282	C18H34O2	Antibacterial

RT: Retention time; MW: Molecular weight; MF: Molecular formula. ** Sources: Mujeeb *et al*. (2014); Abubakar and Majinda (2016); Shapla *et al*. (2018); Suresh and Nadumane (2021)

4.98, 5.55, 6.24, 9.26, 10.93, 21.48, 23.10, 23.61 and 24.52 min. The most predominant constituents were 9-Octadecenoic acid (26.76%) followed by, 2-Furancarboxaldehyde,5- hydroxymethyl (25.73%), 4H-Pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl (12.85%), hexadecanoic acid (11.52%), 5-Methyl-1,3-benzenediol (11.27%), and 4H-pyran-4-one,3-hydroxy-2-methyl (5.27%).

Pomegranate molasses and pomegranate peels aqueous extract normalized sodium nitrate-induced elevation in serum hepatic enzymes of rats

As shown in Figure 1, there were no significant differences in the activities of serum ALT and AST among the control, PM and PPAE groups. Exposure of rats to sodium nitrate in drinking water for 10 successive weeks at 500 ppm significantly ($p < 0.05$) elevated serum ALT and AST activities, compared to control group. Conversely, concomitant administration of either PM (0.5 ml/rat) or PPAE (100 mg/kg) with sodium nitrate normalized the activities of the elevated enzymes.

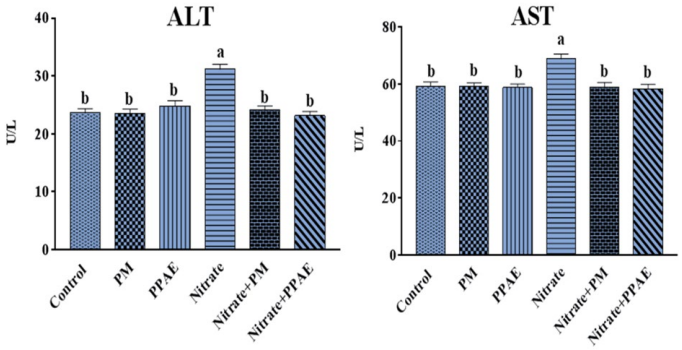

Fig. 1. Effect of sodium nitrate and/or pomegranate molasses and pomegranate peels aqueous extract on serum hepatic enzymes. Values are expressed as mean ± SE, (n = 10). Different superscripts (a,b) indicate significant differences at $p < 0.05$. PM: Pomegranate molasses, PPAE: pomegranate peels aqueous extract, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Pomegranate molasses and pomegranate peels aqueous extract ameliorated sodium nitrate-induced alterations in hepatic oxidant/antioxidant biomarkers of rats

Figure 2 shows no significant ($p < 0.05$) changes in hepatic oxidant/antioxidant biomarkers among the control, PM and PPAE groups. However, significant elevation of MDA, NO and H_2O_2 levels along with significant decrease of GSH content and CAT activity were recorded in liver tissues of sodium nitrate-exposed rats with respect to the control group. Contrariwise, the concomitant administration of either PM or PPAE with sodium nitrate significantly ameliorated the recorded hepatic oxidant/antioxidant alterations induced by sodium nitrate as evidenced by restoring the normal control values of MDA, NO, H_2O_2, GSH, and CAT in the liver.

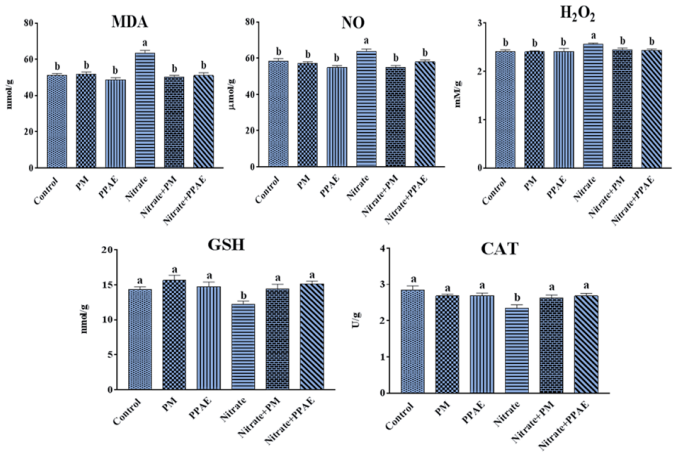

Fig. 2. Effect of sodium nitrate and/or pomegranate molasses and pomegranate peels aqueous extract on hepatic oxidant/antioxidant biomarkers. Values are expressed as mean ± SE, (n= 10). Different superscripts (a,b) indicate significant differences at $p < 0.05$. PM: Pomegranate molasses, PPAE: pomegranate peels aqueous extract, MDA: malondialdehyde, NO: nitric oxide, H_2O_2: hydrogen peroxide, GSH: reduced glutathione and CAT: catalase.

Pomegranate molasses and pomegranate peels aqueous extract improved sodium nitrate-induced alterations in hepatic architectures of rats

The hepatic histopathological findings of different groups are illustrated in Table 3 and Figure 3. Liver sections of control (Fig. 3A), PM (Fig. 3B), and PPAE (Fig. 3C) groups showed normal histological structures. Marked histopathological alterations were demonstrated in liver sections of the nitrate-intoxicated group, including severe vascular changes as congestion of central vein and hepatic sinusoids associated with severe degenerative and necrotic changes as hydropic degeneration, and focal areas of necrosis with mononuclear cell infiltrations (Fig. 3D). Interesting-

Table 3. The main hepatic histopathological changes of the different treated groups.

Hepatic lesion	Experimental groups					
	Control	PM	PPAE	Nitrate	Nitrate+PM	Nitrate+PPAE
Congestions	-	-	-	+++	++	++
Hydropic degeneration	-	-	-	++++	+	+
Coagulative necrosis	-	-	-	+++	+	-
Inflammatory cells infiltrations	-	-	-	++	-	-

The histopathological changes are graded as follows: (−) indicates normal appearance, (+) indicates mild changes, (++) indicates moderate changes, (+++) indicates severe changes, and (++++) indicates very severe changes. PM: Pomegranate molasses, PPAE: pomegranate peels aqueous extract.

Table 4. Semi-quantitative analysis of caspase-3, Bax, Bcl-2, Bax/Bcl-2 ratio, TNF-α, and GFAP immuno-staining in the livers of different treated groups.

IHC	Experimental groups					
	Control	PM	PPAE	Nitrate	Nitrate+PM	Nitrate+PPAE
Caspase-3 (% of positive cells/HPF)	2.56±0.33^{c}	1.63±0.31^{c}	1.59±0.20^{c}	55.14±1.59^{a}	16.54±0.58^{b}	14.53±0.59^{b}
Bax (% of positive cells/HPF)	8.64±0.39^{c}	6.59±0.49^{c}	9.33±0.67^{c}	45.88±1.78^{a}	16.66±0.74^{b}	16.69±0.77^{b}
Bcl-2 (% of positive cells/HPF)	35.17±0.57^{a}	35.98±0.72^{a}	36.82±0.66^{a}	12.41±0.48^{c}	21.51±0.66^{b}	22.44±0.63^{b}
Bax/Bcl-2 Ratio (% of positive cells/HPF)	0.246±0.01^{c}	0.189±0.01^{c}	0.187±0.01^{c}	3.70±0.19^{a}	0.899±0.03^{b}	0.748±0.04^{b}
TNF-α (% of positive cells/HPF)	5.96±0.48^{c}	5.32±0.34^{c}	4.98±0.57^{c}	41.69±1.93^{a}	17.78±1.03^{b}	14.05±1.09^{b}
GFAP (% of positive cells/HPF)	4.43±0.19^{c}	4.08±0.11^{c}	3.87±0.19^{c}	64.48±1.52^{a}	20.59±0.82^{b}	19.09±0.99^{b}

The values are expressed as means ± SE of immunoreactive hepatic cells; different letters in the same row indicate significant differences at $p < 0.05$. PM: Pomegranate molasses, PPAE: pomegranate peels aqueous extract. IHC: immunohistochemistry, Bax (Bcl-2-associated X), and Bcl-2 (B-cell lymphoma 2), TNF-α (tumor necrosis factor-alpha) and GFAP (Glial fibrillary acidic protein).

ly, co-administration either of PM or PPAE with sodium nitrate improved the liver tissue architecture, unless for mild vascular and degenerative changes (Fig. 3E, 3F, respectively).

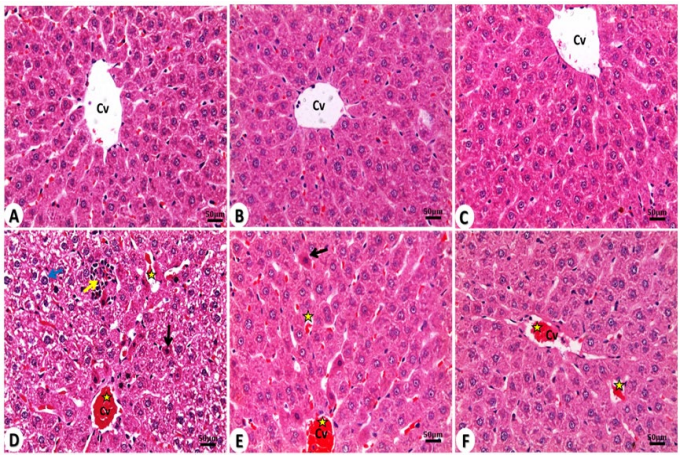

Fig. 3. Representative photomicrographs of hepatic histopathological changes of different groups (Haematoxylin and Eosin stain X20. Scale bar 50 µm); star: congestions, blue arrows: hydropic degeneration of hepatocytes, black arrows: coagulative necrosis of hepatocytes and yellow arrow: inflammatory cell infiltrations. CV: central vein. (A) Control group, (B) PM group, and (C) PPAE group showing normal hepatic architectures. (D) Nitrate group showing congestions of central vein and hepatic sinusoids, hydropic degeneration, and focal area of necrosis with mononuclear cell infiltrations. (E) Nitrate + PM group showing mild congestion and single-cell necrosis. (F) Nitrate + PPAE group showing mild congestion.

Pomegranate molasses and pomegranate peels aqueous extract modulated sodium nitrate-increased caspase-3, Bax/Bcl-2 ratio, TNF-α and GFAP expressions in hepatic tissues of rats

Table 4 and Figures 4–8 illustrate the immune staining targeting caspase-3, Bax, Bcl-2, TNF-α, and GFAP, respectively, in the livers of the different treated groups. Mild immune staining of caspase-3, Bax, TNF-α, and GFAP along with marked Bcl-2 immuno-staining were present in hepatic tissues of the control, PM, and PPAE groups. However, hepatic tissues of the nitrate-intoxicated group exhibited significant increases in the immune-staining for caspase-3, Bax, TNF-α, and GFAP with a marked decrease in Bcl-2 immuno-staining. The concomitant administration of either PM or PPAE with sodium nitrate attenuated these changes, compared to the nitrate group but was still significantly different from control values. Moreover, the calculated ratio of Bax to Bcl-2 is illustrated in Table 4, which showed no significant variations among the control, PM, and PPAE with the highest Bax/Bcl-2 ratio in the nitrate group. Conversely, a significant decrease was recorded in the Bax/Bcl-2 ratio of nitrate+PM and nitrate +PPAE groups, compared to nitrate group.

Discussion

Recently, research papers were directed to study the health benefits

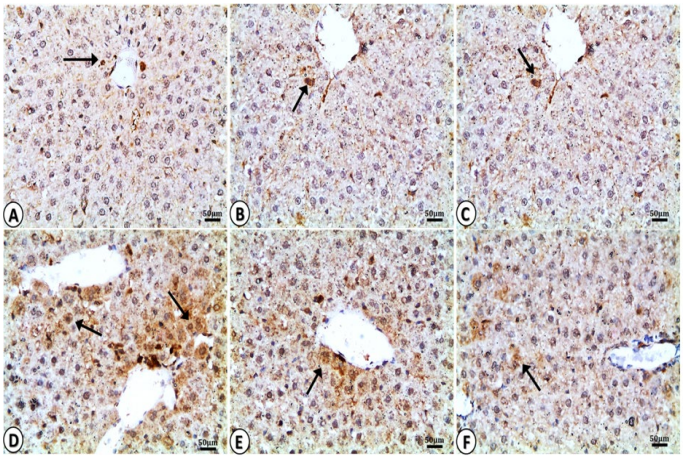

Fig. 4. Representative photomicrographs of immuno-histochemical staining of caspase-3 in liver sections of different groups (Caspase-3 IHC; scale bar = 50 µm), arrows: immune expressions of caspase-3 in hepatic cells. (A) Control group, (B) PM group and (C) PPAE group showing mild immuno-staining caspase-3 in hepatic cells. (D) Nitrate group showing marked immuno-staining caspase-3 in hepatic cells. (E) Nitrate + PM group and (F) Nitrate + PPAE group showing moderate immuno-staining caspase-3 in hepatic cells.

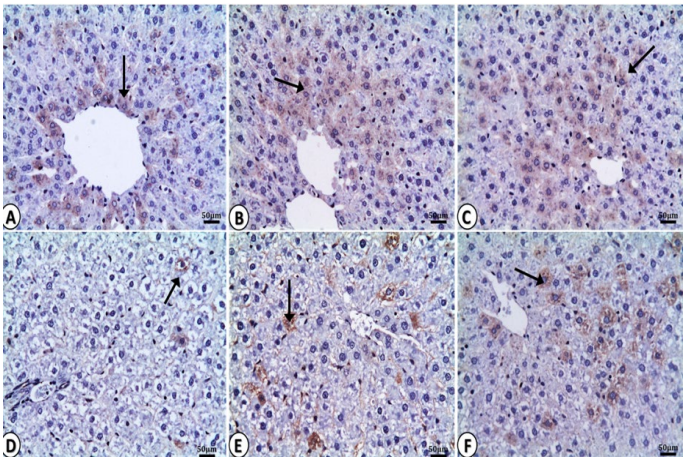

Fig. 6. Representative photomicrographs of immuno-histochemical staining of Bcl-2 in liver sections of different groups (Bcl-2 IHC; scale bar = 50 µm), arrows: immune expressions of Bcl-2 in hepatic cells. (A) Control group, (B) PM group and (C) PPAE group showing marked immuno-staining Bcl-2 in hepatic cells. (D) Nitrate group showing mild immuno-staining Bcl-2 in hepatic cells. (E) Nitrate + PM group and (F) Nitrate + PPAE group showing moderate immuno-staining Bcl-2 in hepatic cells.

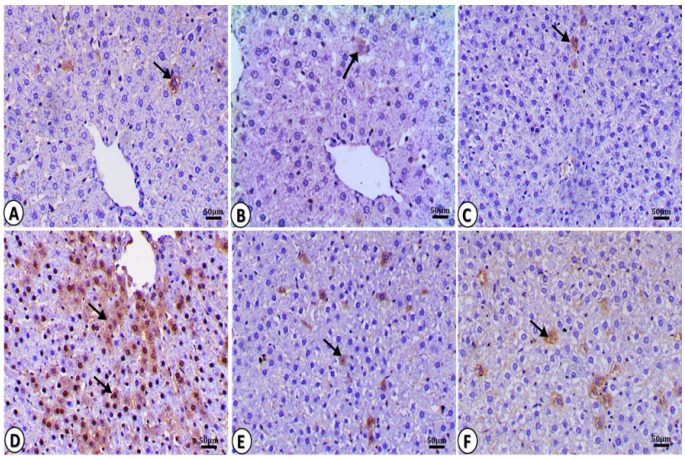

Fig. 5. Representative photomicrographs of immuno-histochemical staining of Bax in liver sections of different groups (Bax IHC; scale bar = 50 µm), arrows: immune expressions of Bax in hepatic cells. (A) Control group, (B) PM group and (C) PPAE group showing mild immuno-staining Bax in hepatic cells. (D) Nitrate group showing marked immuno-staining Bax in hepatic cells. (E) Nitrate + PM group and (F) Nitrate + PPAE group showing moderate immuno-staining Bax in hepatic cells.

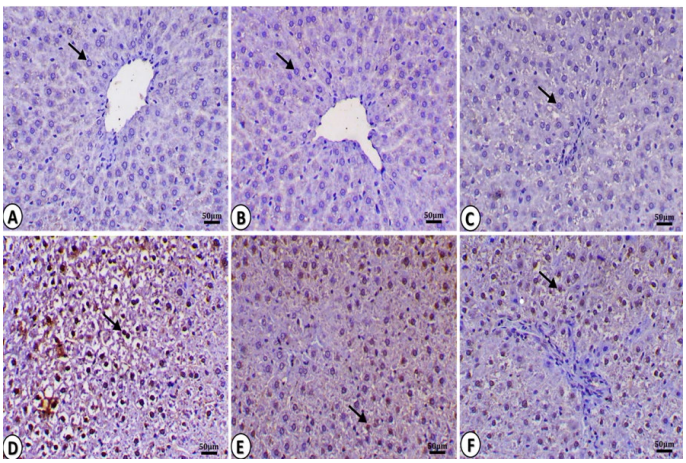

Fig. 7. Representative photomicrographs of immuno-histochemical staining of TNF-α in liver sections of different groups (TNF-α IHC; scale bar = 50 µm), arrows: immune expressions of TNF-α in hepatic cells. (A) Control group, (B) PM group and (C) PPAE group showing mild immuno-staining TNF-α in hepatic cells. (D) Nitrate group showing marked immuno-staining TNF-α in hepatic cells. (E) Nitrate + PM group and (F) Nitrate + PPAE group showing moderate immuno-staining TNF-α in hepatic cells.

not only of the edible parts of the fruit but also to the phenolic by-products of fruit processing such as seeds, peels, and hulls. The food and agricultural products processing industries yield remarkable phenolic-rich by-products such as pomegranate molasses, which is an essential source of natural antioxidant compounds. Therefore, the current research aimed to assess the prospective efficacy of PM versus PPAE against hepatotoxicity of sodium nitrate, focusing on the identification of the mechanisms associated with the protective effects of pomegranate and its by-products.

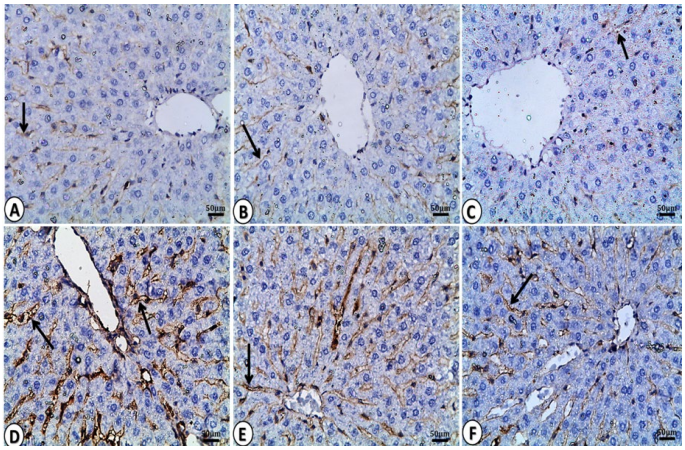

Fig. 8. Representative photomicrographs of immuno-histochemical staining of GFAP in liver sections of different groups (GFAP IHC; scale bar = 50 µm), arrows: immune expressions of GFAP in hepatic cells. (A) Control group, (B) PM group and (C) PPAE group showing mild immuno-staining GFAP in hepatic cells. (D) Nitrate group showing marked immuno-staining GFAP in hepatic cells. (E) Nitrate + PM group and (F) Nitrate + PPAE group showing moderate immuno-staining GFAP in hepatic cells.

The current findings highlight the disturbance in hepatic functions following the exposure to 500 ppm sodium nitrate in drinking water for 10 weeks, manifested by a significant increment in the serum activities of ALT and AST. Transaminases are sensitive hepatic biomarkers that correlate directly with the extent of cellular damage (El-Sharaky *et al*., 2007). The recorded elevation in the serum ALT and AST activities may be due to changes in membrane permeability and cell damage, leading to leakage of transaminases from the hepatocytes (Kasarala and Tillmann, 2016). This assumption was ascertained by the observed histopathological findings, which were in parallel with Bouaziz-Ketata *et al*. (2014) and Rouag *et al*. (2020).

The evidence from relevant studies suggested oxidative stress as a predominant molecular mechanism of sodium nitrate- induced hepatic damage. In this line, our results showed enhancement of lipid peroxidation and oxidative damage in liver of sodium nitrate- exposed rats, evidenced by significant increase in hepatic MDA, NO and H_2O_2 levels associated with significant decrease in GSH content and CAT activity. The same findings were obtained in previous studies (Bouaziz-Ketata *et al*., 2014; Anwar and Mohamed, 2015; Rouag *et al*., 2020). Noteworthy, nitrates are the main precursor of NO, which directly reacts with superoxide producing highly reactive peroxynitrite (ONOO-) radicals, that cause damage to many biological molecules, mainly proteins, lipids, and nucleic acids, and subsequently increase lipid peroxidation (El-Sheikh and Khalil 2011). Moreover, both NO and ONOO- could react further to antioxidant enzymes, resulting in inactivation and disruption of the cellular antioxidant enzyme system (Pacher *et al*., 2007). Herein, the disturbance of oxidant/antioxidant status in the liver of sodium nitrate-intoxicated rats may reflect the over-generation of free radicals and the depletion of antioxidant enzymes, which leads to cytostasis and cytotoxicity, and finally, causes alterations in hepatocellular function and structure.

Moreover, the hepatic immunohistochemistry findings clearly suggested the promotion of cell death pathway in sodium nitrate- intoxicated rats, evidenced by alterations in the balance of the apoptosis regulatory proteins, caspase-3, Bax, and Bcl-2. To our knowledge, no available data have been recorded about the effect of sodium nitrate on caspase-3, Bax, and Bcl-2 expressions in liver tissue. However, accumulating evidence has been proved the ability of sodium nitrite to activate cell death pathways in the liver and other organs (Ogur *et al*., 2005; Al-Rasheed *et al*., 2017; Hamdan *et al*., 2019). In the same line, El-Nabarawy *et al*. (2020) observed significant increases in the immune-expressions of caspase-3 and Bax with significant reduction in Bcl-2, in a dose-dependent manner, in the liver of sodium nitrite-intoxicated rats. Indeed, antecedent studies confirmed that nitrate is endogenously reduced to the more toxic nitrite in saliva and gastrointestinal tract by either bacterial or mammalian metabolic pathways (Sobko *et al*., 2005; Hord *et al*., 2009; Tiso and Schechter, 2015). Apoptosis is a complex biological process that regulates cell survival and eliminates diseased cells (Kuranaga, 2012). During this process, Bax, pro-apoptotic, and Bcl-2, anti-apoptotic proteins interact together to control the cell survival or death and to regulate the apoptosis process (Singh *et al*., 2015). It has been established that oxidative stress is often implicated in the enhancement of mitochondrial signal transduction of apoptotic factors, reduction of Bcl-2 expression, and in activation of caspase-3 protein (Takahashi *et al*., 2003). Indeed, the progressive generation of NO, resulting from nitrite intoxication, mediates apoptosis by altering the balance of pro- and anti-apoptotic proteins, resulting in the liberation of the mitochondrial cytochrome c that in turn, mediates the subsequent activation of caspase-3, which is responsible for apoptotic cell death (Kim *et al*., 2001; Yuan *et al*., 2003). Herein, the up-regulation of Bax and caspases-3 and the downregulation of Bcl-2 resulted in imbalance between pro- and anti-apoptotic proteins that can explain the increase in hepatic apoptotic cells of sodium nitrate- intoxicated rats.

Additionally, our results showed that sodium nitrate induced a notable increase in the expression of TNF-α protein in liver tissue. However, no previous literature studied the effect of sodium nitrate on TNF-α. This finding is supported by those of Eissa *et al*.(2021); Hamdan *et al*. (2019); Alyoussef and Al-Gayyar (2017) and Elsherbiny *et al*. (2017), who stated that intoxication of rats with sodium nitrite increased TNF-α level in serum and various organs. Further, the increase of the expression in TNF-α protein, a pro-inflammatory cytokine, results in tissue damage (Al-Gayyar *et al*., 2014). Indeed, oxidative stress and inflammation are evidently interrelated because oxidative stress induces tissue damage that triggers an inflammatory response, which in turn, may be a direct inducer of oxidative stress by generating free radicles as a characteristic response of the activated immune cells to any stimuli (Hulsmans and Holvoet, 2010; Seyedsadjadi and Grant, 2021). Thus, the increment of the TNF-α protein expression in the liver of the sodium nitrate- intoxicated rats may be attributed to and mediated through the increased oxidative stress recorded in this study.

The glial fibrillary acidic protein (GFAP) is an intermediate filament protein of astrocytes that is essential for maintaining their mechanical strength and structure (Middeldorp and Hol, 2011). GFAP is not limited only to the central nervous system but detected also in muscles (Van Dyke *et al*., 2016) and liver (Elbastawisy and Mohamed, 2020). Interestingly, GFAP is expressed in hepatic stellate cells, the principal fibrogenic cell in the liver; the over expression of GFAP in the liver is supposedly linked to early stage of hepatic fibrosis and inflammatory infiltration, as activated hepatic stellate cells are able to release cytokines that can attract the inflammatory cells (Lotowska *et al*., 2014; Tennakoon *et al*., 2015). Our immunehistochemical investigations demonstrated an increase in GFAP immunoreactivity in liver of sodium nitrate- intoxicated rats. Up till now, no obtainable data have been recorded about the impact of nitrate on GFAP expression in hepatic tissue. However, recent studies recorded that GFAP is expressed in the liver of mice with prolonged exposure to thioacetamide (Tennakoon *et al*., 2015). A previous study suggested that the activation of nuclear factor-kappa B (NF-κB) is involved in NO-mediated increase in GFAP expression in astrocytes that may participate in the pathogenesis of various neurodegenerative disorders (Brahmachari *et al*., 2006). Oxidative stress activates the redox-sensitive transcription factors, activator protein-1 and NF-κB (Janssen-Heininger *et al*., 2008) that strongly suggest that oxidative stress is closely related to the recorded

increase in the expression of GFAP in the liver of nitrate- exposed rats, mainly via the overproduction of NO following nitrate intoxication, and thereby, stimulates the expression of pro-inflammatory and pro-fibrotic molecules (Brahmachari *et al*., 2006).

Pomegranates possess nutritional and pharmacological values due to the high contents of various bioactive phytochemicals (Russo *et al*., 2018, Puneeth and Chandra, 2020). Regarding the GC/MS findings, both PM and PPAE contain almost the same phytochemical compounds with different percentages, namely 9-Octadecenoic acid; 2-Furancarboxaldehyde,5- hydroxymethyl; hexadecanoic acid; and 5-Methyl-1,3-benzenediol. The identified chemical compounds are of medicinal value due to their antimicrobial, antioxidant, anti-inflammatory, anti-proliferative and anticancer activities (Mujeeb *et al*., 2014; Abubakar and Majinda, 2016; Shapla *et al*., 2018; Suresh and Nadumane, 2021)

Similarly, these biologically active phytochemicals have been successfully detected by the GC-MS analysis of pomegranate peel and seeds (Al-Tai and Al-Mayyahi, 2021). Notably, these compounds may be responsible for the recorded hepatoprotective effect of either PM or PPAE against sodium nitrate- induced liver damage, mainly due to their antioxidant and anti-inflammatory activities.

On the other hand, the findings of the current study proved the ameliorative and cytoprotective effects of PM and PPAE against sodium nitrate- induced hepatotoxicity. Daily oral co-administration of PM (0.5 ml/rat) or PPAE (100 mg/kg) with sodium nitrate for 10 weeks significantly reduced the elevated serum activities of ALT and AST with marked improvement in hepatic histoarchitecture, reflecting the cytoprotective effect of both PM and PPAE. In the same line, Mahmood *et al*. (2015) reported that PM reduced serum ALT and AST activities in cispltin- intoxicated rats. However, the literature about the heptoprotective effect of PM is limited, numerous studies reported the heptoprotective effect of PPAE in obese (Sadeghipour *et al*., 2014) and diabetic (Faddladdeen and Ojaimi, 2019) rats. Further, pomegranate extracts ameliorated the hepatotoxic effect of aflatoxins- (Tohame *et al*., 2010), ethanol- (Luangpirom *et al*., 2013), carbon tetrachloride- (Wei *et al*., 2019), and vancomycin- (El Bohi *et al*., 2021) intoxicated rats. Interestingly, pomegranates exert potent nutritional values and immense health benefits because of the high content of punicalagins, which are extremely potent antioxidants found in pomegranate peel and juice, in addition to its contents of polyphenols, especially anthocyanins and vitamins (Suman and Bhatnagar, 2019). Based on the key role of oxidative stress in the hepatotoxic effect of sodium nitrate, the hepatoprotective effect of both PM and PPAE observed in this study could be attributed to the potent antioxidant effect of pomegranates (Abd Elmonem, 2014; Mahmood *et al*., 2015; Wei *et al*., 2019, El Bohi *et al*., 2021) as manifested by the recorded reduction of MDA, NO, H_2O_2 levels and the elevation of GSH content and CAT activity in hepatic tissue. The activation of Nrf2–ARE pathway could preserve cells from oxidative stress-triggered cell death (Jeffrey *et al*., 2009). Going in line with this hypothesis, it was suggested that flavonoids can activate the Nrf2–ARE pathway that is responsible for increasing the endogenous antioxidant enzymes and, consequently, improves the antioxidant capacity (Saw *et al*., 2014). In addition, pomegranate extract provided a marked antioxidant activity in the vascular endothelium cell line by suppressing the intracellular ROS generation, released in the pro-oxidative and pro-inflammatory conditions (Dițescu *et al*., 2019). Importantly, the antioxidant properties of pomegranates may be attributed not only to the free radicals scavenging activity and the prevention of ROS generation, but also to their ability to inhibit LDL oxidation and to form metal chelates (Li *et al*., 2006; Kulkarni *et al*., 2007), that have been closely-related to the remarkable amounts of phytochemicals and bioactive molecules, including punicalagin, anthocyanins, flavonoids, phenolic acids, and hydrolyzable tannins detected in PM (Nasser *et al*., 2017) and PPAE (Russo *et al*., 2018).

Based on the hypothesis that oxidative stress plays a substantial role in triggering and aggravation of inflammation and apoptosis as a response to exposure to various stimuli, the close correlation between oxidative damage and inflammatory/apoptotic responses was considered in this investigation. Our immunohistochemical findings revealed that PM and PPAE decreased Bax/Bcl-2 ratio that is related to the down-regulation of caspase-3 in liver tissue, which may be explained by the anti-apoptotic effect of pomegranate, possibly via its potent antioxidant properties (Morvaridzadeh *et al*., 2020). These findings may suggest that the regulation of Bax, Bcl-2, and caspase-3 play a pivotal role in the repairing process of the damaged hepatocytes (Basile *et al*., 1997). Similarly, punicalagin, a hydrolysable tannin compound in *Punica granatum* L, showed antiapoptotic effect, manifested by up-regulation of Bcl-2 and down-regulation of Bax and caspase-3 proteins expressions in cerebral cortex tissues (Yaidikar and Thakur, 2015) and liver (Fouad *et al*., 2016). Moreover, El Bohi *et al*. (2021) reported up-regulation of caspase-3 and down-regulation of Bcl-2 in liver and kidney tissues of rats following administration of pomegranate ethanolic extract. Additionally, the level of caspase-3 protein was found to be substantially reduced following pomegranate extract treatment (Foroutanfar *et al*., 2020). Besides, the reduction in TNF-α expression in the liver of rats co-administrated PM or PPAE with sodium nitrate recorded in the current study may reflect the anti-inflammatory effect of pomegranates as recorded previously by El Bohi *et al*. (2021); Fouad *et al*. (2016) and Larrosa *et al*. (2010). The activation of p38-mitogen-activated protein kinase (p38-MAPK) and NF-kB is directly- associated with increased gene expression of numerous inflammatory mediators, mainly TNF-α (Hayden and Ghosh, 2004). Previous studies have shown the inhibitory effect of pomegranates on the production of pro-inflammatory cytokines via inhibition of the p38-MAPK and NF-kB pathways (Hayden and Ghosh, 2004; Larrosa *et al*., 2010). These results may explain the antiapoptotic and anti-inflammatory properties of pomegranates, particularly via reducing lipid peroxidation and improving the antioxidant status, as well as blocking inflammatory and apoptotic pathways (Fouad *et al*., 2016). In congruence with earlier study, Hassan *et al*. (2018) recorded that pomegranate juice reduced the expression of GFAP in rats fed on high-fat diet. Based on recent studies relating hepatic stellate cells activation to oxidative stress and stimulation of the expression of pro-inflammatory and pro-fibrotic molecules, the current reduction in GFAP expression in liver of sodium nitrate- intoxicated rats and treated with either PM or PPAE could be strongly attested to the antioxidant, anti-fibrotic and anti-inflammatory activities of pomegranates (Hassan *et al*., 2018).

Conclusion

Sodium nitrate induces hepatic damage, as manifested by the increase in liver function biomarkers and disturbance of the oxidant/antioxidant status alongside remarkable pathological alterations and activation of pro-apoptotic, pro-inflammatory and pro-fibrotic pathways, resulting in the imbalance of Bax/Bcl-2 ratio that positively correlates with the up-regulation of caspase-3, TNF-α, and GFAP in liver tissue. Nevertheless, pomegranate molasses and pomegranate peel aqueous extract showed nearly similar potent antioxidant, anti-apoptotic, anti-inflammatory and anti-fibrotic properties against sodium nitrate- induced hepatotoxicity. Our findings provide new evidence for the promising health benefits not only of pomegranate peel extract but also pomegranate molasses as a commercial pomegranate processing product.

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Conflict of interest

The authors have no conflict of interest to declare.

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