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Hypolipidemic and antioxidant effects of the juice and water seed extracts of two pomegranate species in high-cholesterol diet fed rats

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Abstract

This study investigated the hypolipidemic and antioxidant effects of the juice and water seed extracts of two pomegranate species from Saudi Arabia and Egypt, and compared them with each other as well as with ellagic acid (EA) in high-cholesterol diet (HCD) fed rats. Compared with the case in rats fed HCD alone, EA and juice or water seed extracts of both pomegranate types significantly reduced the levels of total cholesterol, triglycerides, LDL-c, and GSH, increased the level of HDL-c, increased the activity of SOD and CAT, and decreased the level of MDA in the serum of HCD-fed rats. There were more profound effects of the juice or water seed extract of both the Saudi and Egyptian pomegranates on serum HDL-c and GSH levels and the activities of SOD and CAT than those of EA. In conclusion, this study showed that water seed extracts or juice of both types of pomegranate ameliorated HCD-induced hyperlipidemia, lipid peroxidation, and changes of enzymatic and non-enzymatic antioxidants in the serum of HCD-fed rats.

Keywords: hyperlipidemia; hypercholesterolemia; pomegranate; oxidative stress.

Pratica Application: Providing a potential solution to reduce the harm caused by consuming high-fat diet with water seed extracts or juice of both types of pomegranate.

1 Introduction

Cardiovascular disorders (CVDs) remain the leading cause of death worldwide, with very high incidence and mortality rates (Ashoor et al., 2019). Oxidative stress is known to be a key player in the development of coronary heart disease and has been well described as the mechanism driving a range of health conditions, such as hypertension, dyslipidemia, metabolic syndrome, diabetes mellitus, and obesity (Pignatelli et al., 2018). Against this background, it is well established that the impairments of several pro-oxidant and antioxidant enzymes or mediators including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase, glutathione reductase (GSH), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the major mechanisms behind initiation of the oxidative stress response in CVDs (Pignatelli et al., 2018).

Among the various risk factors, there have been extensive studies and reports on the significant contribution of hyperlipidemia to the development and progression of oxidative-damage-induced CVDs (Manolio et al., 1992; Lloyd-Jones et al., 1999). It has been shown that hyperlipidemia is the leading cause of atherosclerosis in humans and animals by increasing reactive oxygen species (ROS) and subsequent oxidation of low-density lipoproteins (LDL), giving rise to oxidized LDL (ox-LDL) (Olea et al., 2014). In this regard, higher serum levels of ROS, lipid peroxides, and malondialdehyde (MDA) were identified in hyperlipidemic patients and animal models fed a high-fat diet (HFD) (Pignatelli et al., 2018). Hypercholesterolemia is a condition characterized by significant increases in circulatory total cholesterol and LDL cholesterol (Olea et al., 2014).

Cholesterol is an important cellular component predominantly found in cell membranes; it plays crucial roles in the structure of cells and the regulation of cell signaling (Ondrejovičová et al., 2010). In patients and animal models, hypercholesterolemia may develop as an inherited disorder (e.g., familial hypercholesterolemia) or as a consequence of an unbalanced HCD or other metabolic disorders (e.g., obesity) (Csonka et al., 2016). Studies have identified hypercholesterolemia as an independent risk factor for the development of atherosclerosis, myocardial ischemia, and other forms of CVD (Csonka et al., 2016). Extensive analyses have been performed within this field and have shown that the effect of hypercholesterolemia is mainly mediated by the systemic and organ-specific generation of ROS (Csonka et al., 2016; Chtourou et al., 2015). Indeed, hypercholesterolemia was found to induce ROS and reactive nitrogen species in a variety of organs, whereas cholesterol-lowering drugs significantly attenuated hypercholesterolemia-induced oxidative/nitrative stress in patients (Chtourou et al., 2015) (Cai et al., 2014;

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Csonka et al., 2016). At the mechanistic level, the pro-oxidant effects of hypercholesterolemia are mainly due to oxidation of the lipid bilayer, mitochondrial damage, and upregulation of the majority of ROS-generating enzymes, antioxidant depletion, and the activation of inflammation (Napoli & Lerman, 2001).

Nonetheless, scientists have continued to be fascinated by the roles of medicinal plants in lowering cholesterol levels and protecting against hypercholesterolemia-induced ROS and lipid peroxidation. Ellagic acid (EA), a natural polyphenolic compound predominantly found in fruit including raspberries, grapes, blackcurrants, and pomegranates, was shown to have health benefits by lowering cholesterol levels and subsequently reducing ROS generation and improving the health of the vascular system (Rozentsvit et al., 2017; Mo et al., 2014; Chao et al., 2009). Most of EA's benefits were attributed to its potent anti-inflammatory, antioxidant, anti-proliferation, and anti-angiogenic properties (Rogerio et al., 2008; Lee et al., 2010). Indeed, PE was reported to reduce the levels of ox-LDL uptake by macrophages, reduce serum levels of cholesterol, and attenuate oxidative stress in a variety of HFD-fed animal models (Yu et al., 2005; Park et al., 2011; Liu et al., 2015). In addition, in some species, EA was shown to have more significant cholesterol-lowering effects than simvastatin (Liu et al., 2015).

EA is the major constituent of the fruit pomegranate (Negi et al., 2003). Pomegranate's antioxidant and anti-atherosclerotic effects have already been described and were indicated to be mainly due to its high levels of phenolic compounds (Morais et al., 2020; Yoshime et al., 2019) such as EA (Negi et al., 2003; Pérez-Vicente et al., 2002; Mertens-Talcott et al., 2006). The antioxidant potential of pomegranate juice or seed extract is about two- to threefold higher than that of green tea (Gil et al., 2000). Notably, pomegranate juice was found to lower serum cholesterol and LDL-c levels in T2DM and hyperlipidemia patients (Esmaillzadeh et al., 2006). It was also shown to exert a potent anti-obesity effect in rats fed an HFD (Lei et al., 2007). In addition, it protected the brains of rats from high-fructose diet induced oxidative stress by decreasing MDA and protein carbonylation increasing levels and the activities of serum activity levels of superoxide dismutase (SOD) and GPx (Amri et al., 2017).

However, the hypocholesterolemic effect of either the juice or the seeds of pomegranate with respect to its antioxidant activity is poorly described at the levels of clinical or experimental science. In one study, Egyptian pomegranate seed oils significantly ameliorated lipid parameters in hypercholesterolemic rats (Elbandy & Ashoush, 2012). However, there are limited data regarding the water extract of this plant. Hence, in this study, we studied and compared the anti-hypercholesteremic and antioxidant effects of Egyptian and Saudi-derived pomegranate juice and water seed extracts and compare them with the effects of pure PE in a rat model fed a high-cholesterol diet (HCD).

2 Materials and methods

2.1 Animals

Seventy Healthy adult male Wistar albino rats were used in this study. They were supplied by and maintained at the animal facility of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. During the adaptation and experimental procedure, all animals were kept under controlled conditions (temperature of 25 °C, 12-h light/dark cycle, and relative humidity of 60 ± 5%).The experiment was conducted at the Experimental Animal Care Center, College of Pharmacy, King Saud University and All experimental procedures conducted in this study were approved by the institutional review board (Ref. No. KSU-SE-17-21), King Saud University, Riyadh, Saudi Arabia in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 80-23; 1996).

2.2 Preparation of the pomegranate juice and seed solution

Ripened fresh Egyptian and Saudi pomegranates were purchased at local markets in Saudi Arabia. The entire pomegranate fruits were peeled manually, squeezed, and filtered with a 15-μm membrane. The resultant juice was collected and stored at 4°C. In addition, the seeds were separated, dried in an oven for 84 h at 40 °C, ground into a fine powder, and then immersed in water at a ratio of 1:9 (w/w) at 4 °C for 24 h to obtain an aqueous extract, which was also stored at 4 °C until use. In both cases, the pomegranate juice and seed extracts were diluted at a ratio of 0.625% for use in the experiment. This preparation was performed every week and the obtained sample was used within 6 days.

2.3 Experimental design

Rats (100 ± 5 g) were randomly selected and divided into seven groups (n=10/group) as follows: 1) control: fed a normal diet (AIN-93M purified diet); 2) HCD: fed an HCD [normal diet mixed with cholesterol (1.5 g/kg) (Dyets Inc., Bethlehem, USA)]; 3) HCD + PE: fed an HCD and administered EA solution (Cat. No. 2250; Sigma Aldrich, St. Louis, MO, USA) (1.13% solution); 4) HCD + Saudi pomegranate juice: fed an HCD and administered Saudi pomegranate juice; 5) HCD + Saudi pomegranate seed extract: Fed HFD and administered Saudi pomegranate seed extract; 6) HFD + Egyptian pomegranate juice: fed an HCD and treated with Egyptian pomegranate juice; and 7) HCD + Egyptian pomegranate seed extract: fed an HCD and treated with Egyptian pomegranate juice. All pomegranate juices and seed extracts of both pomegranate types were administered orally at a final concentration of 0.625%, in accordance with a previously reported procedure (de Nigris et al., 2007). HCD feeding and all treatments were carried out for 60 days on a daily basis. The major constituents of the AIN-93 control diet and HCD are shown in Table 1.

At the end of day 60, an intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (100 mg/kg) was used to anesthetize the rats (Kim et al., 2015). Their chests were opened and blood samples (2 mL) were collected from the carotid artery into plain tubes, centrifuged at 4000 rpm for 15 min, and then stored at -20 °C for further analysis.

2.4 Biochemical analysis

The SOD, catalase (CAT), and glutathione reductase (GSH) as well as the level of malondialdehyde (MDA) were

Table 1. Formulation of the control diet and high-cholesterol diet (HCD) of rats.

Components	Standard diet	HCD
	(g/kg diet)	(g/kg diet)
Corn starch	465.692	450.692
Casein	140	140
Maltodextrin	145	145
Sucrose	100	100
Soy oil	50	50
Cellulose	50	50
Mineral mix (AIN-93-MX)	35	35
Vitamin mix (AIN-93-VX)	10	10
L-Cystine	1.8	1.8
Choline bitartrate	2.5	2.5
TBHQ: tert-Butylhydroquinone	0.008	0.008
Cholesterol	0	15

measured using assay kits (Cat. Nos. 703102, 706002, 700910, and 10009055; Cayman Chemicals, Ann Arbor, MI, USA). Serum levels of cholesterol, high-density lipoprotein (HDL-c), LDL-c, and triglyceride (TG) were measured using commercially available kits (Cat. Nos. ab65390, ab65390, and ab65336; Abcam, Cambridge, UK). All procedures were performed in accordance with the manufacturer's instructions.

2.5 Histology

The rats were sacrificed, and their livers were quickly removed under anesthesia and fixed in 10% buffered formalin for 72 h. The fixed specimens were processed, cleared, and impregnated using an automatic tissue processor (Sakura, Japan). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan). Sections of 4-µm thickness were cut using a rotary microtome (RM2245; Leica, Germany) and an Autostainer (5020; Leica, Germany) was used for hematoxylin and eosin staining. The stained sections were observed under a light microscope (Eclipse BOi; Nikon, Japan) and images were acquired with a digital microscope-mounted camera (OMX1200C; Nikon, Japan).

2.6 Statistical analysis

Data analysis was performed with SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests. Values were considered significant at $P<0.05$. The results are expressed as mean ± SD.

3 Results

3.1 Changes in liver histology

Changes in liver structures are shown in Figures 1 and 2. HCD-fed rats showed an increase in the accumulation of hepatic fat droplets with increases in infiltrating leukocytes at the portal vein and between the hepatocytes, compared with the findings in control rats fed a low-fat diet (Figure 1A-C). However, the number of fat droplets significantly decreased in the liver of all rats under all treatments (Figure 1D and Figure 2A-D), with a pronounced improvement seen in the HCD + EA-fed rats, which retained some infiltrating leukocytes in their portal vein (Figure 1D). In addition, increased dilation in the hepatic sinusoids was seen in the HCD + Egyptian pomegranate seed group (Figure 2C).

3.2 Changes in lipid profile

There were significant increases of serum levels of total cholesterol, TG, and LDL-c, whereas the serum levels of HDL-c were significantly increased in the serum of HCD-fed rats compared with that in control rats fed a normal diet (Figures 3 and 4). However, the levels of cholesterol, TG, and LDL-c were significantly decreased but the level of HDL-c was significantly increased in HCD-fed rats treated with EA or with the juice or seed extract of either Saudi or Egyptian pomegranate (Figures 3 and 4). Notably, the decreases in cholesterol and TG and the increase in HDL-c were significantly more profound in the HCD + EA-treated group than in the other groups. However, the effects of all treatments on the increase in HDL-c were similar (Figure 4).

3.3 Changes in oxidative stress markers

Serum levels of SOD and GSH were significantly decreased and serum levels of MDA were significantly increased in HFD-fed rats (Figures 5 and 6). The changes of all of these markers were ameliorated in HCD-fed rats treated with EA or with the juice or seed extract of either Saudi or Egyptian pomegranate (Figures 5 and 6). Notably, the decrease in the level of MDA was most significant in the HCD + EA-treated rats (Figure 6). However, the increases in the activities of SOD and CAT and the GSH levels were significantly more profound in the serum of the group with HCD treated with the juice or seed extract of either Saudi or Egyptian pomegranate, with no differences found among them (Figures 5 and 6). These findings suggest that EA may have a more profound ROS scavenging ability but less of an effect on the synthesis and upregulation of antioxidants.

4 Discussion

This study showed that chronic administration of both Egyptian and Saudi pomegranate juice and seed water extracts could reverse the accumulation of hepatic fats, reduce the serum levels of cholesterol, TG, and LDL-c, and increase the serum level of HDL in a rat model of hypercholesterolemia. These extracts also mitigated the HCD-induced systemic oxidative stress response by increasing the serum levels of SOD, CAT, and GSH, and suppressing the serum level of MDA. Notably, the effects of these plant fractions of both types of pomegranate were comparable to that of EA.

In this study, our initial aim was to validate our HCD animal model from the perspective of its effect on hepatic lipid accumulation and serum lipid profile. Our findings showed

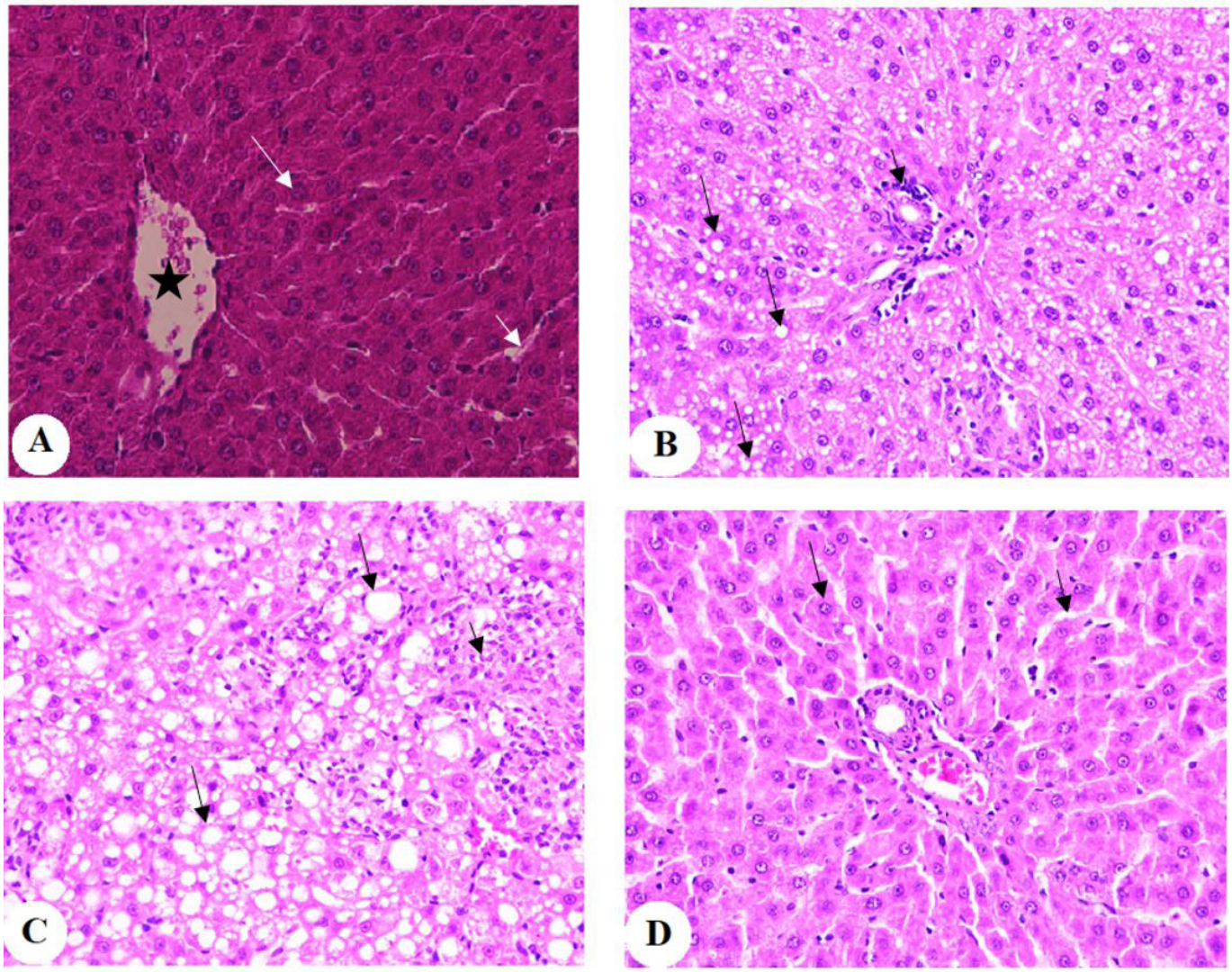

Figure 1. Liver histopathology of some experimental groups as shown by hematoxylin and eosin staining. A: Image from a control rat showing healthy hepatocytes (long arrow) with normal sinusoids (short arrow) that are radiating from a central vein (star). B and C: Images from high-cholesterol fed rats (HCD) showing the accumulation of fat droplets of all sizes (long arrow). Infiltrating leukocytes in the portal vein and in the normal tissue were also evident (short arrow). D: An image from an HCD + ellagic acid (EA)-fed rat showing almost normal architecture of the hepatocytes (long arrow) with normal sinusoids (short arrow). However, infiltrating leukocytes remained present in the portal vein.

that feeding on an HCD for 60 days significantly increased fat accumulation, increased the serum levels of cholesterol, TGs, and LDL-c, and decreased the serum level of HDL-c. These results match the previously described classical markers of hypercholesterolemia in experimental animals, as well as in humans (Csonka et al., 2016). Indeed, serum levels of HDL-c were reported to be significantly decreased in HFD- and HCD-fed animal models (Csonka et al., 2016; Elbandy & Ashoush, 2012); Kamal & Mohamed, 2009; Noeman et al., 2011). In line with this, levels of LDL-c were significantly increased in HCD-fed animals (Woo et al., 2008). These findings prompted us to continue work in this field in the present study.

The seed water extracts or juice of either Saudi or Egyptian pomegranate effectively (and to the same degree) reduced HCD-induced hepatic lipid accumulation, increased TGs, cholesterol, and LDL-c, and decreased HDL. Notably, their effects were comparable to those of EA. These findings suggest the pomegranates of both types, including both their juice and seed extracts, exert potent cholesterol-lowering effects in HCD-fed rats. Generally, lipid-lowering drugs mainly act by decreasing the levels of cholesterol either by decreasing its intestinal absorption or stimulating the decomposition of cholesterol into bile in the liver by regulating several transcription factors and transporters, namely, LXRα, RXRα, PPARα, PPARγ, and ATP-binding cassette transporter A1 (ABCA1) (Liu et al., 2015).

The hypolipidemic effect of EA is well reported. In hamsters, this occurs mainly by stimulating the fecal removal of bile acid and upregulating the LXR/PPAR-ABCA1 axis (Liu et al., 2015). This may explain the reductions in cholesterol, TG, and LDL-c in the serum of HCF + EA-fed rats. On the other hand, several studies support our findings that both pomegranate juice and seeds have potent hypolipidemic effects under healthy conditions and upon feeding on an HFD and HCD. For example, seed oils of Egyptian pomegranate significantly

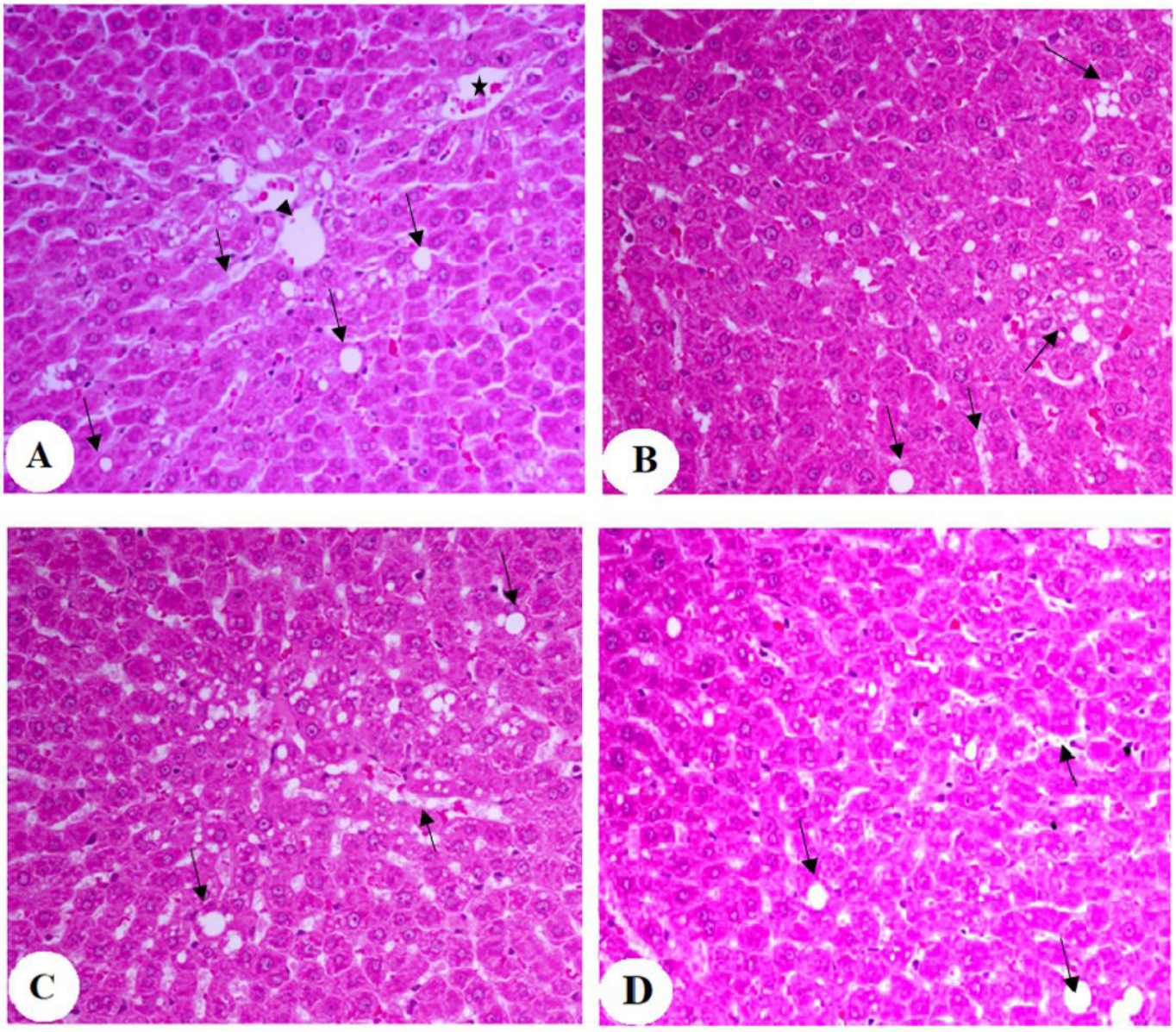

Figure 2. Liver histopathology of some experimental groups as shown by hematoxylin and eosin staining. A and B: Images from the groups with a high-cholesterol diet (HCD) + Saudi pomegranate juice or seeds, respectively, showing very few fat droplets (long arrow) and moderately dilated sinusoids (short arrow). C: An image from the HCD + Egyptian pomegranate seed group showing a reduced number of fat droplets with the presence of largely dilated sinusoids in some areas (short arrow). D: An image from the HCD + Egyptian pomegranate juice group showing very few fat droplets (long arrow) and normal sized sinusoids (short arrow).

lowered total cholesterol, TG, and LDL-c and increased HDL-c in hypercholesterolemic rats (Elbandy & Ashoush, 2012). In addition, in isolated macrophages, pomegranate juice significantly reduced the uptake of ox-LDL-c (Park et al., 2011). Moreover, in healthy individuals, regular consumption of pomegranatess was found to significantly lower LDL aggregates, which is an initial step toward the formation of foam cells and atherosclerosis (Kaplan et al., 2001). Moreover, in diabetic patients with hyperlipidemia, the consumption of concentrated pomegranate juice for 8 weeks significantly reduced the serum level of total cholesterol LDL-c and the ratio of LDL-c/HDL-c, while having no effect on the serum level of HDL-c (Esmaillzadeh et al., 2006).

Unfortunately, in this study, we could not perform phytochemical screening to precisely describe the exact constituents of the pomegranate juice and seeds responsible for their hypocholesterolemic effects. However, among the phytochemicals present in pomegranate is punicic acid, which was shown to have potent anti-inflammatory, hypolipidemic, and anti-obesity effects through activating PPAR-γ (Hontecillas et al., 2009). In addition, catalpic acid, another phytochemical in pomegranate, can increase HDL-c and decrease triglyceride and cholesterol levels via a PPAR-α-dependent mechanism (Hontecillas et al., 2008). Moreover, gallic acid, EA, and linoleic acid are phytochemicals well known to lower total cholesterol, triglycerides, and LDL-c in obese mice (Jang et al., 2008). Furthermore, and as

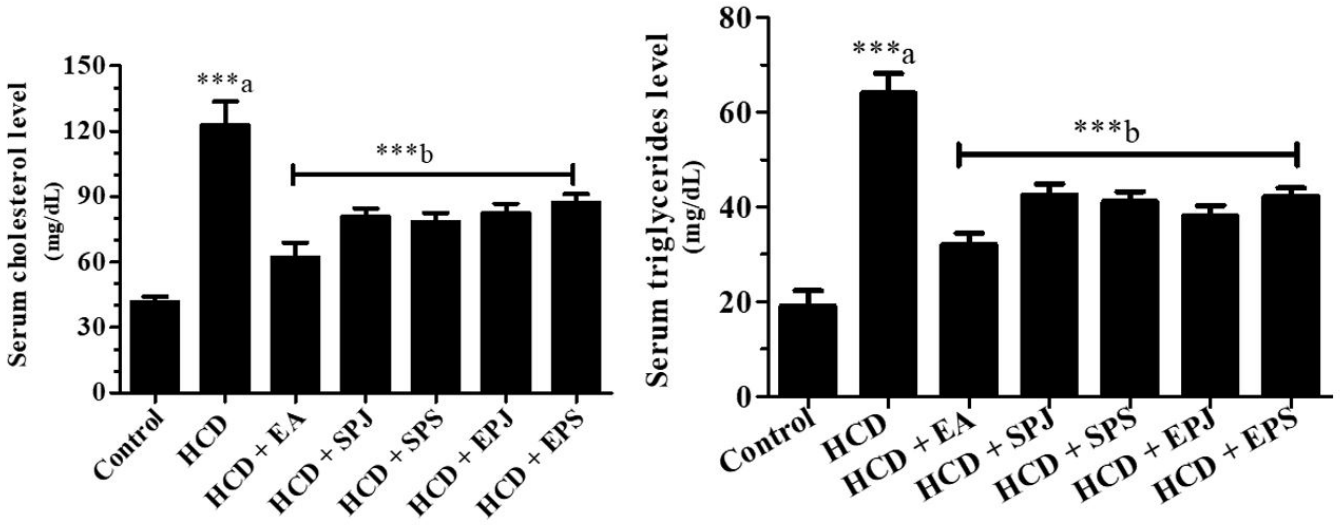

Figure 3. The effects of EPS, EPJ, SPJ, SPS, and EA on total cholesterol and triglycerides levels in the serum of rats. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. P> 0.05 was considered significant (* P> 0.05, ** P> 0.01, *** P> 0.001). a: Comparison of control vs. HCD. b: Comparison of HCD vs. treated groups.

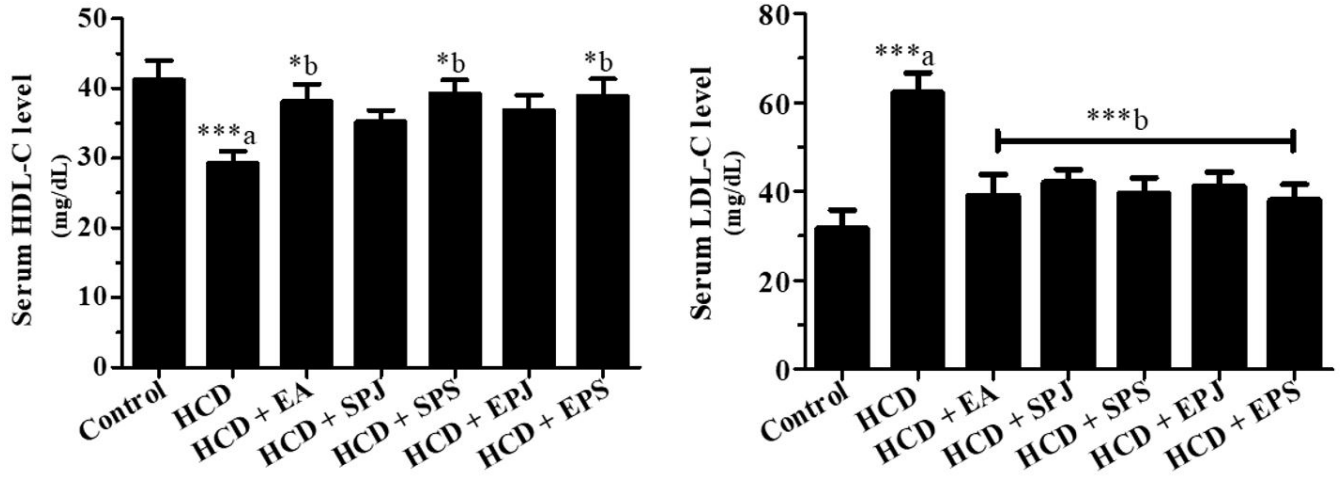

Figure 4. The effects of EPS, EPJ, SPJ, SPS, and EA on HDL-c and LDL-c t levels in the serum of rats. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. P> 0.05 was considered significant (* P> 0.05, ** P> 0.01, *** P> 0.001). a: Comparison of control vs. HCD. b: Comparison of HCD vs. treated groups.

discussed above, EA can suppress serum cholesterol levels by stimulating the fecal removal of bile acid and upregulating the LXR/PPAR-ABCA1 axis (Liu et al., 2015). At this stage, further studies should be performed to delineate the exact mechanism by which pomegranate exerts its hypercholesteremic effects.

HCD is associated with increased oxidative stress in organs, which ultimately leads to tissue lipid peroxidation, further generation of ROS, and cell damage (Otunola et al., 2010). Overactivation of several ROS-generating enzymes including NADPH oxidase, lipid peroxidation, mitochondrial damage, inflammation, and the depletion of cellular antioxidants are believed to be the major mechanisms by which hypercholesterolemia induces ROS (Napoli & Lerman, 2001). Supporting these findings and associated with high cholesterol, TG, and LDL-c levels, HCD-fed rats show a systemic oxidative stress response that is characterized by an increased serum level of MDA and suppression of several antioxidant enzymes including SOD, CAT, and GSH.

On the other hand, in this study, EA and juice or seed extracts of both Saudi and Egyptian pomegranates significantly reduced the serum level of MDA and significantly increased the serum levels of SOD, CAT, and GSH, suggesting an antioxidant protective effect. However, there were no significant differences in the levels of GSH and antioxidant enzyme activities between the juices or seed extract of both Saudi and Egyptian pomegranates. In addition, there was no significant difference when the effects of Saudi and Egyptian pomegranates on these biochemical markers were compared with each other. Supporting our data, the antioxidant potential of both the seeds and the juice of pomegranate has been well reported in healthy, diabetic, and hyperlipidemic individuals

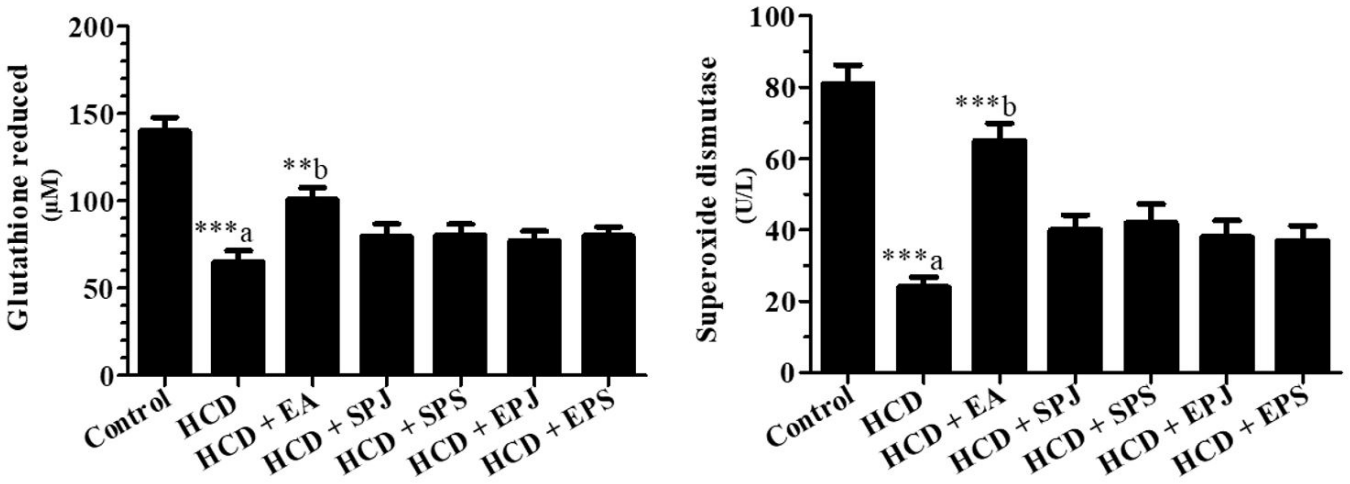

Figure 5. The effects of EPS, EPJ, SPJ, SPS, and EA on glutathione reductase (GSH) and superoxide dismutase (SOD) levels in serum of rats. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. P> 0.05 was considered significant (* P> 0.05, ** P> 0.01, *** P> 0.001). a: Comparison of control vs. HCD. b: Comparison of HCD vs. treated groups.

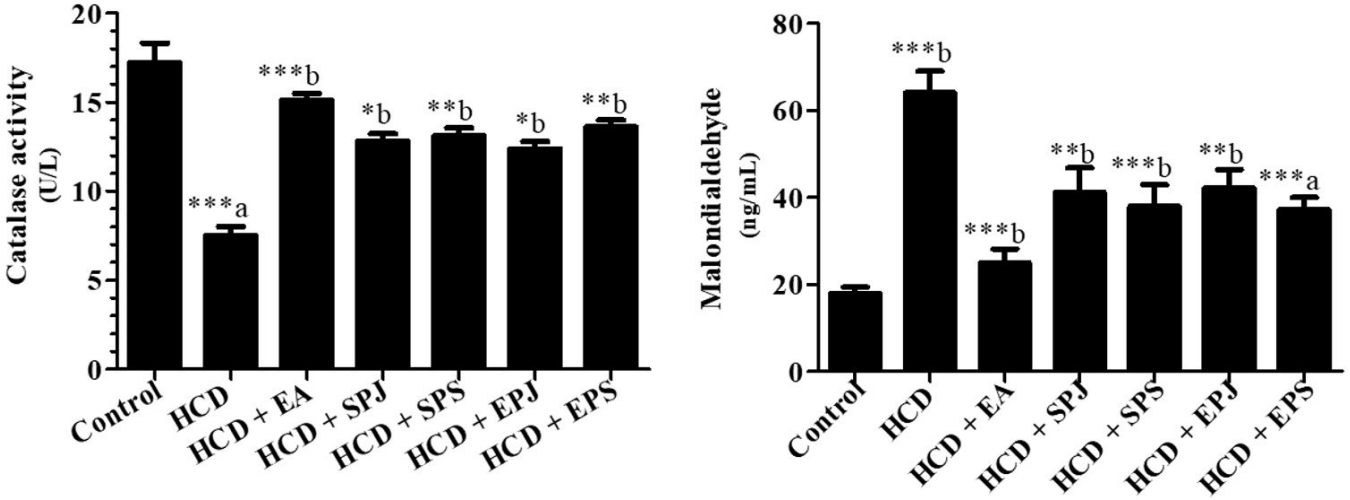

Figure 6. The effects of EPS, EPJ, SPJ, SPS, and EA on catalase activity (CAT) and malondialdehyde (MDA) level in serum of rats. Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. P> 0.05 was considered significant (* P> 0.05, ** P> 0.01, *** P> 0.001). a: Comparison of control vs. HCD. b: Comparison of HCD vs. treated groups.

or animals (Rosenblat et al., 2006; Viuda-Martos et al., 2010; Aboonabi et al., 2014; Matthaiou et al., 2014; Manthou et al., 2017).

The level of antioxidants in pomegranate juice was discovered to be higher than that in other natural fruit juices such as orange, blueberry, and cranberry juice (Tugcu et al., 2017). In diabetic rats, pomegranate juice, seed solution, and their mixture increased the serum activities of SOD and CAT (Aboonabi et al., 2014). Similarly, 2 weeks of intervention with 240 ml enhanced the circulatory SOD and CAT activities, as well as the level of GSH, while also reducing the MDA levels in healthy individuals (Naghizadeh-Baghi et al., 2015).

However, our data fail to reveal the mechanism by which pomegranate juice or seeds can stimulate antioxidation. This could occur in a manner secondary to the hypolipidemic effect or primarily due to the ability to scavenge ROS and synthesize antioxidant enzymes, an effect that can be concluded from these data. However, it was shown that the polyphenol, tannin, and punicic acid components of pomegranate juice or oil have potent antioxidant abilities that can scavenge ROS, inhibit inflammation, and stimulates the synthesis of antioxidants (Boussetta et al., 2009; Lee et al., 2010). However, this needs further investigation.

5 Conclusions

The data obtained here show that juice and water seed extracts of both Egyptian and Saudi pomegranates reduce cholesterol, TG, LDL-c, and MDA, and increase HDL-c and antioxidant enzymes and GSH in the serum of hypercholesteremic rats. Further studies at the clinical level are warranted to confirm pomegranate as a promising therapeutic option as an anti-hyperlipidemic agent.

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