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Phytochemicals in Pomegranate Seeds and Their Effect as Hypolipidemic Agent in Hypercholesterolemic Rats

[1]M.A. Elbandy and [2]I.S. Ashoush

[1]Food Science and Technology Dept., Fac. of Environ. Agric. Sci.,
Suez Canal Univ., El Arish, North Sinai, Egypt
[2]Food Science Dept., Fac. of Agric., Ain Shams Univ., Cairo, Egypt

Abstract: In the present study, pomegranate seed oils (PSO) were extracted and analyzed for their fatty acid profiles and total tocopherols. The defatted pomegranate seed residue (PSR) was evaluated for its total phenolic and scavenging capacities against DPPH. Dietary effect of pomegranate seed oil, defatted seed residue and their mixture (PSOR) on hypercholesterolemic rats was investigated. The results revealed that, pomegranate seed oil was rich in polyunsaturated fatty acid (88.4%) and total tocopherols (905.5 µg/100g). Meanwhile, the pomegranate seed residue contained amount of total phenolics (14.8 mg/g) and exhibited high scavenging activity (98.2%). After 28 days of feeding period, atherogenic rats group (AC) revealed an increasing in level of total cholesterol, triglyceride, low density lipoprotein cholesterol (LDL-C), risk ratio, atherogenic index and lipid peroxidation compared with normal control rats group (NC). Supplemented diet with 5% pomegranate seed oil, 10% pomegranate seed residue and 15% of their mixture at the ratio of 2:1 respectively improved lipid profile of plasma as compared to AC group. Furthermore, the supplementation diets induced a significant increase in levels of reduced glutathione and a reduction in malondialdehyde level (MDA) when compared to AC group. In addition, the histopathological examination of the heart and aorta in rats group fed on hypercholesterolemic diet showed severe histological changes as compared to supplemented PSO, PSR and PSOR groups, these results reflect the protective effect of pomegranate seed fractions (oil and residue) against atherosclerosis and it have more potential as a health supplement rich in natural phytochemicals.

Key words: Pomegranate seed oil · Defatted residue · DPPH · Hypolipidemic · Lipid profile · Oxidative stress · Atherogenic rats

INTRODUCTION

The high prevalence of obesity, atherosclerosis and cardiovascular disease (CVD) is largely attributable to the contemporary lifestyle that is often sedentary and includes a diet high in saturated fats and sugars and low ingestion polyunsaturated fatty acids (PUFAs), fruit, vegetables and fiber [1]. Pomegranate fruit (*Punica granatum* L.) is used from ancient times and reports of its therapeutic qualities has echoed throughout the ages. Both *in vitro* and *in vivo* studies have demonstrated how this fruit acts as antioxidant, antidiabetic and hypolipidemic as well as improves cardiovascular health. The health benefits of pomegranate have been attributed to its wide range of phytochemicals [2]. Pomegranate seeds are by-products of pomegranate juice industry are about 20% (w/w) of the whole fruit. Since, they can be useful for food applications (especially in juice and beverage industries) as a functional agent Tehranifar *et al*. [3] and Mohagheghi *et al*. [4]. Recent studies found that pomegranate seed may have the potential to be a good source of nutrients and antioxidants. It has been suggested that dietary supplementation with pomegranate seeds may reduce weight gain and type 2 diabetes risk [5], reduce the risk of cancer and alleviate menopausal symptoms [6]. The beneficial effects of pomegranate seeds may be related to the presence of a variety of biologically active compounds, particularly polyphenols, which have been studied for their antioxidant effects [7]. Significant levels of phenolic content were detected in pomegranate seeds recommended by Pande and Akoh [8], Elfalleh *et al*. [9].

Corresponding Author: Ihab S. Ashoush, Department of Food Science, Faculty of Agriculture, Ain Shams Univ. Cairo, Egypt.

Pomegranate seed oil (PSO) is a major source of polyunsaturated fatty acids (PUFAs) including conjugated linolenic acid (CLA) isomers as well as a low saturated fatty acid which is important for therapeutic uses in human health [10].

The present study was designed to investigate the phytochemical composition of pomegranate seed oil and its residue. Also, assess the hypolipidemic effects of pomegranate seed oil and its residue on the lipid profile, oxidative stress parameter and histopathological alteration in rats fed hypercholesterolemic diet.

MATERIALS AND METHODS

Materials

Plant Material: Full ripened pomegranate fruits (*Punica granatum* L.) Wonderful variety was obtained from the local market. Pomegranate seeds from the rest of the fruit were separated from the juice and washed carefully to remove sugars and other adhering materials. Separated seeds were dried at 35-37°C in an oven for 12 h. The dried, clean seeds were then finely ground and oil was extracted by steeping in hexane as a solvent overnight in dark place with gentle shaking at 20°C. The resultant oil and defatted seed powder were stored at -18°C until use.

Chemical: Sodium carbonate, hexane and methanol were obtained from El-Gomhoreya Co., Cairo, Egypt. 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), Folin-Ciocalteus phenol reagent and Gallic acid were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Commercial kits used for determining glucose, total cholesterol, triglycerides (TG), high density lipoprotein (HDL), malonaldehyde (MDA) and reduced glutathione (GSH) were purchased from Biodiagnostic Co. Dokki, Egypt.

Animals: Thirty male Wistar rats with an average weight of 140 g were obtained from the Organization of Biological Products and Vaccines (Helwan Farm, Cairo, Egypt).

Methods of Analysis

Fatty Acid Composition and Vitamin E Content: Fatty acids were determined using gas chromatography (GC Hewlett Packard 6890) according to the method described in AOAC [11]. Vitamin E in pomegranate seed oil was determined using HPLC system (Hewlett Packard series 1100) measured at 292 nm according to Pyka and Sliwiok [12].

Total Phenolic Content: The total phenolic content in the methanolic extract of pomegranate seed residue (PSR) was determined according to Singleton *et al*. [13]. The reaction mixture contained 0.5 ml of Folin-Ciocalteu reagent, 0.5 ml of 7.5% Na_2CO_3 and 0.5 ml of extract. The absorbance was measured at 765 nm. Results were expressed as gallic acid equivalents (GAE) per gram pomegranate seed residue powder (mg GAE/g powder) using gallic acid standard curve.

DPPH Radical Scavenge Activity%: The ability of the methanolic extract of pomegranate seed residue (PSR) to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was determined using the method described by Singh *et al*. [14].

Biological Experiment: The rats were housed in screen-bottomed aluminum cages in room maintained at 25±1°C with alternating cycles of light and dark of 12h duration. The animals were fed on the control diet for three consecutive days. Rats were then randomly divided into five groups, each of which consists of 6, the first group (the control) was fed on the normal control (NC) diet, the second was atherogenic control (AC) group fed on hypercholesterolemic diet, the third was fed on hypercholesterolemic diet supplemented with pomegranate oil at 5% (PSO), which was equal to the no observable adverse effect level (NOAEL) as reported by Meerts *et al*. [15], the fourth was fed on hypercholesterolemic diet supplemented with pomegranate seed residue at 10% (PSR) and the fifth was fed on hypercholesterolemic diet supplemented with a mixture of 5% pomegranate seed oil and 10% its residue (PSOR) for 28 days. The composition of the experimental diets is shown in Table 1 according to AIN-93 guidelines [16]. The changes in body weight were recorded weekly.

Table 1: Compositions of the experimental diets (%)

Component%	NC	AC	PSO	PSR	PSOR
Corn starch	62.5	61.2	61.2	51.2	51.2
Casein	18.0	18.0	18.0	18.0	18.0
Corn oil	10.0	10.0	5.00	10.0	5.00
Cellulose	5.00	5.00	5.00	5.00	5.00
Salt mixture	3.50	3.50	3.50	3.50	3.50
Vitamin mixture	1.00	1.00	1.00	1.00	1.00
PSO	-	-	5.00	-	5.00
PSR	-	-	-	10.0	10.0
Cholic acid	-	0.30	0.30	0.30	0.30
Cholesterol	-	1.00	1.00	1.00	1.00

Blood samples were also obtained from the retro-orbital plexus of the eyes from all animals of each group at the end of the experiment; the organ (heart) was excised immediately after bleeding for weight. Plasma was obtained from blood samples by centrifugation at 1500 rpm for 15 min at ambient temperature.

Glucose and Lipid Profiles: Enzymatic determination of glucose in plasma was carried out colorimetrically at 510 nm according to Trinder [17]. Fully enzymatic determination of total cholesterol (TC) was carried out according to Allain [18], total triglycerides (TG) in plasma was measured colorimetrically at 546 nm, according to Fossati and Principe [19], low density lipoprotein cholesterol (LDL-C) was determined by enzymatic methods of Wieland and Seidel [20] and the high density lipoprotein cholesterol (HDL-C) was determined according to the method of Lopez-Virella *et al*. [21].

Oxidative Stress: The extent of lipid peroxidation in plasma was determined by measurement of malondialdehyde (MDA) formation at 534 nm using the thiobarbituric acid reactive substances (TBARS) method as described by Ohkawa *et al*. [22]. The reduced glutathione (GSH) in the plasma was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB) that gave a yellow colored complex with maximum absorption at 412 nm, according to the method described by Beutler *et al*. [23].

Histopathology: Autopsy samples were taken from the heart and aorta of the different groups of rats and fixed in 10% formol saline for twenty four hours. Washing was done with tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) for dehydration. Specimens were cleared in xylene embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stains for histopathological examinations through the light microscope [24].

Statistical Analysis: All data were expressed as mean values±SD for 6 rats in each group. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test with $P < 0.05$ being considered statistically significant. Statistical analysis was conducted with SAS program [25].

RESULTS AND DISCUSSION

Vitamin E and Fatty acid Chemical Composition of Pomegranate Seed Oil: Results in Table 2 show that the seed oil of pomegranate contained high amounts of tocopherols (vitamin E) which recently have attracted scientific attention for its antiaging, anticancer and antiatherosclerosis effect. Because of its richness in vitamin E, the seed oil of pomegranate would be a potential natural source for nutritional and medicinal uses as mentioned by Elfalleh *et al*. [9] and Jing *et al*. [26]. Fatty acids composition of pomegranate seed oil (PSO) presented in Table 2 revealed that the oil is rich in polyunsaturated fatty acids (PUFAs), the most predominant PUFA was linolenic acid C18:3. Concerning the saturated fatty acids/unsaturated fatty acids ratio (SEA/USFA) were very low. Therefore, consumption of pomegranate seed oil, rich in highly poly unsaturated fatty acids (PUFAs), protects against cardiovascular disease as reported by Jing *et al*. [26] and Parashar *et al*. [27].

Total Phenolics Content and Antioxidant Activity of Pomegranate Seed Residue (PSR): From the data presented in Table 3, it could be noticed that the pomegranate seed residue (the defatted pomegranate seed) is a good source of total phenolics content and had a great free radical scavenging activity. This finding showed that PSR could be utilized as nutraceutical resource [28].

Table 2: Vitamin E and fatty acid composition of pomegranate seed oil:

Parameters	
Vitamin E (µg/100g)	905.53
Fatty acid analysis (%)	
C16:0	2.96
C16:1	0.03
C17:0	0.07
C17:1	0.01
C18:0	1.88
C18:1	4.86
C18:2	4.79
C18:3	83.58
C20:0	0.822
C20:1	0.994
SFA	5.732
MUFA	5.899
PUFA	88.37
SEA/USFA	0.065

Table 3: Total phenolics content and antioxidant activity of pomegranate seed residue

Components	
Total phenolics (mg/g)	14.76
Antioxidant activity%	98.2

Table 4: Effects of experimental diets on body weight gain and relative hearts weight of rats fed on hypercholesterolemic diets.

Parameters	NC	AC	PSO	PSR	PSOR
Body weight (g)					
Initial	141.6±1.1[a]	141.45±1.2[a]	140.1±1.4[a]	138.25±1.4[a]	138.2±1.2[a]
Final	203.6±2.3[a]	170.8±1.4[b]	181.9±1.6[b]	212.3±1.7[a]	206.9±1.8[a]
Gain	61.8±1.9[ab]	29.3±1.3[c]	41.8±1.9[bc]	74.1±1.7[a]	68.7±1.7[a]
Relative heart weight (g/100 g BW)	0.90±0.25[a]	0.85±0.19[a]	0.88±0.09[a]	1.05±0.12[a]	1.02±0.19[a]

Means having different letters in the same row are significantly different (P * 0.05).

Table 5: Effect of experimental diets on plasma lipids profiles in rats fed on hypercholesterolemic diets

Parameters	NC	AC	PSO	PSR	PSOR
Triglycerides (mg/dl)	45.92±1.27[c]	163.41±2.93[a]	36.32±0.8[e]	47.92±0.8[b]	43.21±1.02[d]
Total cholesterol (mg/dl)	65.93±1.9[cb]	135.3±2.75[a]	55.51±5.28[c]	75.72±9.26[b]	63.83±3.67[cb]
HDL-cholesterol (mg/dl)	45.41±0.86[d]	24.04±0.67[e]	62.23±0.67[b]	60.71±0.6[c]	70.68±1.48[a]
LDL-cholesterol (mg/dl)	11.84±1.71[b]	78.59±2.95[a]	13.98±4.1[b]	15.49±2.33[b]	10.47±2.63[b]
Risk ratio[1]	1.450±0.28[b]	5.640±0.58[a]	0.89±0.08[c]	1.25±0.15[cb]	0.90±0.035[c]
Atherogenic index[2]	0.45±0.28[b]	4.640±0.58[a]	0.11±0.08[b]	0.26±0.11[b]	0.10±0.03[b]

[1]Risk ratio= Total cholesterol / HDL-cholesterol

[2]Atherogenic index = [(Total cholesterol - HDL-cholesterol)/HDL-cholesterol]

Means having different letters in the same raw are significantly different (P * 0.05).

Effect of Experimental Diets on Body Weight Gain and Relative Hearts Weight of Rats: The initial body weights of all rat groups were not significantly different, however, after 28 days of feeding; the body weight gain were significantly reduced in atherogenic control (AC) group fed on hypercholesterolemic diet as compared to other groups (Table 4). Also, animals fed on diet supplemented as replacer with pomegranate seed oil (PSO) which contained polyunsaturated fatty acids significantly lesser the body weight gain among all groups due to the reduction of abdominal fat accumulation as reported by Arao *et al*. [29] meanwhile, this diet enhanced the weight gain contrary to AC group. On the other hand, rats fed on diets supplemented with pomegranate seed residue (PSR) and the PSOR mixture caused significantly higher increased in body weight gain compared to NC, AC and PSO groups. These results hypothesized that the pomegranate seed residue may improve appetite and enhance weight gain. The relative weight of hearts was not significantly affected by the type of diets fed to rat groups (Table 4).

Lipid Profile Parameters: The present study showed a significant increase in the concentration of plasma total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) in the atherogenic control (AC) rats group fed on hypercholesterolemic diet compared to other groups. While, the PSO, PSR and PSOR-supplemented diets significantly reduced plasma TC concentration by 58.9, 44 and 52.8% and LDL-C by 82.2, 80.3 and 86.7.03%, respectively (Table 5). These findings are in agreement with those obtained by Meerts *et al*. [15]. The concentration of TG was found in rats fed on hypercholesterolemic diet (AC) was greater than the other groups indicating that the PSO, PSR and PSOR-supplemented diets significantly prevented the rise of TG in rats plasma fed on hypercholesterolemic diet. On the other hand, plasma HDL-C was significantly lower in rats receiving AC diet contrary to PSO, PSR and PSOR-supplemented diets (Table 5). The plasma total cholesterol/HDL-cholesterol ratio significantly increased in animals fed on AC diet while it decreased significantly in animals fed on PSO, PSR and PSOR - supplemented diets and control group. For this reason, atherogenic index (AI) was significantly higher in the AC rats group in comparison to the NC and PSO, PSR and PSOR groups (Table 5). The risk ratio and atherogenic index are also predictors of coronary risk [30]. The significant increase in total cholesterol/HDL-cholesterol and atherogenic index observed in AC rats has an effect on cardiovascular diseases. PSO, PSR and PSOR may play an important protective role against atherosclerosis and cardiovascular disease in mammals, which was deduced from the reduction of the risk ratio and atherogenic index in PSO, PSR and PSOR animal groups (Table 5).

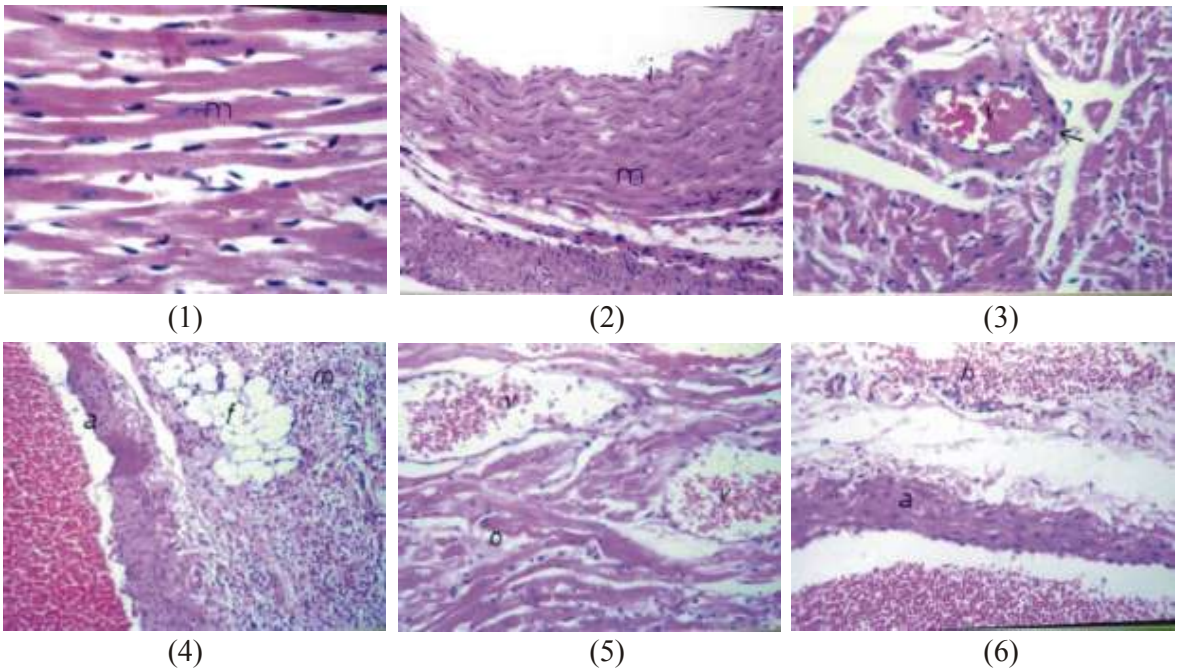
Table 6: Effect of experimental diets on plasma MDA and GSH in rats fed on hypercholesterolemic diets.

Diets	MDA (nmol/ml)	GSH (mg/dl)
NC	4.47±0.15[b]	26.06±0.08[b]
AC	5.30±0.15[a]	12.11±0.05[d]
PSO	3.85±0.03[d]	25.90±0.04[b]
PSR	4.21±0.05[c]	24.03±0.05[c]
PSOR	3.76±0.03[d]	28.10±0.06[a]

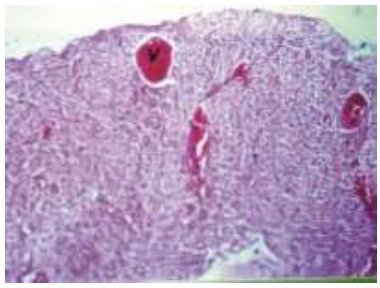
Means having different letters in the same column are significantly different (P * 0.05).

Oxidative Stress: Lipid peroxidation (MDA) is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation and aging [31]. In the present study, plasma Lipid peroxidation concentrations was significantly higher in atherogenic control (AC) group fed on hypercholesterolemic diet as compared to other groups (Table 6). Meanwhile, feeding on PSO, PSR and PSOR-supplemented diets significantly reduced the level of MDA. Changes in reduced glutathione (GSH) level as an antioxidant biomarker content of different groups are shown in Table 6.The levels of non-enzymatic antioxidant (GSH) were significantly lowered in atherogenic control (AC) group fed on hypercholesterolemic diet by about 53.5% as compared to those of normal control group (NC). In contrast, feeding on PSO, PSR and PSOR-supplemented diets significantly improved the level of glutathione compared with AC treated group. Furthermore, the reduction in the levels of MDA and the enhancement of reduced glutathione indicated strong antioxidant properties of pomegranate seed oil, pomegranate seed residue and their mixture.

Histopathological Examination: Micrograph (1) to (10) reveals the histopathological examination of semi-thin sections of heart and aorta of rats stained with hematoxylin and eosin. The group fed on normal control diet (NC) had no histopathological alteration in the myocardium (m), Micrograph (1); as well as in the tunica intima (i), media (m) and tunica adventitia (a) of the aorta (Micrograph 2). Treatment with atherogenic control group (AC) caused severe congestion in myocardial blood vessels (Micrograph 3), associated with thickening and sclerosis in the vascular wall and fat deposition with massive number of inflammatory cells infiltration were detected in the perivascular area of aorta (Micrograph 4).



Micrograph 1: Heart of a normal control rat (H & E, 160X)
Micrograph 2: Aorta of a normal control rat (H & E, 64X)
Micrograph 3: Heart of atherogenic rat (H & E, 80X)
Micrograph 4: Aorta of atherogenic rat (H & E, 64X)
Micrograph 5: Heart of rat treated with pomegranate seed oil (H & E, 64X)
Micrograph 6: Aorta of rat treated with pomegranate seed oil (H & E, 64X)



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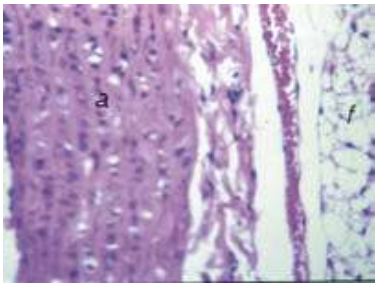

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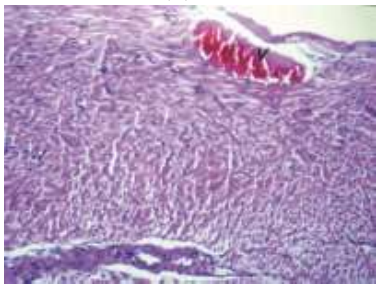

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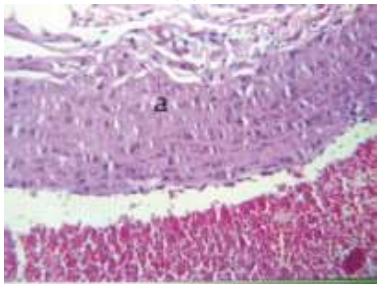

(10)

Micrograph 7: Heart of rat treated with pomegranate seed residue (H & E, 40X)
Micrograph 8: Aorta of rat treated with pomegranate seed residue (H & E, 80X)
Micrograph 9: Heart of rat treated with mixture of pomegranate seed oil and its residue (H & E, 40X)
Micrograph 10: Aorta of rat treated with mixture of pomegranate seed oil and its residue (H & E, 64X)

Rats fed on pomegranate seed oil supplemented diet had severe congestion noticed in the myocardial blood vessels associated with oedema in between the myocardial muscle bundles, (Micrograph 5). There was haemorrhage in the perivascular area of the aorta (Micrograph 6). Rat fed on pomegranate seed residue (PSR) supplemented diet showed congestion in the myocardial blood vessels (Micrograph 7). Fat deposition was detected in the perivascular tissue of the aorta (Micrograph 8). Rats fed on combination of pomegranate seed oil and its residue (PSOR) supplemented diet had mild congestion observed in the myocardial blood vessels (Micrograph 9). There was no histopathological alteration observed in the aorta, as recorded in (Micrograph 10).

CONCLUSION

Based on the above results, it could be concluded that the pomegranate seeds oil and its residue are rich sources of phytochemical and their supplemented diets significantly hindered the increasing in TC, TG, LDL-C, risk ratio, atherogenic index and Lipid peroxidation levels. Therefore, the hypolipidemic and antioxidative effect of pomegranate seed oil and pomegranate seed residue could be utilized in food applications as nutraceutical resource.

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