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Pomegranate Peels Extract Improves Plasma Lipid Profiles in Triton-WR-1339-Induced Hyperlipidemic Mice and Attenuates Lipoprotein Oxidation

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Abstract: This study was undertaken to investigate the hypolipidemic and anti-lipoprotein oxidation activities of an aqueous extract from pomegranate peels using Triton WR-1339-induced hyperlipemic mice as experimental model. Hyperlipidemia was developed by intraperitoneal injection of Triton at a dose of 200 mg/kg body weight. The animals were divided into three groups of eight mice each: normolipidemic control group (NCG), hyperlipidemic control group (HCG) and pomegranate peels extract treated group (PTG). After 10 h treatment, Triton caused a significant increase in plasma lipid parameters (total cholesterol, triglycerides, LDL-cholesterol, Atherogenic index and LDL/HDL ratio). However, the administration of pomegranate peels extract significantly reduced the elevated plasma lipid profile induced by triton injection. Although no significant change of HDL-cholesterol levels was noticed after treatment. Furthermore, the aqueous pomegranate peels extract showed a significant ameliorative action on elevated atherogenic index (AI) and LDL/HDL-C ratios. On the other hand, the extract showed effective antiradical activity (CI_{50}=3.67±0.06 µg/ml) against DPPH and significantly protects lipoproteins obtained from hyperlipidemic mice against copper induced oxidation *in vitro* (P<0.001). The extract contains 394.21±4.55 mg/g total phenolics, 309.27±4.13 mg/g tannins and 67.33±1.26 mg/g flavonoids. This finding indicates that pomegranate peels may contain polar products able to lower plasma lipid concentrations and might be beneficial in treatment of hyperlipidemia and atherosclerosis prevention.

Keywords: Hypocholesterolaemia, Hypotriglyceridaemia, Atherogenic Index, Anti-lipoprotein Oxidation, Pomegranate Peels, Triton WR-1339, Mice

1. Introduction

Many epidemiological and experimental studies have shown that the hypercholesterolemia and oxidative stress could contribute to the development and progression of atherosclerosis and related cardiovascular diseases which are the most common cause of death in the world [1, 2]. Indeed, a number of clinical trials have demonstrated that the increase in plasma low density lipoprotein cholesterol (LDL-C) levels plays a key role in the early development of atherosclerosis. However, high density lipoprotein cholesterol (HDL-C) is an anti-atherogenic parameter [3, 4]. On the other hand, triglycerides represent also a potential risk factor of atherosclerosis especially in diabetic individuals [5].

The logical strategy for management of atherosclerosis and prevention of the incidence of cardiovascular disease events is to target hyperlipidemia by drugs and dietary intervention [6]. For this purpose, efforts to develop effective and safety hypolipidaemic drugs have led to discovery of natural products and have stimulated the search for new lipid-lowering agents especially from foods, food byproducts and medicinal plants.

In Morocco, as in many developing countries, most hyperlipidemic individuals use folk medicine to treat

hyperlipidaemia and prevent atherosclerosis. Therefore, there is a strong interest, locally, in natural hypolipidemic substances derived from food byproducts and medicinal plants. Vast numbers of plants have received attention in this regard and have been shown to lower plasma lipid levels [7].

Pomegranate peels were produced as byproduct in huge amounts by the food industry and are an important source of bioactive compounds including hydrolysable tannins, consisting of gallic acid and ellagic acid esters of core polyol molecules [8]. This byproduct is often used in the east of Morocco by hyperlipidemic subjects as an alternative therapeutic tool to treat hyperlipidaemia and prevent atherosclerosis. In the traditional Chinese medicine, pomegranate peels are considered as a powerful astringent and anti-inflammatory agent and are applied in the treatment of traumatic haemorrhage, ulcers and infections, and disorders of the digestive tract such as diarrhea and dysentery [9]. However, an effect on blood lipid profile has not yet been shown. The present study is designed to evaluate the possible beneficial effect of aqueous pomegranate peels extract on plasma lipid levels in Triton WR-1339-induced hyperlipidemic mice and on the prevention of lipoprotein oxidation.

2. Material and methods

2.1. Preparation of Pomegranate Peels Extract

Pomegranate peels (*Punica granatum* 'variety Sefri') were purchased from an herbalist in Oujda city and reduced into fine powder. The aqueous extract was prepared by the same method used in folk medicine with some improvements. The dried powder was infused for 30 min in boiled distilled water, filtered, and the solution obtained concentrated in a rotatory evaporator under vacuum at 60 °C. The yield of extract in terms of starting dried plant material was of 37.31% (w/w). The resulting extract was suspended in distilled water for experimental tests.

2.2. Dosage of Total Polyphenol Content

Total polyphenols content of pomegranate peels extract was determined by the Folin–Ciocalteu procedure with slight modifications [7]. To aliquots of 0.5 ml were added 0.25 ml of Folin–Ciocalteu reagent and 0.5 ml 20% aqueous sodium carbonate solution. Samples were vortexed and the absorbances of blue colored mixtures were recorded after 30 min at 725 nm against a blank containing 0.5 ml of water, 0.25 ml of Folin–Ciocalteu reagent and 0.5 ml of 20% aqueous sodium carbonate solution. The amount of total polyphenols was calculated as catechin equivalent from a calibration curve of catechin standard solutions and expressed as mg catechin/g dry plant extract. All measurements were done in triplicate.

2.3. Quantification of Flavonoids

Flavonoids content was determined as previously described [10]. To each 5 ml of analyzed solution, 2.5 ml of aluminum chloride ($AlCl_3$) reagent were added (133 mg crystalline aluminium chloride and 400 mg crystalline sodium acetate were dissolved in 100 ml of distilled water) and absorbances were recorded at 430 nm against a blank (5 ml of analyzed solution plus 2.5 ml of water). The flavonoids content was determined as rutin equivalent from acalibration curve of rutin standard solutions and expressed as mg rutin/g of dry plant extract. All measurements were done in triplicate.

2.4. Dosage of Tannins

Total tannins were determined after their adsorption onto BSA (bovine serum albumin/ fraction V, ACROS, New Jersey, USA) [10]. In brief, 20 ml of sample (20 mg/ml) were homogenized with 250 mg of BSA and the mixture was stirred for 30 min, the preparation obtained was stored for 2 h at +4°C. Then the pH was adjusted to 4.6 by 1N HCl solution. After centrifugation at 4000 rpm/15 min, no adsorbed phenolics in the supernatant were determined by the Folin–Ciocalteu procedure as described above. Calculated values were subtracted from total polyphenol content and the amount of total tannins expressed as mg catechin/g dry plant extract. All measurements were carried out in triplicate.

2.5. Animals and Treatments

Twenty four adult male albino mice, weighing 25–30 g, bred in the animal house of the department of biology (Faculty of Sciences, Oujda, Morocco), were provided *ad libitum* access only to tap water throughout the experimental duration (10h). Their housing was maintained at a temperature of 22 ± 02°C with a 12 h light-dark cycle.

2.5.1. Triton Model of Hyperlipideamia

Triton WR-1339 (Tyloxapol, Sigma–Aldrich, USA) was dissolved in normal saline (pH 7.4) and a volume of 0.3 ml was administered intraperitoneally to the mice at a dose of 200 mg/kg in order to develop an acute hyperlipidemia.

2.5.2. Experimental Schedule

Overnight fasted mice were randomly divided into three groups of eight animals each. The first group, serving as normolipidemic control (NCG), received 0.3 ml of normal saline intraperitoneally and 0.5 ml of distilled water by gavage. The second, hyperlipidemic control group (HCG) was treated with 0.3 ml of Triton and gavaged by 0.5 ml of distilled water. The third, pomegranate peels extract treated group (PTG); animals were intraperitoneally injected with 0.3 ml of Triton and gavaged with 0.5 ml of the aqueous extract of pomegranate peels at a dose of 500 mg/kg. After treatment period (10 h), animals were briefly anaesthetized with diethyl ether and blood was taken from their retro-orbital sinus. The blood samples were immediately centrifuged (2500 rpm/10 min) and the plasma was used for lipid analysis.

2.5.3. Analytical Procedures

Plasma total cholesterol, triglycerides, HDL and LDL-cholesterol

Plasma total cholesterol levels were determined by the cholesterol oxidase enzymatic method, using Reactivos GPL Kits (CHEMELIX S.A, Barcelona, Spain); cholesterol esters were hydrolyzed and, in the presence of phenol, the quinoneimine as indicator was formed from hydrogen peroxide and 4-aminoantipyrine under peroxidase catalysis and spectrophotometrically measured at 510 nm.

Triglycerides were also quantified by enzymatic method using Reactivos GPL Kits (CHEMELIX S.A, Barcelona, Spain). Briefly, after enzymatic hydrolysis with lipases, the formation of quinoneimine from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic effect of peroxidase, was followed spectrophotometrically at 520 nm.

HDL-cholesterol concentrations were quantified by the same method used to determine total cholesterol after removal of other lipoproteins by precipitation with a mixture containing phosphotungstic acid (PTA) and $MgCl_2$ (Sigma Diagnostic kit, Inc, USA).

The LDL-cholesterol was calculated by the Friedwald formula as described previously [10]: LDL-Cholesterol = Total cholesterol – (HDL-Cholesterol + (TG/5)).

Atherogenic index (AI) and LDL-C/HDL-C ratio

The atherogenic index was calculated by the following formula:

AI = (total cholesterol - HDL-C)/HDL-C and the LDL-C/ HDL-C ratio was calculated as the ratio of plasma LDL-C to HDL-C levels.

2.6. Measurement of DPPH free Radical Scavenging Activity

As radical form, DPPH (2,2-diphenyl-1-picrylhydrazyl) absorbs visible light at 517 nm and after reduction by antioxidants species, its absorbance decreases. The anti-radical effect pomegranate peels extract was determined by the DPPH test according to the method described by Touiss et al. [11]. 5 µl of pomegranate peels extract at different concentrations (0.5, 10, 25, 50, 100 and 200 µg/ml) were mixed with 2495 µl of methanolic solution containing DPPH radical (0.06 mg/ml). The mixture was shaken vigorously and left to stand for 30 min. The reduction of the DPPH-radical was measured by the decrease of absorption at 517 nm. A positive control was carried out using butylated hydroxyanisole (BHA) as standard antioxidant substance in the same experimental conditions. The radical scavenging activity (RSA) was calculated according to the following formula: RSA (%) = $(A_{blank} - A_{sample} / A_{blank}) *100$. The IC_{50} (concentration providing 50% inhibition) values were calculated from the plotted graph of scavenging activity against concentrations of the samples. All tests were done in triplicate.

2.7. Determination of Lipoprotein-Rich Plasma Oxidation

The thiobarbituric acid reactive substances (TBARS) levels were analyzed as marker of plasma lipoprotein oxidation according to the method described by Touiss et al. [11]. Lipoprotein-rich plasma used as substrate for oxidative process was obtained from mice injected with Triton WR-1339 at a dose of 200 mg/kg for 10 h, the plasma contains 92 ± 10 mg/dl of LDL-cholesterol (analyzed by enzymatic kit as described above). In the control tube, lipoprotein-rich plasma (40 µl) was incubated with distilled water only. In a second control tube, lipoprotein-rich plasma (40 µl) was incubated with 10 µl of copper sulphate ($CuSO_4$-$5H_2O$) solution (0.3 mg/ml). In the third assay, lipoprotein-rich plasma (40 µl) was incubated with 10 µl of copper sulphate and the pomegranate peels extract was added at final concentrations of 2, 8, 32, 72, 128 and 200 µg/ml. The preparations were mixed vigorously and incubated 8 h at 37°C. Then, each tube was added of 500 µl of 20% trichloroacetic acid (pH 3.5) and 500 µl of 0.8% thiobarbituric acid (TBA), the reaction mixture was heated at 95°C for 30 min. After cooling, the resulted color was recorded at 532 nm. The amounts of TBARS (thiobarbituric acid reactive substances) were calculated and expressed as malondialdehyde (MDA) equivalent from the calibration curve of standard solutions. All measurements were done in triplicate.

2.8. Statistical Analysis

Data obtained were analyzed using the Student's t-test and P values less than 0.05 were considered as statistically significant. Our results are expressed as means ± SEM.

3. Results

3.1. Induction of Hyperlipidemia by Triton WR-1339

The levels of plasma total cholesterol and triglycerides after 10 h treatments are reported in figure 1. Comparatively to the normolipidemic control group (NCG), Triton WR-1339 caused a marked increase in plasma total cholesterol and triglyceride concentrations. The plasma total cholesterol was increased by 15 times ($p<0.001$) and triglycerides by more than 20 times ($P<0.001$). HDL and LDL-cholesterol levels are summarized in figure 2, as can be seen, LDL-cholesterol was still far higher in triton-treated group (+820%, $P<0.001$) comparatively to control (NCG). Furthermore, the Triton caused a significant increase in HDL-cholesterol by +460% ($P<0.001$) comparatively to control.

Figure 3 shows the changes of atherogenic index (AI) and LDL-C/HDL-C ratio in control and treated mice. It appears clear from the results that the Triton administration significantly affects these cardiovascular risk markers. Indeed, the AI was statistically increased in HCG (+500%) when compared with values found in normolipidemic control ($P<0.001$). The LDL/HDL-cholesterol ratio was also hindered in HCG comparatively to NCG (+86%, $P<0.05$).

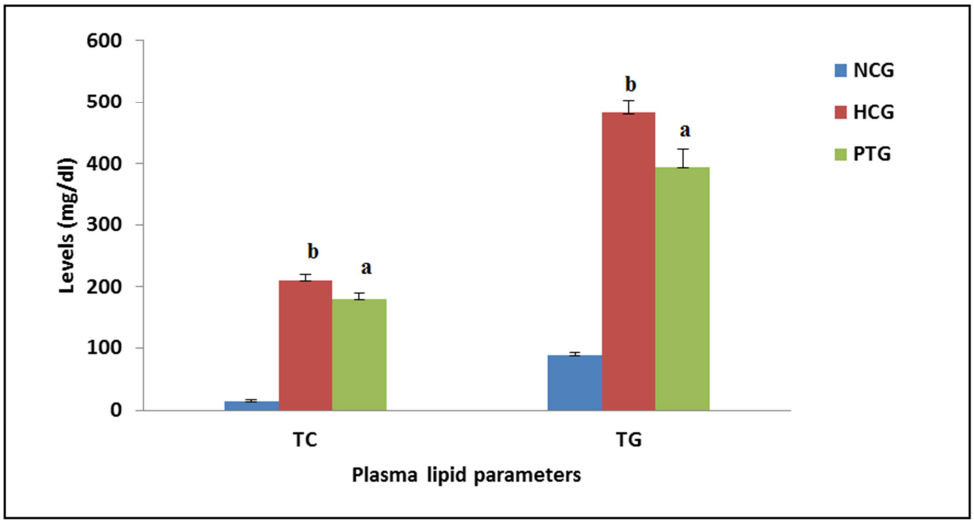

Figure 1. *Effect of pomegranate peels extract on plasma total cholesterol and triglycerides levels in mice.*

TC: total cholesterol; TG: triglycerides.
[a]P<0.05; [b]P<0.001 (HCG *versus* NCG and PTG *versus* HCG).
Values are expressed as mean ± SEM (n=8).

3.2. Effect of Pomegranate Peels Extract on Plasma Lipid Profile in Mice

After 10 h from the administration of aqueous pomegranate peels extract in Triton injected mice (PTG), both plasma total cholesterol and triglycerides were significantly decreased by 14% (P<0.05) and 18% (P=0.02), respectively (Figure 1).

LDL-cholesterol was lowered by 44% (P<0.01) but the increase of HDL cholesterol level (+57%) was not statistically significant (P=0.07) (Figure 2).

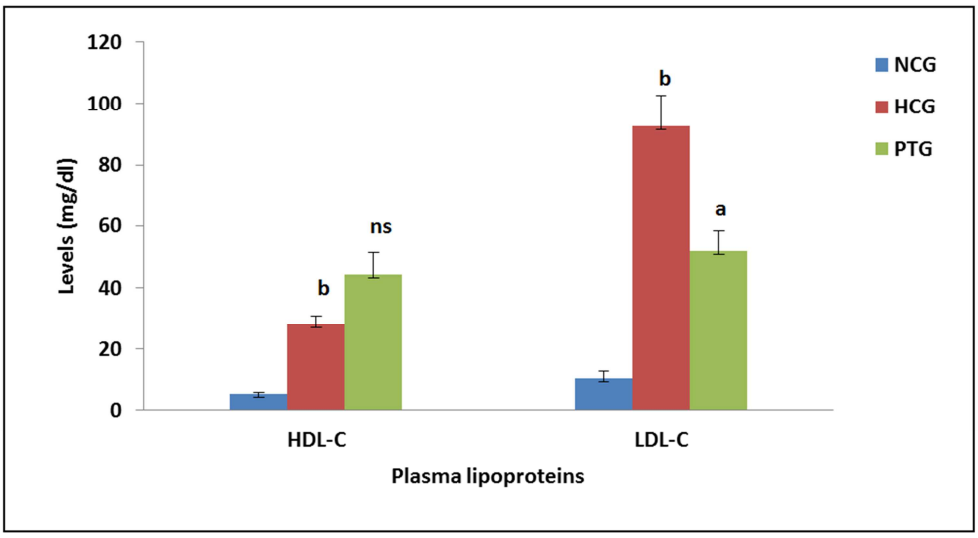

Figure 2. *Effect of pomegranate peels extract on plasma HDL and LDL-cholesterol in mice.*

HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol.
[a]P<0.01; [b]P<0.001; ns: not significant (HCG *versus* NCG and PTG *versus* HCG).
Values are expressed as mean ± SEM (n=8).

Promising results in lowering of the AI by the aqueous extract of pomegranate peels in Triton-induced hyperlipidemic mice were found (Figure 3). This cardiovascular predictive marker in pomegranate peels extract-treated group was significantly reduced (-47%, P<0.05) after 10 h when the data were compared, in the same period, to data found in hyperlipidemic mice.

The ratio of LDL-C to HDL-C is also a predictive indicator of cardiovascular disease incidence. Triton injection produced a significant increase of this marker and the pomegranate extract statistically return the ratio to basal value (-64%, P<0.01) (Figure 3).

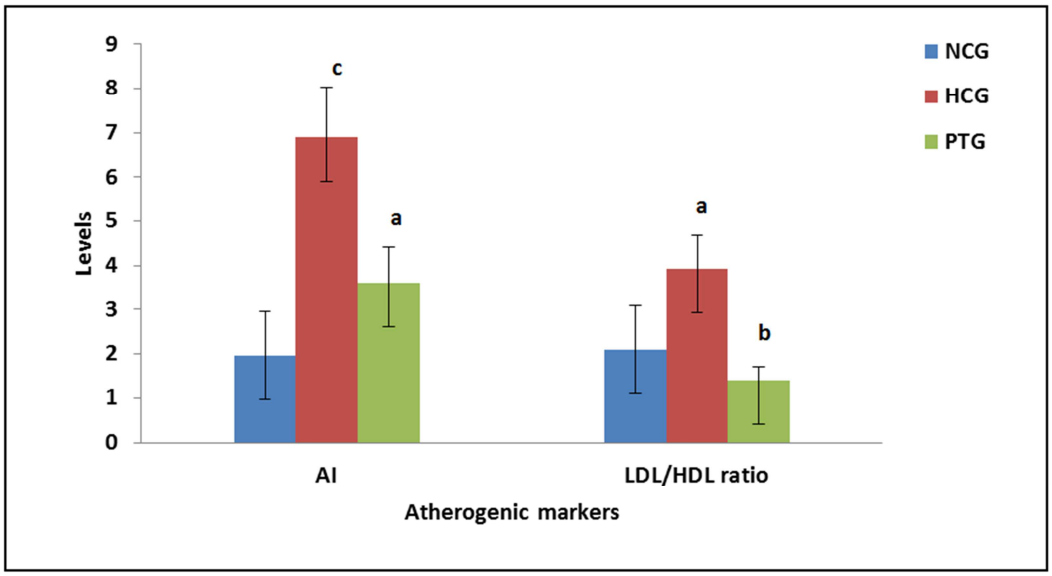

Figure 3. *Effect of pomegranate peels extract on atherogenic markers in mice.*

AI: atherogenic index; LDL/HDL: ratio of LDL-cholesterol to HDL-cholesterol.
[a]P<0.05; [b]P<0.01; [c]P<0.001 (HCG *versus* NCG and PTG *versus* HCG).
Values are expressed as mean ± SEM (n=8).

3.3. Free Radical Scavenging Activity of Pomegranate Peels Extract

The radical scavenging activity of the aqueous pomegranate peels extract was summarized in figure 4. We note that the extract scavenges DPPH radical in a dose-dependent manner. The percentages of inhibition were 13%, 61%, 93%, 97% and 98% at doses of 0.5, 10, 25, 50, 100 and 200 µg/ml, respectively. The BHA is used as standard known antioxidant and it scavenges the free radical by 2%, 25%, 50%, 63%, 77% and 90% at the same concentrations described above. When comparing IC_{50} of the tested compounds, we concluded that the pomegranate peels extract was more efficient against the DPPH radical with an IC_{50}= 3.67±0.06 µg/ml compared to that recorded by BHA; IC_{50}= 8.18±0.1 µg/ml (P<0.001).

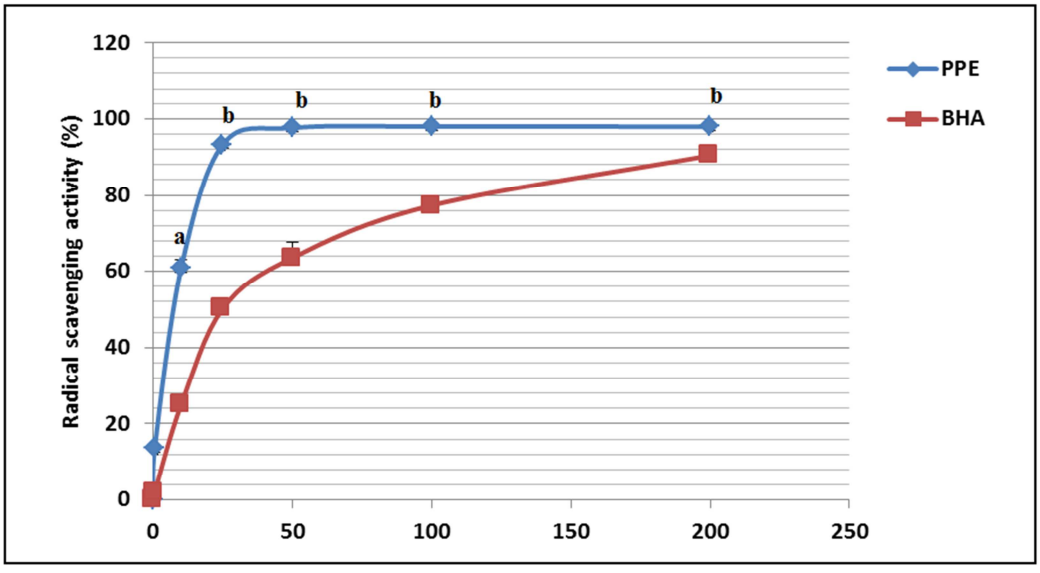

Figure 4. *Free radical scavenging activity of pomegranate extract and BHA.*

PPE: Pomegranate peels extract; BHA: butylated hydroxyanisol.
[a]P<0.05; [b]P<0.001 (PPE *versus* BHA).
Values are expressed as mean ± SEM (n=3).

3.4. Effect of Pomegranate Peels Extract on Plasma Lipoprotein Oxidation

The oxidation of plasma lipoproteins induced by copper, in the presence or absence of pomegranate peels extract, was measured by assaying TBARS at 532 nm. We note that the copper induces a significant increase in plasma lipoprotein oxidation at 37°C when compared to control ($P < 0.001$). However, treatment of copper-added plasma lipoproteins with different concentrations of aqueous pomegranate peels extract shows a significant and dose-dependent decrease of TBARS levels (Figure 5).

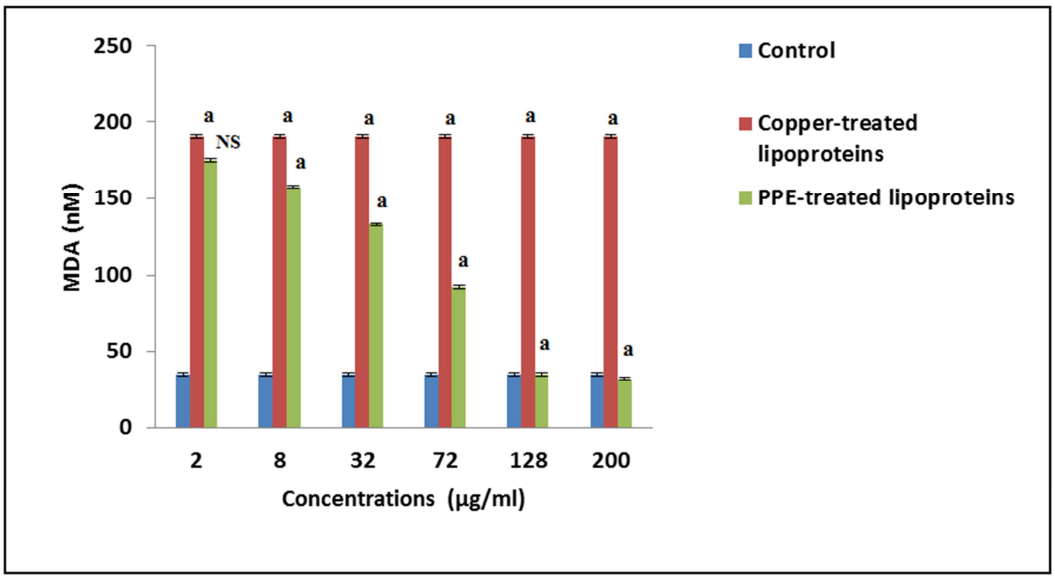

Figure 5. *Effect of pomegranate peels extract on plasma lipoprotein oxidation.*

PPE: Pomegranate peels extract; MDA: molondialdehyde
[a]P<0.001 (copper-treated lipoproteins *versus* control and PPE-treated lipoproteins *versus* copper-treated lipoproteins).
Values are expressed as mean ± SEM (n=3).

3.5. Polyphenol Contents of Pomegranate Peels Extract

The determination of polyphenol composition of pomegranate peels extract was reported in Table 1. The results show that the extract is rich in total phenol compounds which represent 394.21±4.55 mg/g dray extract. The tannins represent 309.27±4.13 mg/g and are the major polyphenol fraction (88.5% of total analyzed phenolic compounds). One gram of dry extract contains also 67.33±1.26 mg of flavonoids (17% of total analyzed phenolic compounds).

Table 1. *Polyphenol content of the aqueous pomegrante peels extract.*

	Total phenol[a]	Tannins[a]	Flavonoids[b]
Pomegranate peels extract	394.21±4.55	309.27±4.13	67.33±1.26

Values are expressed as means ± SEM from three assays.
[a]Expressed as mg catechin/g dry extract.
[b]Expressed as mg rutin/g dry extract.

4. Discussion

Triton WR-1339, a non-ionic detergent (oxyethylate tertiary octyl phenol formaldehyde polymer), has been widely used to inhibit lipoprotein lipase and block the uptake of triacyl glycerol-rich lipoproteins from plasma by peripheral tissues in order to produce acute hyperlipidemia in animal models [10, 11]. In our hands, this model presents an important plasma lipid and lipopritein profile changes, after 10 h from WR-1339 injection. This result demonstrates the feasibility of using such model to investigate the hypolipidemic and anti plasma lipoprotein oxidation effects of aqueous pomegranate peels extract.

It is clear from our results that the extract significantly decreases plasma total cholesterol in a marked manner. This reduction was associated with a decrease of LDL-cholesterol fraction called "bad cholesterol" which is a major modifiable risk factor of cardiovascular diseases and the target of many hypocholesterolemic drugs. This finding let us to suggest that the cholesterol-lowering activity of the extract appears to be due to the enhancement of LDL-C catabolism through hepatic receptors as demonstrated by Khanna et al. [12].

It is also reported that triglycerides play an important role in the regulation of lipoprotein interactions to maintain normal lipid and lipoprotein metabolism. Indeed, hypertriglyceridemia was positively correlated with an

increased incidence of cardiovascular disease [5]. The aqueous extract of pomegranate peels significantly suppressed the elevated blood concentrations of triglycerides. This result indicates that the extract is able to maintain normal triglyceride metabolism by stimulation of lipoprotein lipase activity.

Furthermore, treatment of hyperlipidemic mice with the pomegranate peels extract provides a beneficial action on lipid metabolism in regard to the reduction of atherogenic index. In fact, the elevated index was reduced in the extract-treated group comparatively to control. The result agrees with other works studying the hypolipidemic effect of natural products [13]. We note that this ameliorative action on lipid metabolism was especially due to the plasma lipid-lowering activity of the extract.

On the other hand, lower LDL/HDL-cholesterol ratio is very important to prevent atherogenesis since there is a positive correlation between the ratio and the development of atherosclerosis. The administration of pomegranate peels extract significantly reduced the higher values of LDL-C/HDL-C ratio showing the beneficial effect of this treatment in preventing atherosclerosis incidence.

Besides hyperlipidemia, free radicals were shown to cause oxidative modification of plasma lipoproteins contributing to the atherosclerotic process [14]. The prevention of the LDL oxidation by antioxidants is one of the major targets of many antiatherogenic drugs [15]. In this regards, natural antioxidants are very effective as free radical scavengers and provide protection against lipoprotein oxidation and atherosclerosis progression [16]. Our experimental study demonstrated that the extract from pomegranate peels significantly neutralizes DPPH free radical and prevents lipoprotein-rich plasma against oxidation. The observed activity can be attributed to phenolic compounds contained in the extract which are known to act via three major mechanisms. In the fact, polyphenols are able to scavenge free radicals and then stop chain reaction of lipid oxidation [17], they may also chelate prooxidant metal ion enhancing free radical production and finally preserve the HDL-associated paraoxonase activity preventing low density lipoprotein oxidation [18, 19].

Our results clearly demonstrate that the bioactive compounds contained in the extract have a polar character since they are more soluble in water. This finding is in agreement with previous reports showing that plant water-soluble extracts possess cholesterol-suppressive capacities and ability to attenuate the accelerated development of atherosclerosis in hypercholesterolemic models. In fact, tannins and flavonoids, a heterogeneous group of ubiquitous plant polyphenols, exhibit different pharmacological activities, including hypolipidemic and anti-atherogenic effects [20]. Thus, according to polyphenols quantification, there is ample evidence to suggest that tannins and flavonoids are the major compounds responsible for the hypolipidemic and antioxidant activities of the pomegranate peels extract.

5. Conclusion

Our finding let to consider pomegranate peels extract as an important source of bioactive compounds to treat hyperlipidemia and prevent lipoprotein oxidation leading to atherosclerosis development. So, this food byproduct can be valorized by extracting phenolic compounds or use of peels powder to prepare functional foods.

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