



Antioxidant activity of juice and peel extract of three variety of Pomegranate and the effect of pomegranate juice on the plasma lipids

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Abstract

Pomegranate (*Punica granatum* L.) fruit is widely consumed as fresh fruit and juice. In this experimental and laboratory study, antioxidant properties of pomegranate juice and peel extract of three variety were evaluate. Their antioxidant properties by different method were measured. Results are expressed as mean \pm SD. The statistical examination of the data was performed using the SPSS version 11.5 program. Mean values of antioxidant activity in different cultivars were compared by using analysis of the variance (ANOVA) test. When significant ($p < 0.05$) difference was detected, the results showed that pomegranate juice of malas variety had markedly higher antioxidant capacity than the other. In the present study, the FRAP value of juice of three cultivars of pomegranate was determined in an attempt to make a systematic comparison among their antioxidant activities. Further studies on the effective antioxidants contained in these pomegranate juice and the mechanisms by which they protect against disease development are highly recommended. The results showed that the contents of total phenolics, flavonoids were higher in peel extract of wild soar variety than in malas pomegranate extract. The large amount of phenolics contained in peel extract may cause its strong antioxidant ability. We concluded that shomal soar pomegranate peel extract appeared to have more potential as than malas variety extracts. Moreover, we studied effect of two wariety pj on plasma cholesterol and LDL of hypercholesrolemia patients. The finding showed those patients' total cholesterol and LDL.C in three groups, after consumption showed a significant decrease at $p < 0.01$ the levels of blood total cholesterol and LDL c.

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Introduction

Punica granatum L. (Punicaceae) is a deciduous shrub or small tree originally distributed in Iran and Afghanistan, and was introduced into China in the 2nd century BC. The extracts from this plant (juices, seed oil and peel) have been reported to exhibit strong antioxidant activity (Lansky *et al.*, 2007). Pomegranate leaf extract has shown free radical scavenging activity and antioxidant effects in vitro as well as numerous pharmacological activities, such as antitumor, antibacterial, astringent, anti-obesity and antidiarrheal activity (Adhami *et al.*, 2006; Lei *et al.*, 2007).

Antioxidants from leaf tissue have been isolated and identified and include flavone glycosides and gallo- and ellagitannins. The nutritional and antioxidant characteristics of pomegranate leaves have increased recent interest in their use as a beneficial source of secondary metabolites. As such, pomegranate leaves have been developed into a series of commercial products including green tea and other teas which are consumed in China, and have been included in nutrition capsules in the USA. Physical and chemical changes in pomegranate leaves have been reported (Bacha, 1975).

The seasonal trends in nitrogen and carbohydrate contents in the leaves of 'Banati' pomegranate trees were studied during two successive growing seasons. The nitrogen content (% and mg/leaf) decreased gradually during the growing season of 1972 and 1973, while, the total sugars (%) in the leaves fluctuated and did not show a discernable trend over the 2-year observation period. The starch content in the leaves tended to decrease from May to August and then increased until the end of the growing season in both years (Lihua *et al.*, 2010).

Pomegranate is a good example for this type of fruits. Pomegranate peels constitute approximately 40% of the whole fruit and are rich in ellagic acid derivatives such as the ellagitannins, punicalagin, and penicillin. In addition, some ellagic acid derivatives (ellagic acid hexoside, -pentoside, etc.) are also present, although

in lesser amounts. The most abundant of these polyphenols is punicalagin which is extracted from pomegranate juice during juice processing and which is responsible for more than 50% of the pomegranate juice's potent anti-oxidant activity (Mustafa *et al.*, 2010).

Plant based antioxidants are extracted from raw materials or waste products of food industry by organic solvents such as methanol, diethyl ether, and acetone. Methanol is an effective extractant for a broad range of polyphenols; therefore it is a frequently used solvent for both a laboratory scale and an industrial extraction process. Methanol is cheap and easily accessible and the manufacturing of herbal medicine usually uses methanol as a solvent to extract natural ingredients, a fact that will also concern us about the residual level of methanol in these products (Wang *et al.*, 2004).

Pomegranate juice is nutritionally an important beverage since it is consumed frequently for its phenolics compounds (such as anthocyanins, ellagic acid, phytoestrogenic flavonoids and tannins). In the past decade, numerous studies on the antioxidant activity have shown that pomegranate juice contains high levels of antioxidants - higher than most other fruit juices and beverages. Besides these findings, some clinical research studies suggest that pomegranate juice changes the blood parameters such as LDL, HDL, and cholesterol increase the prostate specific antigen (PSA), and may be helpful against heart disease, Alzheimer's disease. Phenolic compounds including flavonoids and flavonoids are widespread in plants and they are important in contributing the flavor and color of many fruits and vegetables. They are C₁₅ compounds of 2 Phenolic rings connected by a 3-carbon unit, and grouped according to presence of different substituents on the rings and the degree of ring saturation. They are frequently attached with sugars moiety to increase their water-solubility. Flavonoids extracted from pomegranate showed strong anti-oxidant activity (Mustafa *et al.*, 2010; Yunfeng *et al.*, 2006). Currently, there is great interest in finding

antioxidants from natural sources to minimize oxidative damage to cells. Oxidative damage is caused by free radicals and reactive oxygen species, mostly generated endogenously. Pomegranate fruit has valuable compounds in different parts of the fruit whose functional and medicinal effects such as antioxidant, anticancer and anti-atherosclerotic effects have been confirmed. Pomegranate juice is nutritionally an important beverage since it is consumed frequently for its Phenolic compounds (such as anthocyanins, ellagic acid, phytoestrogenic flavonoids and tannins, (Bazzano *et al.*, 2002; Block *et al.*, 1992). Phenolic compounds could be a major determinant of antioxidant potentials of foods, and could therefore be a natural source of antioxidants. Phenolic acids consist of two subgroups, i.e., the hydroxybenzoic and hydroxycinnamic acids (table 1 and table 2). Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which in common have the C₆-C₁ structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C₆-C₃), with caffeic, ferulic, p-coumaric and sinapic acids being the most common (Knekt *et al.*, 1997).

Pomegranate juice consumption for 3 years on patients with carotid artery stenosis and reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Dietary supplementation with polyphenolic antioxidants to animals was shown to be associated with inhibition of LDL oxidation and macrophage foam cell formation, and attenuation of atherosclerosis development (Aviram *et al.*, 2004).

Moreover, we investigated the effects of pomegranate juice (PJ, which contains potent tannins and anthocyanins, pj) consumption by hyperlipidemia patients, the aim of our study is to estimate the effect of two variety pomegranate juice consumption for four weeks by hypercholesterolemia patients and comparing the blood levels of LDL cholesterol in patients before and after pj consumption.

Materials and methods

Plant collection

The pomegranates were collected in November 2010 from one village of Saveh, in the Mrkazy Province of Iran. The area falls within the latitudes 35°.15' and longitudes 49°. 45' and the altitude of area is 1680 m.

Chemicals

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin Ciocalteu reagent, trifluoroacetic acid (TFA) and methanol were purchased from Merck Co. (Germany).

Sample preparation of peel

The pomegranate peel samples were first ground to fine powder. For water extraction, 0.5 g of the fine powder was extracted with 10 ml of ultra-filtered water at 100 °C for 30 min in a water bath. For methanol extraction, 0.5 g of the powder was extracted with 10 ml of 80% methanol at 40 °C for 24 h. The samples were then cooled down to room temperature and centrifuged at 4500 rpm for 15 min. The supernatant was recovered and used for the DPPH assay and total phenolic analysis. We used HPLC analyzing for polyphenol types determination.

Sample preparation of juice

Approximately 5 kg of pomegranate fruit was sampled for each cultivar (Malas and Saveh Black Leather). After discarding injured and sunburnt fruits, pomegranate fruits were peeled and the skins covering the seeds were removed manually. The juice of the seeds was extracted with a pilot plant packaged-type press (Bucher, Switzerland). The juices were kept at -20 °C until analyzed for no longer than three months. Before the experiments, PJs were defrosted and then centrifuged at 4000 g for 15 min at +4°C in order to remove water insoluble particles.

Determination of total phenolic content

The total phenolic content of the pomegranate peel Extracts and juice was determined using the Folin-Ciocalteu reagent (Wolfe *et al.*, 2003). The reaction

mixture contained: 200 µl of diluted peel extract and juice, 800 µl of freshly prepared diluted Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance at 765 nm was measured. Gallic acid was used as standard and the results were expressed as mg gallic acid (GAE)/g peel extract and juice.

Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride (AlCl₃) according to a known method, (Ordon *et al.*, 2006) using quercetin as a standard. The plant extract and juice (0.1 ml) was added to 0.3 ml distilled water followed by 5% NaNO₂ (0.03 ml). After 5 min at 25°C, AlCl₃ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The results were expressed as mg quercetin (QE)/g peel extract and juice.

Antioxidant activity

DPPH (2, 2'-diphenyl-1-picrylhydrazyl)

The antioxidant activity was tested by the DPPH (2, 2'-diphenyl-1-picrylhydrazyl) free radical scavenging method for each pomgranate sample. Then, we also prepared a dilution 1 M of DPPH. The absorbance of a mixture of 1 ml of sample and 1 ml of the DPPH solution was measured at 517 nm. The radical scavenging activity was calculated from the equation: Percentage of radical scavenging activity = (Abs control - Abs sample)/Abs control X 100.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe³⁺ to Fe²⁺ in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe²⁺-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie *et al.*, 1996) 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10

parts of 300 Mm sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl₃. 6H₂O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. FeSO₄ is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol FeSO₄ equivalents per gram of sample. Ascorbic acid, quercetin, can be used as a positive control.

Clinical investigation

In this investigation, patients were divided in three groups with twelve patients for each group. The treatments were including: 1-using malas variety of pomegranate juice 2- black variety of pomegranate juice 3- drug (levustatine). The levels of blood LDL.C in different experiments, before and after consumption of treatments were measured. We used venous blood sampling for fasting blood LDL cholesterol. These samples were collected in before and after consumption treatment. Serum was separated by centrifugation (10 minute, 1500 rpm). Serum LDL cholesterol levels were measured with the use of enzymatic LDL kit (zist shimi) by unic spectrophotometer. Fresh PJ (black and malas variety) was used in this study. Pomegranates were hand-picked, washed, chilled to 4°C. The fruit was then crushed, squeezed. The PJ was filtered, and stored at -20°C until use (only 1-2 day). Administration of levustatine (cholesterol lowering drug by enzyme inhibition) to another group.

Results and discussion

All the analysis was performed in triplicate. Results were expressed as means ± standard deviation. Descriptive statistical analysis, Pearson correlation coefficients, one-way analysis of variance (ANOVA) were performed using SPSS. Extract and juice of three variety of pomegranate showed scavenging effects against DPPH radical. The hierarchy for antioxidant capacity with respect to their EC₅₀ values was peel aqueous extract > aqueous juice. Correlation coefficient showed that total Phenolic

content was responsible for antiradical efficiency in peel extracts. The antioxidant and total Phenolic content levels are also positively and significantly correlated our results strongly suggested peel extracts and juice of pomgranate can be promising

sources of potential antioxidants. The peel extracts of this plant had not significantly difference in ferrous reducing antioxidant power (FRAP) relative to aqueous juice.

Table 1. Comparing (Mean±sd) ,cholesterol and LDLcholesterol in before and after treatment.

Variety of pomegranate	Ch(Mean±sd)before consumption	Ch(Mean±sd)after consumption	LDL(Mean±sd)before consumption	LDL(Mean±sd)after consumption
SBL* pom juice	247.00± 10.758	243.75± 10.758	158.25±10.758	130.50±10.758
Malas pom juice	263.58±10.759	225.75±10.759	150.50±10.758	130.92±10.759
levustatine	265.25±10.758	208.42±10.759	138.33±13.64	111.58±13.30

Table 2. total : Total phenolic ,flavonoids contents and antioxidant activities of juice and aqueous extract of pomegranate.

Variety:wild (soar shomal)	Phenolic content(mg GAL/g)	Flavonoids content((QE)/g)	Antioxidant activity By DPPH (Inhibition%)	Antioxidant (FRAP)
juice	92.2±0.04	36.7 ± 0.05	38.3±0.15	98.02 ± 0.5319
peel extract	330.2±0.04	110.2±0.03	69.12 ±0.15	171.08 ± 0.53

Each value in the table was obtained by calculating average of three experiments a standard deviation.

Table 3. Total phenolic ,flavonoids contents and antioxidant activities of methanol and aqueous extract of Stachys inflata.

Variety:malas pomegranate	Phenolic content(mg GAL/g)	Flavonoids content((QE)/g)	Antioxidant activity By DPPH (Inhibition%)	Antioxidant (FRAP - mmol/l)
juice	79.2±0.04	36.7 ± 0.05	48.3±0.15	109 ± 0.9
Peel extract	271.4±15.2	83.2±3.8	77 ±0.35	141.08 ± 8.53

Each value in the table was obtained by calculating average of three experiments a standard deviation.

Table 4. Total phenolic ,flavonoids contents and antioxidant activities of methanol and aqueous extract of Stachys inflata.

Variety:saveh black	Phenolic content(mg GAL/g)	Flavonoids content((QE)/g)	Antioxidant activity By DPPH (Inhibition%)	Antioxidant (FRAP:mmol/l)
juice	40.2±0.1	16.4 ± 2.5	32±0.52	7.2 ± 9.25
Peel extract	283.2±14.2	69.2±4.2	75±2.15	161± 8.53

Each value in the table was obtained by calculating average of three experiments a standard deviation.

The statistical examination of the data was performed using the SPSS version 11.5 program. Mean values of antioxidant activity in different cultivars were compared by using analysis of the variance (ANOVA) test. When significant ($p < 0.05$)

difference was detected, The results showed that pomegranate juice of malas vareity had markedly higher antioxidant capacity than the other. In the present study, the FRAP value of juice of three cultivars of pomegranate was determined in an

attempt to make a systematic comparison among their antioxidant activities. Further studies on the effective antioxidants contained in these pomegranate juice and the mechanisms by which they protect against disease development are highly recommended. The results showed that the contents of total phenolics, flavonoids were higher in peel extract of wild soar variety than in malas pomegranate extract. The large amount of phenolics contained in peel extract may cause its strong antioxidant ability.

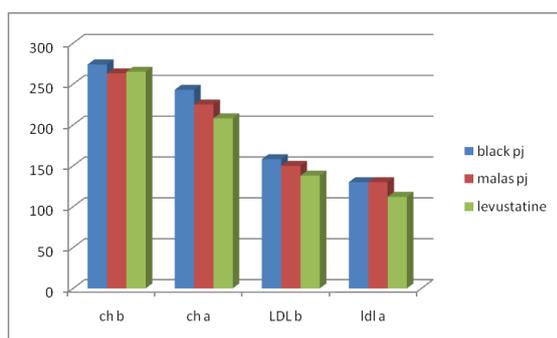


Fig. 1. Comparing (Mean±sd) , LDLcholesterol in before and after treatment.

Administration of (black and malas) PJ to two groups (12 patients in each group) 200 ml/day for 4 week, had significant effect on the plasma lipid, including total cholesterol and LDL-cholesterol (Fig 1) .LDL cholesterol of two groups were measured before and after consumption of treatments. Means of before and after was compared in each group. Black and malas pomegranate juice consumption decreased LDL cholesterol ($P < 0.01$). On statistical analysis, serum LDL after pj consumption by patients was comparatively lesser than before pj using (figure 1). Administration of levustatine (cholesterol lowering drug by enzyme inhibition) to another group, had is significant effect on serum LDL cholesterol. The levels of blood total cholesterol and LDL C in different experiments, before (b) and after (a) consumption of treatments were achieved (Table 1).

Results were analyzed by compare means statistic test. The data indicated that LDL .analysis of results were revealed that patients ' LDL C in three groups, after consumption showed a significant decrease at

$p < 0.01$.the consumption of group one and two were compared with group three, there was no difference between group one and two with group three. As with the drug, the two groups were effective on LDL.C decreasing.

Atherosclerosis is a multi-factorial disease associated with different risk factors. Hypercholesterolemia is a major risk factor for atherosclerosis (Dominiczak, 1998; Sniderman *et al.*, 1980) and reduction in plasma cholesterol concentration by drug therapy has reduced cardiovascular incidence (Gotto *et al.*, 1999). Consumption of natural nutrients, capable of reducing plasma cholesterol, thus, should also reduce development of atherosclerosis. Our study demonstrated that dietary consumption of pomegranate juice by patients with Hypercholesterolemia significantly reduced the levels of plasma cholesterol and LDL cholesterol. The LDL cholesterol lowering effect of pomegranate juice could have possibly resulted, at least in part, from its ant oxidative effects. The anti-atherogenic of pomegranate juice could also be attributed to its direct ant oxidative effects on macrophages as well as on plasma LDL. Arterial wall macrophages play a major role during early parthenogenesis. Oxidative stress induces macrophage responses such as increased capacity to oxidize LDL, increased Ox-LDL cellular uptake, as well as macrophage lipid peroxidationin (Fuhrman *et al.*, 1995). These lipids-per oxidized cells were shown to oxidize LDL even in the absence of transition metal ions, and this process depends on the oxidative state of the LDL and that of the macrophage (Aviram *et al.*, 1988). Pomegranate juice was shown in this study to decrease plasma LDL cholesterol. The LDL oxidation hypothesis of atherosclerosis development suggests that inhibition of LDL oxidation should result in the attenuation of the development of atherosclerotic lesions. We have demonstrated indeed that the reduced development of atherosclerotic lesions in patients that consumed pomegranate juice was associated with reduced LDL oxidative state. This may be related to the fact that

pomegranate juice can act as a free radical scavenger.

We conclude that consumption of pomegranate juice may be proven beneficial in attenuation of atherosclerosis development, since it is associated with reduced oxidation of LDL, reduced uptake of oxidized LDL by macrophages, reduced oxidative state of LDL and reduced LDL aggregation. All these effects lead to a reduced cellular cholesterol accumulation and foam cell formation, the hallmark of early atherosclerosis. Investigations of aviram, furham, esmailzadeh and Rosenblatt compatible with our study. We concluded That shomal soar pomegranate peel extract appeared to have more potential as than malas variety extracts. Moreover ,we studied effect of two wariety pj on plasma cholesterol and LDL of hypercholesrolemia patiens. The finding showed those patients ' total cholesterol and LDL.C in three groups, after consumption showed a significant decrease at $p < 0.01$ the levels of blood total cholesterol and LDL c .

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