Evaluation of antioxidant and antiradical properties of Pomegranate (*Punica granatum* L.) seed and defatted seed extracts

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Abstract Pomegranate seeds are byproducts of the Pomegranate juice industries that contains functional compounds such as phenols. This study was aimed to evaluate the effect of solvents on extraction from Pomegranate seed and Pomegranate defatted seed and to measure the yield extract and phenolic content and antioxidant properties. For this purpose, the seeds and defatted seeds were directly isolated from fruits and seeds by cold pressing respectively, then were crushed and extracted with different solvents, including water, Methanol, Acetone, Ethyl acetate and Hexane and finally the extracts of them were evaluated. Phenolic compounds, ferric reducing-antioxidant power and radicals scavenging property of extracts were measured. The results showed the highest extraction efficiencies were for Hexane and Acetone solvents in extraction of seed and defatted seed respectively. The highest phenolic content was obtained from Methanol seed extract. Reducing activity test proved that the Methanol extracts of Pomegranate seed and Pomegranate defatted seed had the highest reducing strength. Results of radical scavenging activity were similar to reducing activity results. The order of antioxidant capacity of Pomegranate seed and Pomegranate defatted seed were found to be Methanol > Water > Acetone > Butanol > Ethyl acetate > Hexane. It can be concluded Pomegranate seed, which possesses high levels of polyphenols, can be one of the sources of the natural antioxidants. The Methanol extract had a higher antioxidant efficiency than seed and defatted seed extracts.

Keywords Pomegranate seed · Antioxidant · Radical scavenging activity · Reducing power · Oxidative stability

Introduction

Pomegranate (*Punica granatum* L.) is an important fruit of tropical and subtropical regions, which originated in the Middle East and India and has been used for centuries in ancient cultures for its medicinal purposes (Longtin 2003).

Pomegranate is consumed fresh and in processed form as juice, jelly, flavore and extract. It is widely reported that Pomegranate exhibits antiviral, antioxidant and anticancer activities (Faria et al. 2006; Faria et al. 2007; Adhami and Mukhtar 2006).

Over the past few years scientific investigations have laid some credible information for utilization of different parts of Pomegranate fruit (Aslam et al. 2006; Singh et al. 2002). Pomegranate seeds are byproducts of the Pomegranate juice industries. Pomegranate seed has powerful antioxidants, anti-inflammatory compounds, vitamin E, sterols, phenols and high quantities of valuable fatty acids such as punicic acid and natural estrogens (estradiol, estrone and estradiol) (Fadavi et al. 2006; Lansky and Newman 2007). Antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell 1995). Many studies have shown the antioxidants contained in fruits and vegetables, including ascorbic acid, carotenoids, flavonoids, hydrolysable tannins, play an important role in the prevention of several diseases (Huxley and Neil 2003; Knekt et al. 2002; Lampe 1999). In Pomegranate fruit, antioxidant properties may be related to diverse phenolic compounds present in different parts of Pomegranate, including punicalagin isomers, ellagic acid derivatives and anthocyanins (delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides). These compounds are known for their properties in scavenging free radicals and inhibiting lipid oxidation in vitro (Noda et al. 2002). The Pomegranate antioxidant activity is typically

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higher in commercial juices extracted from whole Pomegranates than in experimental juices obtained from the arils only (Gil et al. 2000). Recently, natural antioxidants have become very popular for medical and food applications and are preferred by consumers than synthesized antioxidants, such as BHA and BHT (Lapornik et al. 2005; Landbo and Meyer 2001; Spigno and De Faveri 2007). Extraction is the first step in the commercial isolation of these antioxidant compounds from fruits. Because of the presence of large amounts of certain pharmaceutical, nutraceutical and antioxidant components in different parts of fruits such as Pomegranate, type of solvent to obtain these components is highly demanded in the food industry.

Accordingly, the objective of this study was to evaluate the effect of solvents with different polarity on extraction from Pomegranate seed (PS) and Pomegranate defatted seed (PDS) by measuring yield extract, phenolic content, in-vitro antioxidant activity, and ability to retard oxidation in a food oil model.

Materials and methods

Materials

Ripened Pomegranates (*Punica granatum* L. var. Malas Sabzevar) were collected from trees growing in a Pomegranate garden at Agricultural Research Station of Sabzevar, Khorasan province in Iran. The mean weight of each fruit was 250 g and the taste of fruit juice was sour-sweet. The Pomegranate seeds (PS) were directly isolated from fruits by pressing them with soft cotton and then washed to remove any adhering Pomegranate flesh and finally oven-dried at 25 °C for 24 h. The Pomegranate Defatted Seeds (PDS) were produced from dried Pomegranate seeds after removing their oil by cold press. Then they were stored at −18 °C for subsequent tests.

Refined and antioxidant free Soybean oil was purchased from Shadgol edible oil factory (Neyshabor- Iran).

All chemicals and solvents were analytical reagent grade and were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO).

Moisture content

The moisture content of PS and PDS were calculated using AOAC method No. 984.25 (AOAC 2005).

Fat content

The Soxhlet method as described by AOAC method No. 963.15 was used to extract the oil with petroleum ether (40–60 °C) for 8 h (AOAC 2005).

Acid value (AV)

The AV was determined according to AOCS official method cd 3d-63 (AOCS 1993).

Peroxide value (PV)

The PV was measured with the spectrophotometric method of the International Dairy Federation (thiocyanate method) (Shantha and Decker 1994).

Oxidative stability index (OSI)

The OSI was determined by a Rancimat instrument (Model 743; Metrohm Ltd., Herisau, Switzerland). Oxidation was carried out with 3 g oil sample at temperature of 110 °C at an air flowrate of 15 l/h (Farhoosh 2007).

Total phenols (TP) content

The determination of TP content was done spectrophotometrically using Folin-Ciocalteu's reagent as described by Capannesi et al. (2000). A calibration curve of Gallic acid in Methanol was performed in a concentration range of 0.04–0.40 mg/ml.

Preparation of Pomegranate seed extracts

The clean and dry PS (17.33 % oil) and PDS (2.02 % oil), were crushed to a particle size of less than 0.5 mm. The seeds powder were mixed with solvent at a ratio of 1:10 (w/v). The solvents, were Water, Methanol, Acetone, Ethyl acetate and Hexane. After 24 h of shaking of seeds at room temperature in solvent, liquid was separated from the solid using vacuum filtration through a Whatman No.1 filter paper. The residues were re-extracted by the same solvent. All extracts were concentrated under vacuum at 60 °C, and the concentrates were dried, desiccated and stored at −18 °C for subsequent analysis. Yield extract was calculated using following equation (seed extraction with solvents were done in 3 replications):

$$\text{Extraction yield(\%)} = \frac{(\text{weight of dried extract} \times 100)}{(\text{initial weight of seed powder})}$$

Evaluation of antioxidant properties of Pomegranate seed extracts

Several methods have been developed to measure the antioxidant capacity of pure compounds and plant extracts. One of the most frequently used techniques for measuring antiradical capacity is depletion of the free radicals 2,2-

diphenyl-1-picrylhydrazyl (DPPH) by addition of scavenger compounds. It determines the ability of crude extracts for trapping this unpaired-electron to disappearance of radical color. This will lead to the formation of less reactive species derived from the antioxidant (Brand-Williams et al. 1995). There are many assays which have been used to assess the total antioxidant content of foods, e.g. the ferric-reducing ability of plasma (FRAP). The FRAP assay directly measures antioxidants with a reduction potential below the reduction potential of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple (Halvorsen et al. 2002). Therefore, the antioxidant properties of extracts were determined using two methods, DPPH and FRAP. These methods seem to be rapid and accurate methods for assessing the antioxidant activity of Pomegranate seed extracts.

DPPH radicals scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity of Pomegranate seed extracts was determined according to the method described by Singh et al. (2002) with slight modifications. In brief, 0.1 ml Pomegranate seed extract at various concentrations were respectively added to 0.49 ml of Methanol and 0.39 ml of DPPH Methanolic solution (4 mg/100 ml). Then, the mixtures were vortexed vigorously and allowed to stand in the dark for 60 min. Finally, the absorbance of these mixtures was measured by using a spectrophotometer at 512 nm. The sample concentration providing 50 % of radical scavenging activity (EC_{50}) was obtained through interpolation of linear regression analysis. The lower EC_{50} indicates higher radical scavenging activity.

FRAP assay

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as a novel method for assessing “antioxidant power.” Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. The ferric reducing-antioxidant power test was conducted according to Benzie and Strain (1996). Acetate buffer (0.3 M, pH 3.6) was prepared by dissolving 3.1 g $\text{C}_2\text{H}_3\text{O}_2\text{Na}\cdot 3\text{H}_2\text{O}$ and 16 ml of acetic acid in 1 l of distilled water. TPTZ (2,4,6-tripyridyl-S-triazine) solution was prepared by dissolving 23.4 mg of TPTZ in 7.5 ml of 40 mM HCl solution. Ferric solution (20 mM) was prepared using $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$. The final working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ and ferric solutions at a ratio of 10:1:1, and was warmed to 37 °C. Aliquots (200 μl) of each extract were mixed with 1.8 ml of FRAP reagent and the absorbance of the reaction mixture

was measured at 593 nm after incubation at 37 °C for 10 min (Faithong and Benjakul 2012). Aqueous standard solutions of $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ (0–1,000 $\mu\text{mol/l}$) were used for the calibration curve.

Antioxidant efficiency of seed extract

The same amounts (2 % W/W) of the different Pomegranate seed extracts was added to purified and antioxidant free Soybean oil and then was exposed to the thermal resistance (oxidative stability) test was explained in 2.6 section.

Purification and preparation of the soybean oil

To evaluate antioxidant efficiency of seed extract in model system oil, it is necessary to remove natural antioxidants oil. For this purpose, 200 g of commercial refined Soybean oil without additive synthetic antioxidants, was passed through aluminum oxid 60 (100 g, active, neutral), which had been activated at 200 °C for 3 h immediately before use. The alumina column (25-cm $\times$ 2.5 cm id) and collection vessels were wrapped in aluminum foil, and the oil was drawn through the column by suction without solvent. This procedure was repeated twice to ensure complete elimination of probable antioxidants in soybean oil.

Statistical analysis

Data was reported as mean $\pm$ standard deviation. Each data is mean of 3 replication in chemical analysis of mixed sample of 3 extractions. Analysis of variance (ANOVA) accompanied with Duncan test were conducted to identify the significant difference between samples ($p < 0.01$).

Results and discussion

The moisture and fat contents of PS and some of the characteristics of Pomegranate seed oil (PSO) were presented in Table 1.

Comparison of PS and PDS extraction efficiency

Table 2 depicts the yield of each extract from PS and PDS. Hexane extraction of PS and Acetone extraction of PDS gave maximum yield, whereas water extract gave minimum yield. The PS has considerable amounts of oil, so the highest extract yield belonged to Hexane solvent, after PS oil extraction, the highest extraction yield resulted by Acetone solvent. The polarity of Acetone is intermediate, so it ables solve both polar (phenolic compounds and so on) and in polar (oil and its byproducts) compounds.

Table 1 Chemical properties of Pomegranate seed (PS) and Pomegranate seed oil (PSO)

Measured parameters	Value
PS	
Moisture Content (%)	3.75±0.200 ¹
Fat content (% dwb) ²	17.33±1.330
PSO	
Peroxide value (meq g O ₂ /Kg oil) ³	0.79±0.190
Oxidative stability index (h)	3.03±0.150
Free fatty acid (mg NaOH/g oil)	0.56±0.010

¹ Mean ± SD (n=3)² Dry weight base³ Mili equivalent gram of O₂ in each Kg of oil

Total phenolic content

The total phenolic contents (TPC) of PS extracts were measured (Table 3). In recent studies, it has been reported that the yield of extractable phenolic compounds were the highest in Methanol extract from Pomegranates and Citrus in comparison with solvents such as Ethyl acetate and water (Haminiuk et al. 2012; Jayaprakasha and Patil 2007; Singh et al. 2002; Wang et al. 2011). Furthermore, extraction of phenolic compounds from the fruits is commonly achieved with Methanol or aqueous Methanol (Sultana et al. 2009; Ghasemzadeh et al. 2011). The result of mean comparison (Table 3) shows that Methanol extract of Pomegranate seed was significantly higher than other solvents, that is in agreement with literatures.

Singh et al. (2002) extracted antioxidant-rich fractions from Pomegranate (*Punica granatum*) peels and seeds using Ethyl acetate, Methanol, and Water. They reported that Methanol extract of Pomegranate peel had the highest antioxidant activity among all of the extracts.

Razali et al. (2012) studied the effect of solvents, with different polarities, on the extraction of antioxidant phenolics from the leaves, seeds, veins and skins of *Tamarindus*

Table 3 Total phenolic compounds in Pomegranate seed

Solvent type	Phenolic content (mg/l seed extract)
Water	22.61±0.391 [#] b*
Methanol	27.93±0.301 a
Acetone	3.41±0.043 c
Butanol	0.57±0.002 d
Ethyl acetate	0.37±0.010 d
Hexane	0.29±0.016 d

*Different lower case superscripts in the column indicate significant difference ($P<0.01$)[#] Mean ± SD (n=3)

indica L. They found that the efficiencies of the solvents for extraction of the antioxidant phenolics were in the order: Methanol > Ethyl acetate > Hexane.

According to Table 3, results showed that efficiencies of the solvents for extraction of the phenolic compounds were in the order: Methanol > Water > Acetone > Butanol > Ethyl acetate > Hexane, which confirmed by Razali's and Singh's findings.

It has been observed that the phenol antioxidant index is a combined measurement of the quality and quantity of antioxidants in fruits and vegetables (Vinson et al. 1998). In the present study the responses of the extracts in this assay may be ascribed to the quantity of phenolics found in six extracts from PS.

However, Methanol extract of PS gave highest phenolics and Hexane extract showed the minimum of phenolics. These differences in TPC might be due to the difference between the extraction methods; in fact, phenolic contents of PS extracts are expected to strongly depend on extraction conditions as well as the type of solvent used (Maheshu et al. 2012; Yasoubi et al. 2007). The solubility phenolic compounds are depends on polarity of solvents. They are solved well in polar solvents such as water and Methanol and aren't extracted with nonpolar solvents such as Hexane. Therefore

Table 2 Extract yield, the value of half maximal effective concentration of antioxidant (EC₅₀) and Ferric reduction power of different solvent extracts of Pomegranate seed (PS) and Pomegranate defatted seed (PDS)

Solvent extract	Extract yield (%)		EC ₅₀ (mg/ml)		Ferric reduction power (μMol Fe ²⁺ /l)	
	PS	PDS	PS	PDS	PS	PDS
Water	5.0±0.07 [#] e*	4.5±0.06 F*	0.30±0.015 e	0.32±0.022 D	217.3±0.21 b	207.6±0.23 B
Methanol	6.1±0.06 d	6.3±0.05 E	0.15±0.003 f	0.19±0.006 E	557.0±0.37 a	721.8±0.35 A
Acetone	14.9±0.09 c	10.1±0.11 A	0.34±0.002 d	0.24±0.008 E	40.7±0.29 cd	155.1±0.21 C
Butanol	17.0±0.11 b	9.0±0.08 B	1.77±0.010 c	1.69±0.008 C	59.3±0.27 c	60.5±0.11 D
Ethyl acetate	15.0±0.08 c	8.6±0.09 C	2.01±0.055 b	1.83±0.015 B	22.2±0.19 d	35.1±0.18 DE
Hexane	17.9±0.09 a	7.9±0.06 D	4.23±0.067 a	3.88±0.081 A	6.4±0.11 d	9.9±0.17 E

*Different lower case superscripts in the column of PS and upper case superscripts in the column of PDS indicate significant difference ($P<0.01$)[#] Mean±SD (n=3)

the extracted oil of PS with Hexane solvent, contains a little phenolic compounds (0.29 mg/kg) and the most of phenolic compounds remain in seed. So PDS is the valuable source of phenols. The phenols are secondary plant metabolites with beneficial biology effects, e.g. anti-bacterial, anti-inflammatory and anti-allergic agents (Koshihara et al. 1983). The most important their effects is their documented action as potent antioxidant (Formica and Regelson 1995; Rice-Evans et al. 1996). Hence, it is common practice to measure both phenolic content and antioxidant activities when investigating the antioxidant potential of plants as various studies have shown that plants rich in phenolics are also potent antioxidants (Maisuthisakul et al. 2007; Razali et al. 2008).

Radical-scavenging activity (DPPH assay)

Antiradical activities (free radical-scavenging) of extracts of PS and PDS were determined using the DPPH method, which are presented in Table 2. The results suggest that highest antiradical potential was observed in Methanol extracts of PS and PDS, on a μg analyte basis ($\text{EC}_{50}(\text{PS})=0.14$ and $\text{EC}_{50}(\text{PDS})=0.19 \mu\text{g/g}$). A lower value of EC_{50} indicates a higher antioxidant activity. It was reported by Katalinic et al. (2010) that antioxidants can deactivate or scavenge stable free DPPH radicals by two major mechanisms: by reduction *via* electron transfer or by hydrogen atom transfer that may occur also in parallel and steric accessibility is one of the major determinants of the reaction. Differences for DPPH (EC_{50}) between the extracts of PS could be probably due to differences in polyphenolic contents of analysed extracts.

Recently, Amarowicz et al. (2000) reported that the tannins extracted from canola and rapeseed hulls exhibited a high scavenging efficiency toward DPPH radicals. Such a radical-scavenging activity seed extracts would be related to substitution of hydroxyl groups in the aromatic rings of phenolics, thus contributing to their hydrogen donating ability (Brand-Williams et al. 1995).

Ferric reducing/antioxidant power (FRAP assay)

Antioxidants can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of the other. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. Antioxidant potential of the PS and PDS extracts were estimated from their ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex. The PS and PDS Methanol extracts showed highest FRAP antioxidant activity in all extracts of PS and PDS (Table 2). Increasing concentration of extractable total phenolics exhibited relative efficiency of FRAP activity. Similar results have also been

reported in Guava fruit extracts (Jiménez-Escrig et al. 2001). Yen and Duh 1993 and Siddhuraju and Becker 2003 have reported that the reducing power of bioactive compounds (mainly low and high molecular phenolics) extracted from Peanut hulls and stem bark of Indian laburnum, respectively, were associated with antioxidant activities.

Oxidative stability extracts

In this study, the effects of addition of PS extracts on oxidative stability index (OSI) of antioxidant free Soybean oil, were evaluated. The results showed addition Methanol extract to antioxidant free soybean oil (blank sample) had the highest OSI (6.2 h) (Table 4). The water extract contained polar antioxidant component but it is insoluble in oil, so the OSI of mixture antioxidant free Soybean oil and water extract was lower than OSI of antioxidant free Soybean oil (blank). Addition of the extracts with less polarity such as Acetone, Butanol, Ethyl acetate and Hexane to oil caused to decrease OSI of antioxidant free Soybean oil. The extracts had some PSO. The PSO is an unstable oil (80 % Punicic acid, $\text{C}_{18:3}$). Therefore addition PSO to antioxidant free Soybean oil facilitated oil oxidation and led to product with short shelf life.

Conclusion

The Pomegranate seed is a potential source for the extraction of phenolic compounds. Phenols are the main compounds with high antioxidant properties in Pomegranate seed that remain in the seed after oil extraction. It is concluded Pomegranate seed functionally and nutritionally has a wide application in food, pharmaceutical and cosmetic industries. The Methanol extracts of Pomegranate seed had the highest

Table 4 Oxidative stability of antioxidant free soybean oil accompanied by 2 % (W/W) different seed extracts

Sample	Oxidative stability (h)
Antioxidant free Soybean oil (blank)	4.4±0.01 ^{# c*}
blank + Water extract	3.8±0.02 d
blank + Methanol extract	6.2±0.02 a
blank + Butanol extract	3.7±0.01 d
blank + Acetone extract	3.4±0.06 e
blank + Ethyl acetate extract	3.1±0.03 f
blank + Hexane extract	2.8±0.03 g
blank + BHT**	5.1±0.05 b

*Different lower case superscripts in the column indicate significant difference ($P<0.01$)

[#] Mean ± SD ($n=3$)

**BHT butylated hydroxytoluene, used as a food additive with antioxidant properties in oils/oil products

antioxidant and antiradical activity, these contents being significantly correlated ($p < 0.01$). Our results also confirm that addition of Pomegranate seed Methanol extract to antioxidant free oil led to increase of thermal resistance (oxidative stability). The seed water extract was the second level of important and it is economically viable.

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