Antioxidant Activity of Pericarp Extract from Different Varieties of Pomegranate Fruit

S. Jalili¹, A. Tabatabee Naini², M. Ashrafi¹, and M. Aminlarⁱ

ABSTRACT

The pomegranate *Punica granatum* fruit pericarp, contain polyphenolic compounds including alpha and beta punicalagins and ellagic acid, which exhibit remarkable antioxidant activities. The aim of this study was to purify and quantify the phenolic components from different varieties of Pomegranate Pericarp Extracts (PPEs) and determine their antioxidant properties. Methanolic and aqueous extracts of four pomegranate cultivars (Shahvar, Siahsorfeh, Torshsabz and Abdorahimkhany, from Shiraz, Iran) were prepared and total phenolic content of PPEs was determined. PPE components were further purified by XAD-16 column chromatography followed by LH-20 gel filtration. The eluted components were subjected to HPLC analysis to differentiate and quantify polyphenolic compounds. Antioxidant activity was measured using DPPH assay. The result revealed significant difference in total phenolic contents and phenolic components in four cultivars. Total phenolic content in methanolic PPE was significantly (P< 0.05) higher than aqueous extracts. Shahvar cultivar had the highest total phenols (11.72±0.01 mg mL⁻¹ in water, 17.7±0.12 mg mL⁻¹ in methanol) and exhibited the most antioxidant property among cultivars. Analysis of components of Shahvar PPE by HPLC showed that proportions of different phenolic components were alpha punicalagin (28.34±2.12%), beta punicalagin (39.75±2.14%) and ellagic acid (3.49±0.93%).

INTRODUCTION

Pomegranate has been used as a folk medicine since ancient times and it is a symbol of life (Longtin, 2003), permanence, wellbeing, femaleness, fertility, knowledge, immortality, and holiness. Pomegranate is native to Iran and the surrounding countries (Levin, 1994; Seeram *et al.*, 2005b; Celik *et al.*, 2009; Viuda-Martos *et al.*, 2010). Many researchers have focused on the biological waste part of this wonder fruit, pomegranate, in order to discover the medical effects on human health. The potential therapeutic properties of pomegranate peel are extensive, including treatment and prevention of cancer (Dikmen *et al.*, 2011; Hong *et al.*, 2008), cardiovascular diseases (Basu and Penugonda, 2009; Jurenka, 2008), diabetes (Banihani *et al.*, 2013; Middha *et al.*, 2012), dental conditions (Viuda-Martos *et al.*, 2010), erectile dysfunction (Kanatt *et al.*, 2010), protection from UltraViolet (UV) radiation (Kanatt *et al.*, 2010) and antimicrobial activity (Dahham *et al.*, 2010; Ferreira, 2007; Hayouni *et al.*, 2011). Other potential applications include prevention of infant brain ischemia, Alzheimer’s disease (Kwak *et al.*, 2005; Middha *et al.*, 2012),

1 Department of Biochemistry, Shiraz University, Shiraz, Islamic Republic of Iran.

2 Department of Surgery, School of Veterinary Medicine, Shiraz University, Shiraz, Islamic Republic of Iran.

*Corresponding author; e-mail: aminlari@shirazu.ac.ir
Figure 1. The chemical structure of punicalagin (Kraszni et al., 2013).
However, the antioxidant activity of purified phenolic compounds of PPE from different varieties and the relative contribution of each phenol component have not been compared yet. This communication reports a comparative study on the antioxidant activity of pericarp components from four cultivars of pomegranate.

**MATERIALS AND METHODS**

Punicalagin and ellagic acid (as a standard), formic and acetic acids, Folin-Ciocalteu reagent, DiPhenyl-2-PicrylHydrazyl (DPPH), Sephadex Lipophilic LH-20 and Amberlite XAD-16 resins, tryptic were purchased from Sigma Aldrich. All solvents were HPLC grade.

**Preparation of Pericarp Extract**

Fruits of four pomegranate cultivars (Shahvar, Abdorahimkhany, Siahsorfeh, and Torshsabz), crop of 2014, were purchased from local markets in Shiraz, Iran. After removing the thin outer layer, their pericarps were manually separated from the aril and used for extraction. To prepare samples, 20 g fresh pericarps were soaked in 60 mL methanol or water and placed in shaker at 150 rpm for 4 hours. Sample were left in dark at room temperature for 12 hours, homogenized using a laboratory homogenizer and centrifuged at 9,000 rpm for 15 minutes. Supernatant were labeled as PPE and used for further studies (Basiri et al., 2015).

**Analytical Studies**

Total phenol content of extracts was determined using Folin-Ciocalteu reagent and gallic acid as standard (Sadasivam and Manickam, 1996). Approximately 40 μL of PPEs were mixed with 1.25 mL of 20% aqueous sodium carbonate solution. After 3 minutes, 250 μL Folin-Ciocalteau’s phenol reagent was added to the mixture. After 30 minutes of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of the calibration curve of gallic acid. A linear calibration curve was obtained in the range of 20-140 μg mL⁻¹ gallic acid in methanol, with regression equation y = 0.0712x+0.0106 and linear regression coefficient r² = 0.999. This assay was performed in triplicate and the results were reported as mean±standard deviation. Antioxidant activity of water and methanol PPEs was evaluated by 1,1-DiPhenyl-2-PicrylHydrazyl (DPPH) assay (Kulkarni et al., 2004). Different concentrations of each PPE (0-15 μg mL⁻¹) were added to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 515 nm.

The results were recorded as A₅₁₅₅₅ nm vs phenolic content and percentage of Radical Scavenging Activity (RSA%) was calculated based on the following equation:

\[
\text{DPPH Scavenging activity (％)} = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

Where, A blank and A sample are Absorbance of the control (containing all reagents except the extract) and extract, respectively. Tert-ButylHydroQuinone (TBHQ), a synthetic phenolic antioxidant, was used as positive control (0-15 μg mL⁻¹).

**Purification of Polyphenols**

Purification of Total Pomegranate Tannin (TPT) from PPEs was performed by the method of Seeram et al. (2005b) with minor modifications. PPEs were dried using a drying oven at 50°C. The resultant semi-solid residues were re-dissolved in 20 mL water and loaded onto a 3x40 cm chromatography column packed with Amberlite XAD-16 resins (a hydrophobic polyaromatic resin normally used for removing hydrophobic compounds up to 40,000 MW). The column was washed with 2 L water. Ellagitannins were recovered.
from the column using 400 mL methanol and were dried on rotary evaporator. This fraction was labeled TPT. One hundred mg TPT powder was dissolved in Sephadex lipophilic LH-20 gel filtration resin that was pre-equilibrated with H2O and eluted with stepwise (0, 20, 40, 60, 80, 100%) methanol to give six fractions. Each fraction was analyzed by HPLC.

Before injection, samples were centrifuged (4 min at 5000 rpm) and the supernatant was filtered through a 0.4 µm filter. A Knauer reversed phase HPLC system (Germany) with a 4.6×250 mm C18 separation column at 20°C was used. The HPLC method was performed as described by Hayouni et al. (2011) with minor modifications, in which two solvents were used as mobile phase including solvent A consisting of 0.1% formic acid in water and solvent B consisting of 100% methanol (Hayouni et al., 2011). The gradient was initially 1% B in A for 5 minutes followed by 1 to 20% B in A for 15 minutes, 20 to 40% B in A for 10 minutes, 40% for 5 minutes, 40 to 95% B in A for 5 minutes, and finally, 15% B in A for the next 15 minutes. Absorption was detected at 254 nm.

Ellagitannin (punicaladin and ellagic acid) rich fraction was evaporated and re-chromatographed, this time with stepwise increasing (0, 20, 40, 60, 80, 100%) concentration of ethanol (six fractions). The punicalagin-rich fraction was filtered through 0.45-µm filter and 100 µL was injected on the HPLC column for separation of punicalagin. Pure alpha and beta punicalagins were collected manually. To detect alpha and beta punicalagin and ellagic acid, their standard samples were used as external standard and then area percentage was calculated.

**Statistical Analysis**

Triplicate experiments were performed and means were compared using SPSS statistical software (SPSS version 10.0.0; SPSS Inc., Chicago, IL). One-way Analysis Of Variance (ANOVA) and Tukey’s post hoc tests were carried out to compare means. P-values less than 0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

Table 1 presents total phenolic contents in pericarp of four pomegranate cultivars. Shahvar cultivar had the highest (0.74±0.005 mg GAE mg–1 pericarp) and Abdorahimkhany cultivar the lowest (0.23±0.040 mg GAE mg–1 pericarp) phenolic content in methanolic extracts. The aqueous extract of Shahvar contained the highest (0.49±0.004 mg GAE mg–1 pericarp) and Siahsorfeh the lowest (0.30±0.501 mg GAE mg–1 pericarp) phenolic content. The aqueous extract of Shahvar contained the highest (0.49±0.004 mg GAE mg–1 pericarp) and Siahsorfeh the lowest (0.30±0.501 mg GAE mg–1 pericarp) phenolic content.

Interestingly, Table 1 shows that the water extract of Abdorahimkhany had a higher phenolic content than methanolic extract. The reason for this phenomenon is not known at present. It can be related to the

<table>
<thead>
<tr>
<th>Pomegranate cultivars</th>
<th>Methanolic extract (mg GAE mg–1 pericarp)</th>
<th>Aqueous extract (mg GAE mg–1 pericarp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shahvar</td>
<td>0.74±0.005</td>
<td>0.49±0.004</td>
</tr>
<tr>
<td>Siahsorfeh</td>
<td>0.37±0.016</td>
<td>0.25±0.024</td>
</tr>
<tr>
<td>Torshsaz</td>
<td>0.48±0.019</td>
<td>0.35±0.021</td>
</tr>
<tr>
<td>Abdorahimkhany</td>
<td>0.23±0.040</td>
<td>0.30±0.501</td>
</tr>
</tbody>
</table>

* Values are mean±SD.
difference in chemical and structural properties of each cultivar, which probably facilitated more extraction in water.

The results indicated that there were significant differences in total phenolic contents among the tested cultivars. It has been recognized that phenolic compounds show antioxidant activity and their effects on human nutrition and health are considerable (Kalaycıoğlu and Erim, 2017). Phenolic compounds are a class of antioxidant agents that act as free radical terminators (Shahidi et al., 1992; Rahmanian et al., 2015; Kalaycıoğlu and Erim, 2017). Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS (Kim et al., 2015). Antioxidants through their scavenging power are useful for the management of those diseases (Taghvaei and Jafari, 2015). It has been reported that the peel extract of pomegranate has the highest antioxidant activity amongst extracts from eight types of fruits (Okonogi et al., 2007). Few studies have been conducted on pomegranate pericarp. A study illustrated that components of pomegranate pericarps have antioxidant activity (Zhou et al., 2014). The antioxidant activity of PPEs from different cultivars is illustrated in Figure 2, which shows the decrease in absorbance of DPPH reagent due to the free radical scavenging ability of the PPEs. Four pomegranate pericarp cultivars (methanolic and aqueous extracts) showed different antioxidant activities. The result of the present study showed that the PPE of Shahvar cultivar, which contained the highest amount of phenolic compounds, exhibited the greatest antioxidant activity in aqueous and methanolic extracts.

![Figure 2. DPPH scavenging activity of methanolic (A) and aqueous (B) extracts of pericarp from four pomegranate cultivars.](image-url)
approaching those of TBHQ. The PPE of Siahsorfeh cultivar had the lowest antioxidant activity in both extracts (Figure 2). Previous studies also showed that the antioxidant activities of different pomegranate peels are different (Hajimahmood, et al., 2008; Ozgen et al., 2008). Ardekani et al. (2011) examined antioxidant activity of nine different cultivars of Iranian pomegranate peels and pulp. They found that the highest antioxidant activity and phenolic contents were in Sour summer cultivar for pulp and peel extracts, but the flavonoid content was not very high in pulp extract of this cultivar. Also, the antioxidant activity of pomegranate peel extract was 10 times higher than the pulp extract (Ardekani et al., 2011). Hajimahmoodi et al. (2008) reported the antioxidant activity of ten Persian pomegranate cultivar hydro-extracts. Based on their findings, the highest FRAP value for pulp and peel was observed in Sour alac. Suitable properties for the selection of antioxidants include low concentration (0.010–0.01%), lack of undesirable effects on food properties, non-toxic, compatibility, and economical. This data could be useful to the pomegranate industry in identifying and developing cultivars having commercial value.

Pomegranate pericarp is an important source of phenolic active compounds that are valuable to human health. Among phenolic compounds, punicalagin (α and β) and ellagic acid can be useful as antioxidants in preventing many diseases (Zhou et al., 2014). HPLC results showed significant differences in alpha and beta punicalagin isomers and ellagic acid in HPLC profile of four cultivars (both methanolic and aqueous extracts) (Figure 3). Alpha and beta punicalagin profiles and their elution order were similar in all four cultivars, but the peak areas varied significantly among them. As shown, the most polar phenolic compound in these cultivars was alpha punicalagin, which eluted first, followed by beta punicalagin and ellagic acid (Figure 3). The major phenolic components in PPEs were beta punicalagin, alpha punicalagin and ellagic acid. Shahvar cultivar had the highest content of beta punicalagin both in methanol (46.10±4.012%) and aqueous extracts (39.57±2.143%) (Table 2). Siahsorfeh cultivar in methanol extract (34.22±2.122%) and Abdolrahimkhany in aqueous extract (29.89±1.154%) had the highest alpha punicalagin, respectively. Punicalagin constant ratio (K = [β]/[α]) was different in the four cultivars (Table 2). This ratio was the highest in Shahvar cultivar (1.85 in methanol extract and 1.39 in aqueous extract) and the lowest in Siahsorfeh (1.06 in methanol extract and 0.94 in aqueous extract). Also, the highest content of punicalagins (sum of alpha and beta) in extracts was found in Shahvar cultivar (67.95% in aqueous extract), which is nearly 1.3 fold higher than in Siahsorfeh (51.35% in aqueous extract). The results of this study indicate that the amount of punicalagins is significantly different among cultivars.

HPLC analysis showed that ellagic acid is the most non-polar component in the four cultivars of pomegranate pericarp. Amount of ellagic acid was also different among cultivars. Torshsabz cultivar had the highest ellagic acid in methanolic extract (5.98±0.64%) and Shahvar cultivar had the highest ellagic acid in aqueous extract (3.49±0.92%) (Table 2). These results predict that the punicalagin, especially β isoform, is responsible for the antioxidant activities of PPEs. To confirm this data, we purified components of PPE and determined their antioxidant activities. Further purification of the components was performed as described in the Materials and Methods section. Purification of pomegranate pericarp components was performed by, first, XAD-16 column chromatography using 100% methanol for separating TPT’s. HPLC profile of TPT’s showed four peaks corresponding to four ellagitannins (Figure 4-A). Each peak was identified based on the external standard. Peaks 1 and 2 were identified as alpha and beta punicalagin anomers and the 4th peak was ellagic acid. TPT’s contain 50–71%
Figure 3. HPLC chromatogram of methanolic (A) and aqueous (B) extracts of pericarps from different pomegranate cultivars (1: Shahvar, 2: Siahsorfeh, 3: Torshsabz and 4: Abdorahimkhany). Absorbencies are recorded at 254 nm (a: Alpha punicalagin, b: Beta punicalagin, c: Ellagic acid)
Table 2. HPLC-analysis of methanolic and aqueous extract of pericarp from pomegranate cultivars.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>%α</th>
<th>%β</th>
<th>% (α+β)</th>
<th>(β /α)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shahvar</td>
<td>24.84±1.78</td>
<td>46.10±4.01</td>
<td>70.92±5.79</td>
<td>1.85</td>
<td>3.35±0.84</td>
</tr>
<tr>
<td>Siahsorfeh</td>
<td>34.22±1.34</td>
<td>36.46±1.34</td>
<td>70.68±3.46</td>
<td>1.06</td>
<td>4.65±0.39</td>
</tr>
<tr>
<td>Torshsabz</td>
<td>27.26±1.34</td>
<td>42.35±1.55</td>
<td>69.62±2.89</td>
<td>1.55</td>
<td>5.98±0.64</td>
</tr>
<tr>
<td>Abdorahimkhany</td>
<td>28.87±0.79</td>
<td>42.06±1.29</td>
<td>70.92±2.09</td>
<td>1.42</td>
<td>4.52±0.46</td>
</tr>
<tr>
<td>Aqueous Extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shahvar</td>
<td>28.38±2.12</td>
<td>39.57±2.14</td>
<td>67.95±4.26</td>
<td>1.39</td>
<td>3.49±0.92</td>
</tr>
<tr>
<td>Siahsorfeh</td>
<td>26.52±4.92</td>
<td>24.83±2.05</td>
<td>51.35±6.97</td>
<td>0.94</td>
<td>2.70±0.53</td>
</tr>
<tr>
<td>Torshsabz</td>
<td>28.96±2.74</td>
<td>32.14±3.37</td>
<td>61.10±6.18</td>
<td>1.11</td>
<td>2.92±0.17</td>
</tr>
<tr>
<td>Abdorahimkhany</td>
<td>29.89±1.15</td>
<td>32.30±1.40</td>
<td>62.19±2.56</td>
<td>1.05</td>
<td>2.70±0.21</td>
</tr>
</tbody>
</table>

w/w of punicalagin anomers and 2-6% w/w ellagic acid. More purification was done by loading TPT’s on LH-20 Sephadex column so that fraction of 65-75% of methanol had the highest amounts of punicalagin and ellagic acid (Figure 4-B). Punicalagin and ellagic acid were separated from each other by reloading this fraction on the LH-20 column and increasing the gradient of ethanol, as described in Materials and Methods section. Alpha and beta punicalagin isomers were isolated manually after loading on HPLC system (Figures 4-E and -D).

Antioxidant activity of purified pomegranate pericarp components was determined. Antioxidant activity of alpha punicalagin and beta punicalagin, their mixture and ellagic acid is shown in Table 3. As expected, beta punicalagin had the highest antioxidant activity (60.5±8.46%) while ellagic acid showed the least antioxidant activity (27.0±3.28%). The antioxidant activities of alpha and beta punicalagins were statistically different. This result confirms the HPLC result. The HPLC profile showed different composition of phenolic compounds in the four studied cultivars. The constant ratio (K = [β]/[α]) of punicalagins were different in the four cultivars and it was higher in methanolic extract as compared with water extract. In Shahvar cultivar, constant ratio K = [β]/[α] is 1.4 in aqueous and 1.85 in methanolic extract. The equilibrium constant was reported to be equal to 1 in methanol and 4 in water (Doig et al., 1999). The result of another study showed a constant ratio of about 1.6 in different solvents (Jurenka et al., 2008). Alpha and beta punicalagins interconvert.

Recently, there have also been numerous reports on the in vitro and in vivo antioxidant activities of punicalagin and ellagic acid, (Rosenblat et al., 2013; Seeram et al., 2005a; Xu et al., 2015), but their antioxidant activity has not been compared. We purified alpha and beta punicalagins as well as ellagic acid in order to identify the main component of pomegranate pericarp with highest antioxidant activity. DPPH antioxidant assay showed that alpha and beta punicalagins have different antioxidant activities. This is the first report on the antioxidant activity of punicalagin anomers. Therefore, our finding further confirms that beta punicalagin is the major component of PPE that is responsible for antioxidant potential of pomegranate pericarp.

The result of antioxidant activity of beta punicalagin is almost equivalent to antioxidant activity of TBHQ, a synthetic antioxidant that it used in foods (Table 1). Since there are considerable discrepancies regarding the safety of this and other synthetic antioxidants for human and animals, replacing them with natural antioxidants such as those reported in this study seems to be a desirable goal.

CONCLUSIONS

Our study showed that pomegranate pericarp extract from different cultivars have
Figure 4. HPLC chromatogram of pomegranate pericarp extracts (A), TPT (B), alpha punicalagin, beta punicalagin and ellagic acid (C), alpha and beta punicalagin isomers (D) and ellagic acid (E).

Table 3. DPPH scavenging activity of alpha and beta punicalagin isomers, mixture of punicalagin (alpha+beta), ellagic acid, and TBHQ.

<table>
<thead>
<tr>
<th>Phenol component</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>α Punicalagin</td>
<td>50.8±3.8</td>
</tr>
<tr>
<td>β Punicalagin</td>
<td>60.5±8.5</td>
</tr>
<tr>
<td>α+β Punicalagins</td>
<td>56.0±3.2</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>27.0±3.3</td>
</tr>
<tr>
<td>TBHQ</td>
<td>66.9±6.4</td>
</tr>
</tbody>
</table>
different antioxidant activities. The difference between antioxidant activities of the four studied cultivars was related to
difference in phenolic contents and composition. Among the cultivar studied,
Shahvar was found to contain the highest
phenolic constituents and highest antioxidant activity. Purified phenol
compounds have different antioxidant
activities. Our results confirmed that the
punicalagin is the substance responsible for
the antioxidant activity of the pomegranate pericarp. The therapeutic use of medicinal
plants is becoming popular because of the
absence of side effects and, in this regard,
Shahvar cultivar can potentially replace the
current synthetic antioxidants in food and
pharmaceutical industries.

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فعالیت آنتی اکسیدانی عصاره پریکارپ رقم های مختلف میوه انار

چکیده
پریکارپ میوه انار حاوی ترکیبات پلی فنولی شامل آلفا و بتا پونیکالاژین والاژیک اسیداست که فعالیت آنتی اکسیدانی قابل توجهی را نشان می دهد. هدف از این مطالعه، بررسی و ارزیابی ترکیبات پلی فنولی عصاره پریکارپ رقم های مختلف انار (PPE) و تعریف خواص آنتی اکسیدانی آن هاست. عصاره های متانولی و آبی از پریکارپ چهار رقم مختلف انار (شهوار، سیاه سرفه، ترش سبز و عبدالرحیم خانی) تهیه شدند. سپس این عصاره ها به صورت ستونی در سطح ژل فیلتراسیون و پس از فیلتراسیون خالص شدند. ترکیبات پلی فنولی خارج شده از سطح های کروماتوگرافی از طریق'HPLC' مورد بررسی قرار گرفتند. فعالیت آنتی اکسیدانی اکسیدانی با استفاده از آزمون DPPH مورد بررسی قرار گرفت. نتایج نشان داد که اختلاف معنی داری در محتوای فنول تام در عصاره پریکارپ چهار رقم مختلف موجود بود. مقدار فنول تام در عصاره های متانولی به طور معنی‌دار تر از عصاره های آبی بود. در عصاره های آبی به ترتیب 0.71 ± 0.27 و 0.71 ± 0.27 درصد و در عصاره های متانولی به ترتیب 0.71 ± 0.27 و 0.71 ± 0.27 درصد از تعداد کل میزان پونیکالاژین با عنوان بازدارنده میزان انحلال اکسیدانی را منبع عالی آنتی اکسیدانی طبیعی است و می تواند به طور بالقوه جایگزین آنتی اکسیدان های مصنوعی در صنایع غذایی شود.