Evaluation of Antioxidant Activity, Phenolic and Flavonoid Content in *Punica granatum* var. Isfahan Malas Flowers

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**ABSTRACT:** Pomegranate is a native Persian fruit that is grown around the world; the present study is the report of the research on different extracts of different polarity from flowers regarding their antioxidant activity. *Punica granatum* L. var. Isfahan Malas flowers were extracted with petroleum ether. The dried petroleum extract was suspended in ethanol %80 and successively partitioned with n-butanol. The total phenolic, flavonoid content and antioxidant capability of different extracts of *Punica granatum* in two systems (DPPH, \(\beta\)-carotene), were evaluated in this work. As a result of the present study, the antioxidant activities of Pomegranate flowers have not direct relationship with phenolic compounds and flavonoids content. Extract concentration have significant relationship in different tests. Antioxidant activity of plant extracts is not limited to phenolics; there is a wide grade of variation between different phenolic compounds in their effectiveness as antioxidant. Type and polarity of the extracting solvent, concentration, pH and the chemical structure of phenolics could also play a role in their antioxidant activity.

**Keywords:** Antioxidant, Flavonoid, Flower, Phenolic, *Punica granatum*.

**INTRODUCTION**

Free radicals (reactive oxygen and nitrogen species, ROS/RNS) are produced in normal and pathological cell metabolism and they are controlled by endogenous enzymes such as superoxide dismutase, glutathione peroxidase, catalase or chemical compounds such as \(\alpha\)-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione. The large production of free radicals results in the onset of numerous diseases and accelerates (Matkowski et al., 2009). Natural products such as fruits and vegetables, herbs, cereals, sprouts, seeds and edible mushrooms can also constitute an important source of antioxidants and they may be used to help the human body to reduce oxidative damage (Talcott et al., 2006). Therefore, there is an interest in substances containing antioxidant ingredients protective to human and animal organisms as food components or as specific preventive pharmaceuticals (Matkowski et al., 2009). The plant kingdom is a wide range of natural antioxidants. In the category of secondary plant metabolites, antioxidant phenolics are commonly found in plants and they have indicated providing a defense against oxidative stress. Plants have antioxidative and pharmacological properties related to the presence of phenolic compounds, especially phenolic acids and flavonoids (Matkowski et al., 2009). Medicinal plants are very important for the healthy lives of most of the people across the world. The several classes of biologically active compounds of these plants are include alkaloids, flavonoids, tannins and phenolic compounds (Alamzeb et al., 2013). Phenolics are a class of secondary metabolites found in most land plants; moreover, they protect plants against ultraviolet radiation, pathogens, and herbivores. Flavonoids in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, quenching of free radicals and chelating abilities, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Yoshimora et al., 2005). Polyphenols are also known for their ability to prevent fatty acids from oxidative decay; in some cases they are used as food ingredients since they are nutritionally rich in Ellagic acid, for instance. The botanical family Punicaceae includes a large number of plant species that are well known for their high ellagic acid content and excellent antioxidant properties. Plants of the
genus Punica from the Punicaceae are rich sources of flavonoids, tannins, alkaloids and organic acids (Wang et al., 2010). Therefore, some of them are used in various regions and folk or traditional medical systems as a food supplement or a medicine. *Punica granatum* Linn. known as pomegranate, a deciduous small tree and known locally as *Golnar-e-farsi*, is an important medicinal plant in Iran whose flowers are used as astringent, hemostatic, antibacterial, antifungal, antiviral and as a treatment for bronchitis, diarrhea, digestive problems, man sex power reconstituent, dermal infected wounds and diabetes in Unani medicinal (Iranian Traditional Medicine) literature (Ghasemi-Pirbalouti et al., 2010). This flower was also used for the treatment of injuries from falls and grey hair of young men in the traditional Chinese medicine (Ghasemi-Pirbalouti et al., 2010). Numerous studies have demonstrated the in vitro antioxidant activity and polyphenol content of *Punica granatum* of foreign origin but data about Iranian pomegranate are insufficient.

The present study is a part of an investigation on the antioxidant potential and the contribution of polyphenols to the antioxidant activity of Iranian pomegranate (Isfahan Malas *Punica granatum*).

**MATERIALS AND METHODS**

**Sampling**

*Punica granatum* L. (Punicaceae) flowers were collected locally in September 2010 from botanical garden in South Esfahan, Iran. The plant was identified in herbarium of Research Institute of Esfahan Forests and Rangelands. The plant materials were dried under shade. The dried flowers were homogenized to fine powder using electric blender and were further subjected to extraction.

**Preparation of flower extracts**

Powdered plant materials were extracted with different solvents. *Punica* flowers (100g) were extracted with petroleum ether at room temperature for 24 h prior to removal of the solvent in vacuum. The plant residue was further extracted similarly with aqueous ethanol (75%) for 5 days and the combined ethanol extracts were taken to dryness. The crude extract was suspended in distilled water and extracted successively with ethyl acetate and n-butanol. After removal of solvents in vacuum, yields of ethyl acetate extract and butanol extract were obtained (Salahuddin et al., 2005; Ghasemian et al., 2006).

**Antioxidant Studies**

The antioxidant activity of the samples and standards was determined by the radical scavenging activity method using 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH). About 0.1 ml of methanolic solutions of the samples or standards at different concentrations was each added to 3.9 ml of a DPPH methanolic solution (0.2 mM). These concentrations were selected due the linearity range of DPPH solutions. The blank sample consisted of 0.1 ml of methanol added to 3.9 ml of DPPH. The tests were carried out in triplicate. After a 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm (Gil et al., 2000).

The antioxidant activity for the samples and standards was modified from method described *β*-carotene-linoleic acid. One milliliter of *β*-carotene (2 mg in 20 ml of chloroform) was added to a conical flask with 40 mg of linoleic acid and 400 mg of Tween-40. Chloroform was removed using a rotary evaporator. To the resulting residue, 100 ml of oxygenated distilled water were added and mixed; 3 ml of the oxygenated *β*-carotene emulsion were placed in a tube containing 0.2 ml of the extracts (0.2 mg/ml) and the absorbance read at 470 nm immediately, against a blank consisting of the emulsion without the *β*-carotene. Absorbance of a control consisted of 0.2 ml of distilled water instead of the extract was also monitored (Yoshimora et al., 2005; Kawaii et al., 2004).

**Determination of Total Phenolics**

The concentrations of total phenolics (TP) in extracts were determined by the Folin–Ciocalteau colorimetric method and external calibration with gallic acid. Therefore, 0.2 ml of extract solution in a test tube and 0.2 ml of Folin–Ciocalteau reagent was added and the contents mixed thoroughly. After 4 min, 1 ml of 15% sodium carbonate (Na₂CO₃) was added. The mixture was allowed to stand for 2 h at room temperature in the dark before the absorbance was measured at 760 nm spectrophotometrically. The concentration of the total phenolics was determined as mg of gallic acid equivalents by using an equation obtained from the gallic acid calibration curve (Yoshimora et al., 2005).

**Determination of Total Flavonoids**

Total flavonoid content was measured according to a colorimetric assay (Zhishen et al., 1999). A 1ml of catechin at different concentrations was added to 10-ml volumetric flasks containing 4ml water. At the onset of the
experiment, 0.3 ml of 5% NaNO₂ was added to the flask. After 5 min, 0.3 ml of 10% AlCl₃ was added. At 6 min, 2 ml of 1M NaOH was added to the mixture. The solution was diluted to a final volume of 10 ml with water and mixed. Absorbance of the mixture was determined at 510 nm versus the prepared blanks. Total flavonoid content in extracts was expressed as mg catechin per 100 g dry weight (Afzal et al., 2010).

**Statistical Analysis**

All experiments were performed in triplicate. The data were expressed as means ± standard deviations (SD) and one-way analysis of variance (ANOVA) was carried out to assess for any significant differences between the means. Differences among means were determined by the Least Significance Difference Test with significance defined at P < 0.001.

**RESULTS AND DISCUSSION**

The tests used to evaluate the potency of the extracts as antioxidants were β-carotene–linoleate system and 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH). The amount of total phenolics and flavonoids measured by Folin-Ciocalteau and Colorimetric assay, varied in different extracts and ranged from 348.808 to 944.75 mgGA/100g dry weight (dw) for total phenol and from 98.399 to 359.6 mgCA/100g dry weight (dw) for total flavonoid [Table 1]. Ethyl acetate extract of flowers (944.75 mgGA/100g dw, 359.6 mgCA/100g dw) had very high levels of phenolics and flavonoids [Table 1].

A variation in antioxidant activities in β-carotene test ranging from 90.87 to 92.16 mg/ml and in DPPH test ranging from 197.46 to 938.62 µmol/gr were observed. Hydro ethanol extract of Punica flowers inhibited linoleic acid oxidation to the greatest extent and had the greatest DPPH antioxidant capacities relative to other extracts. Ethyl acetate extract had more gallic acid than other extracts. Each extract showed a concentration-dependent scavenging effect on the two radicals tested (P < 0.001). Effects of extract concentrations on β-carotene and DPPH tests are shown in [Table 2], in which extract concentration was significant on antioxidant activity in β-carotene and DPPH test (P < 0.001), but extract concentration was not significant on total phenols and flavonoids content.

A free radical in each molecule is determined as an unpaired electron that occupies an atomic or molecular orbital on its own. This reactive molecule is to another electron to pair, this instep an uncontrolled chain reaction that can damage the natural function of the living cell, resulting in different diseases (Zhishen et al., 1999). Almost all organisms are well protected against free radical harm by oxidative enzymes similar superoxide dismutase and catalase or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione (Matkowski et al., 2009). Many fruits and vegetables, herbs, cereals, seeds that contain natural antioxidants can abstract the lone electron from free-radical molecules and help humans to keep control on these harmful species. Most of these antioxidants in plants are highly colored anthocyanines, proanthocyanidins, flavans, flavonoids, and their glycosides, carotenoids, like β-carotene and lycopene (Matkowski et al., 2009). Isolation of antioxidants from plants depends on the polarity of these compounds. First distribution of antioxidants between a polar (aqueous, hydro ethanol) and a semi-polar solvent (n-butanol, ethyl acetate) can be used to determine the distribution factor of the compounds between phases (Matkowski et al., 2009). Therefore, natural antioxidants can be divided into three main categories; 1: Water-soluble antioxidants: these include ascorbic acid, anthocyanidins, catechins, epicatechins, flavonoids and other phenolic glycosides. 2: Fat-soluble antioxidants: these include vitamins A and E, carotenoids, including β-carotene, lycopene and many quinonoid compounds. 3: Antioxidant metals such as selenium are also found in many plants like onion and garlic (Zhishen et al., 1999).

Flavonoids as antioxidant compounds in our study reported in range of 98-359 mg CA/100g dw. Viera et al. (2003) reported Ocimum americanum L. cultivars had 10-749 mg CA/100g dw (Viera et al., 2003). This difference may be attributed to differences in extraction, hydrolysis time, temperature, climate, geography or agricultural practices (Andarwulan et al., 2010). In our study, flavonoids content were not correlated with antioxidant activity in the DPPH and β-Carotene assays. This impact could also be associated with metal antioxidants in our extracts. Andarwulan et al. (2010) reported flavonoid content of vegetables from Indonesia had not correlated with antioxidant activity in the DPPH, ABTS and reducing power assays, similar to the present study. Annual and geographical climate differences, soil conditions and pesticide or herbicide use may contribute to variations in antioxidant activity and flavonoid content of plants (Andarwulan et al., 2010).

The ratio of phenolic compounds to extraction yield can react as the measurement of the adaptability of concentrating phenols in the plant extracts. Punica granatum is an important medicinal plant in Iran whose flowers are used as astringent, hemostatic, antibacterial, antifungal, antiviral and as a remedy for cut wound, bronchitis,
Antioxidant activity of plant extracts also depends on the type and polarity of the extracting solvent (Ismail et al., 2004). Data suggest that ethyl acetate extracts of *Punica granatum* have a rather high content of polyphenol and flavonoid since the ethyl acetate is a semi-polar solvent. Mathkowski et al. (2009) have indicated that maximum amount of the phenolic compounds could be found in the ethyl acetate. It can be assumed that most of the antioxidant compounds can be rather separated with this solvent; however, as a polar solvent, hydro ethanol represented higher antioxidant activity. Regarding this, Wang et al. (2010) indicated that the *Punica granatum* flowers have tannin compounds including Ellagic acid (skin-whitening), gallic acid (anti-inflammatory, anti-mutagenic), ethyl brevifolincarboxylate, and pomegranatante (Wang et al., 2010). Thus, flavonoid compounds include punicaflavone and triterpene compounds include ursolic acid, oleanolic acid, maslinic acid, purcanolic acid, friedelin, betulinic acid and asiatic acid (Wang et al., 2010). It is known that tannins (Wojdyło et al., 2007) have the strongest radical-scavenging power among all natural phenolic compounds. Moreover, it is a potent antioxidant against lipid peroxidation in mitochondrion and microsome (Wang et al., 2010); therefore, 80% ethanol, as a polar solvent, was the better extraction solvent for antioxidant capacity in this study. More et al. (2001) reported that apolar solvents are among the most employed solvents for removing polyphenols from water (Moure et al., 2001). Several studies have reported on the relationships between phenolic content and antioxidant activity (Ismail et al., 2004). Velioglu et al. (1998) reported a strong relationship between total phenolic content and antioxidant activity in selected fruits and vegetables. Thus, no correlation between antioxidant activity and phenolic content was found in the study, by Kahkonen et al. (1999), on some plant extracts containing phenolic compounds (Velioglu et al., 1998; Kähkönen et al., 1999). In our study, the findings do not show any relationship between antioxidant activity and total phenolic and total flavonoid contents.

There is a wide grade of variation between different phenolic compounds in their effectiveness as antioxidant (Robards et al., 1999). In hence, concentration and PH can also play role in the antioxidant activity of phenolics (Bouayed et al., 2011). In addition, the chemical structure of phenolics play a role in the free radical-scavenging activity, mainly depending on the number and position of hydrogen donating hydroxyl groups on the aromatic rings of the phenolic molecules (Bouayed et al., 2011).

The yield of extracted phenols was correlated with the plant cell wall separation caused by pectinases and cellulases, although these latter did not cause the degradation of polysaccharides. Particle size decrease significantly increased the antioxidant activity as a result of both increased extractability and enhanced enzymatic degradation of polysaccharides (Moure et al., 2001). Increased polyphenol improvement was reported by Weinberg et al. (1999) (Weinberg et al., 1999). The temperature during drying and extraction, affects the compound stability due to chemical and enzymatic degradation, casualties by volatilization or thermal analysis, these latter have been suggested to be the main mechanism causing the reduction in polyphenol content (Moure et al., 2001). Also, for synthetic antioxidants, evaporation and analysis were the main mechanisms for the loss of activity. Of course, the temperature during extraction can affect the extractable compounds differently: boiling and static increased the total phenol content in *Quercus suber*; on the other hand, proanthocyanidin content decreased. The antioxidant activity depends on the extract concentration. As a general tendency, increased antioxidant activity was found with increasing extract concentration, but the concentration leading to maximum antioxidant activity is mainly dependent on the antioxidant activity test (Moure et al., 2001).

The results of the present study showed that *Punica granatum* flowers are rich in phenolic constituents and demonstrate good antioxidant activity measured by different methods. This plant, rich in flavonoids and phenolic acids could be a good source of natural antioxidant. The present study demonstrated that *Punica granatum* flower extracts containing high amounts of phenolic compounds so that these compounds are efficient free radical scavengers. Therefore, qualitative and quantitative analysis of major individual phenolics in *Punica granatum* flowers could be useful for explaining the relationships between total antioxidant capacity and total phenolic contents in this plant. This provides a supplementary prophylactic value for this antioxidant plant and supports its gaining popularity as a botanical food supplement.
Table 1. Total phenols, total flavonoids and antioxidant activity (DPPH, β-Carotene) of different extracts of *Punica granatum* (Data is mean± SD of three determinations).

<table>
<thead>
<tr>
<th>Solution Test</th>
<th>Hydroethanol</th>
<th>Aqueous</th>
<th>n-Butanol</th>
<th>Ethyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene (mg/ml)</td>
<td>92.16± 8.74</td>
<td>91.23± 9.81</td>
<td>90.87± 13.08</td>
<td>90.89± 8.01</td>
</tr>
<tr>
<td>DPPH (µmol/gr)</td>
<td>197.46± 194.33</td>
<td>613.01± 273.46</td>
<td>509.52± 185.22</td>
<td>938.62± 3.46</td>
</tr>
<tr>
<td>Polyphenol (mgGA/gr)</td>
<td>348.81± 5.58</td>
<td>509.83± 11.61</td>
<td>866.47± 26.61</td>
<td>944.75± 8.27</td>
</tr>
<tr>
<td>Flavonoid (mgCA/gr)</td>
<td>225.77± 2.93</td>
<td>98.39± 1.15</td>
<td>258.12± 16.19</td>
<td>359.6± 13.91</td>
</tr>
</tbody>
</table>

Table 2. The effect of different concentrations of extract on antioxidant assay, total flavonoids and total polyphenols content (Data is mean± SD of three determinations).

<table>
<thead>
<tr>
<th>Concentration Test</th>
<th>125(µg/ml)</th>
<th>250(µg/ml)</th>
<th>500(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carotene</td>
<td>72.07± 14.39</td>
<td>86.55± 17.11</td>
<td>92.41± 15.79</td>
</tr>
<tr>
<td>DPPH</td>
<td>1057.18± 559.36</td>
<td>926.62± 715.66</td>
<td>915.15± 1091.67</td>
</tr>
<tr>
<td>Polyphenol</td>
<td>483.24± 267.01</td>
<td>483.24± 267.01</td>
<td>483.24± 267.01</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>205.04±173.07</td>
<td>205.04±173.0</td>
<td>205.04±173.07</td>
</tr>
</tbody>
</table>

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REFERENCES


