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Original article

Antioxidant activity of leaf different extracts in *Punica granatum*

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ABSTRACT

Background: Pomegranate *(Punica granatum L.)* is an edible fruit native to Persia that is grown and consumed around the world, including the Iran; it has been revered through the ages for its medicinal properties. In the present project, we reported the research on different extracts of different polarity from leaves for their antioxidant activity. Methods: Pomegranate leaves were extracted with petroleum ether. The dried petroleum extract was suspended in ethanol %80 and successively partitioned with n-butanol. Three different bioassays were used, namely scavenging of the β-carotene-linoleic acid bleaching method, nitric oxide scavenging and hydroxyl oxide cavenging. Results: Total phenolic content was quantified as well. All statistical analyses were carried out with P values <0.001 were considered significant. The results of this study suggested that the antioxidant activities of Pomegranate leaves have direct relationship with phenolic compounds. Thus, extract concentration have significant relationship in different tests. Conclusions: Antioxidant activity of plant extracts is not limited to phenolics; the presence of different antioxidant components in the extracts that function as hydrogen donors, may contribute to the concentration of the total phenols determined. Therefore, there is no relationship between the concentration of polyphenol and the antioxidant activity when comparing plant extracts.

1. Introduction

Antioxidants are the compounds that when added to food products, especially to lipids and lipid-containing foods, can increase the life by retarding the process of lipid peroxidation, with is one of the major reasons for deterioration of food products during processing and storage [1]. Plant-derived polyphenols, present in fruits, leaves, and vegetables have attracted much attention recently due to their beneficial health effects in several disease models [2].

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 [3]. 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, is a deciduous small tree and known locally as ìGolnar-e-farsiî, 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 [3]. 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 aim of this study were to prepare antioxidant-rich fractions from Iranian pomegranate (Isfahan Malas *Punica granatum*) leaves extracts and to evaluate their antioxidant activity using various in vitro models.

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2.Materials and Methods

Punica granatum L. (Punicaceae) flowers were collected locally in septamber 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 leaves were homogenized to fine powder using electric blender and further subjected to extraction.

Powdered plant materials were extracted with different solvents. Punica flowers (100 g) were extracted with petroleum ether at room temperature for 24 h prior to removal of the solvent in vacuo.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 vacuo, yields of ethyl acetate extract and butanol extract were obtained [4].

The antioxidant activity of the samples and standards was determined by way of 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 were each added to 3.9 ml of a DPPH methanolic solution (0.2mM). 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. The radical scavenging activity was calculated as follows: I% = [(Abs0ɜAbs1)/Abs0] ɜ100, where Abs0 was the absorbance of the blank and Abs1 was the absorbance in the presence of the test compound at different concentrations [5].

The antioxidant activity for the samples and standards was modified from method described b-carotene-linoleic acid. One millilitre of b-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 b-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 b-carotene. Absorbance of a control consisted of 0.2 ml of distilled water instead of the extract was also monitored. The bleaching rate (R) of b-carotene was calculated using the equation:

$$R=In(a/b)\times 1/t$$

where ln = natural log, a = initial absorbance (470 nm), b = absor-bance at 120 min interval and t = time (min). The antioxidant activ-ity (AA) was determined as percent inhibition relative to control sample using the equation:

$$AA\ (\%)=[(Rcontrol-Rsample)/Rcontrol]\times 100$$

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 (Na_2CO_3) 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 [6].

All experiments were performed in triplicate. The data were expressed as means ± standard deviations (SD) and oneway 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 $P < 0.001$.

3.Results

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 results of the leaf antioxidant activity and total phenols analysis are summarized in 1. The n-butanol extract in β–carotene test and ethyl aqueous extract in DPPH test possessed high antioxidant activity . A variation in antioxidant activities in β–carotene test ranging from 35.84 to 92.44 mg/ml and in DPPH test ranging from 277.18 to 835.13 μmol/gr were observed.

The results showed that the total phenolic content of the different extracts varied from 218.68 to 492.91 mg galic acid/g dry weight (Punica). There was significant difference between the means of the antioxidant effects from different tests with respect to total phenolic contents. Ethyl acetate extract had more gallic acid than other extracts. Each extract showed a concentration-dependent scavenging effect on the two radicals tested ($P < 0.001$), in which ethyl acetate extract was significantly greater than other extracts in scavenging two radicals ($P < 0.001$). Effects of different extract concentrations on β–carotene and DPPH tests are shown in 2, in which extract concentration was significant on antioxidant activity in β–carotene and DPPH ($P < 0.001$),but extract concentration was not significant on phenols content.

Table 1: Results of the antioxidant assay and quantitative polyphenol analysis (X±SD)

Solution	Hydroethanol	Aqueous	n-Butanol	Ethylasetat
β-Carotene (mg/ml)	35.84± 7.39	89.28± 13.59	92.44± 9.09	91.17± 6.38
DPPH (µmol/gr)	364.23± 319.01	277.18± 54.88	672.41± 249.45	835.13± 61.25
Polyphenol (mg CA/gr)	218.68± 7.09	269.58± 9.57	259.21± 8.52	492.91± 35.99

Table 2: Different concentration of extract in antioxidant and polyphenolic tests (X±SD)

Concentration	125(µg/ml)	250(µg/ml)	500(µg/ml)
β-Carotene (mg/ml)ol	72.07± 14.39	86.55± 17.11	92.41± 15.79
DPPH (µmol/gr)	1057.18± 559.36	926.62± 715.66	915.15± 1091.67
Polyphenol (mg CA/gr)	483.24± 267.01	483.24± 267.01	83.24± 267.01

4.Discussion:

Isolation of antioxidants from plants depends on the polarity of these compounds. First distribution of antioxidants between a polar (aqueous, hydroethanol) and a medium polar solvent (n-butanol, ethylacetate) can be used to determine the distribution coefficient of the compounds between phases. Therfore, natural antioxidants can be divided into three main classes: 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 is also found in many plants like onion and garlic [7].

Ellagic acid is considered to play an important role in the antioxidant activity in pomegranate leaves, which can react with free radicals due to its ability to chelate with metal ions,is a potent antioxidant against lipid peroxidation in mitochondrion and microsome [3].

Mathkowski et al.(2009) have shown in the ethylacetate extract exist maximum of the phenolic compounds. it can be assumed that most of the antioxidant compounds can be rather separated with this solvent [8].

In case of antioxidant compounds, some polyhenols can be actually water-insoluble and some can be water-soluble, Hence, the lack of correlation to both of the other assays can result from the difference in mechanism involved [3]. Therefore, maximum of the leaf phenolic compounds was in ethylacetate extract. The present study demonstrated that Punica granatum leaf extract containing high amounts of ellagic acid, so that ellagic acid is an efficient free radical scavenger. This provides a complementary preventive value for this antioxidant plant and supports its gaining popularity as a botanical food supplement.

5.Conclusion

The present study demonstrated that Punica granatum leaf extract containing amounts of ellagic acid, so that ellagic acid is an efficient free radical scavenger. The n-butanol extract, ethyl acetate, hydroethanol and aqueous extracts differ in the response of two in vitro antioxidant assays, most likely due to the differences in polyphenol content and composition. There are in the ethyl acetate extract, polyphenols other than alternative extracts.

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