SUPPLEMENTARY MATERIAL

Antioxidant activity of different parts of *Pistacia khinjuk* Stocks fruit and its correlation to phenolic composition

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Abstract

The fruits of *Pistacia khinjuk* Stocks were collected from Ilam province, Iran. The aim of this study was to analyse antioxidant capacity and phenolic composition of different parts of *Pistacia khinjuk* fruit. The antioxidant capacity of extracts were measured using different assays: FRAP, DPPH and nitric oxide radical scavenging. The phenolic composition of *Pistacia khinjuk* fruit is reported for the first time. Among different parts of the fruit analysed in this study, hull extract contained the highest total phenolic and flavonoids contents. We observed a high correlation between different antioxidant activity and total phenolic and flavonoid contents. Therefore, antioxidant capacity can be related to total phenolic and flavonoid contents. A correlation analysis revealed that ascorbic acid, gallic acid, rutin, caffeic acid, ferulic acid and sinapic acid were the phenolic compounds mainly responsible for antioxidant power of the fruit extracts.

Keywords: Fruit; Antioxidant capacity; Phenolic composition; Hull extract

Experimental

Plant material and preparation of methanolic extract

The plant sample was identified by Dr. Mohammad Ali Rajamand at Urmia University and the voucher specimen (No. UU-DB-2012-19) had been deposited at the herbarium of the Department of Biology, Faculty of Science, Urmia University. The ripen fruits of *P.khinjuk* Stocks were collected from Ilam province at August- September 2012. *P.khinjuk* fruits were picked up by hand in Ghalajeh mountain of Ilam province (Altitude: 1909 m, latitude (N): 33°57', longitude (E): 46°20').

The samples were manually separated and then they were air-dried in shadow at room temperature for 48 h and reduced to fine powder. A fine dried powder of samples (2 g) was extracted using pure methanol (50 ml) in a soxhlet apparatus at 60°C for 30 min (Wijeratne et al. 2006). The supernatant was filtered through filter paper.

Determination of total phenolic and flavonoid

Phenolic concentration in all extracts was determined using the Folin-Ciocalteu colorimetric method (Singleton et al.1999; Tsantili et al. 2010), with some modification. Basically, 0.2 ml of extract was added to distilled water (2.6 ml) and 0.2 ml of Folin- Ciocalteu Reagent (FCR) and mixed thoroughly. The reaction was neutralized with 2 ml of sodium carbonate (7%). The absorbance at 750 nm was recorded after 90 min by using a spectrophotometer (Biowave, WPA S2100, UK). A mixture of distilled water and reagents was used as a blank. All the tests were carried out in triplicate. The concentration of phenolic compounds was expressed as gallic acid (mg) equivalents (GAEs) per 1 g of sample.

The flavonoid content was quantified using the colorimetric assay according to method of Lenucci et al. (2006) with a minor modification. Briefly, 0.1 ml of the extracts was diluted with distilled water up to 1 ml and subsequently 0.050 ml of sodium nitrate solution (5 %) was added and allowed to react for 5 min. Then 10 % aluminum chloride solution (0.1 ml) was added. Finally, after 6 min, 0.5 ml of 1 M sodium hydroxide and 1 ml of distilled water were added in to the mixture. The absorbance of the mixture was immediately recorded at 510 nm and the flavonoid content was expressed as mg of catechin equivalents (CEs) per 1 g of sample. All the tests were carried out in triplicate.

Antioxidant activity

FRAP assay

The ferric reducing antioxidant power (FRAP) assay was determined as described by Benzie, Iris and Wilsson (Benzie & Strain 1996; Iris et al.1999; Nilsson et al. 2005). The FRAP reagent was prepared by mixing acetate buffer (0.3 M, pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM Fe₂(SO₄)₃.7H₂O. Acetate buffer was prepared by dissolving 3.1 g sodium acetate trihydrate, plus 16 ml of acetic acid in 1 L of distilled water. The final working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ and Fe₂(SO₄)₃.7H₂O (10:1:1).

In brief, 50 µl of extracts was added to the diluted FRAP reagent in methanol (1 ml FRAP reagent mixed with 2 ml methanol) and absorbance at 593 nm was recorded after 30 min at room temperature against a blank (FRAP diluted reagent previously prepared without the extract). Data were calculated according to following equation that was obtained with ascorbic acid from calibration curve and then expressed as ascorbic acid equivalents AEAC. All the tests were carried out in triplicate.

DPPH radical scavenging activity assay

DPPH radical scavenging activity assay was performed as described by Wu et al. (2003) with a minor modification. 0.1 ml of extracts was mixed with 1.5 ml of methanolic solution containing 0.1 mmol of DPPH (2, 2-

diphenyl- 1- picrylhydrazyl). The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min (until stable absorbance values were obtained). The reaction of the DPPH radical was estimated by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation:

$$\text{RAS\%} = (\text{A0} - \text{A1}) / \text{A0} \times 100$$

Where A0 is the absorbance of control reaction (containing all reagents except the extract) and A1 is the absorbance of solution when the sample extract has been added. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph plotting inhibition percentage against the corresponding extract concentration. BHA was used as the reference compound.

Nitric oxide radical inhibition assay

Nitric oxide can be determined using of Griess Illosvoy reaction according to the method of Garrat (1964). In this investigation, Griess Illosvoy reaction was modified by using naphthyl ethylenediamine dihydrochloride (0.1 % w/v) instead of 1- naphthylamine (5%). The reaction mixture was prepared by mixing 2 ml of sodium nitroprusside (10 mmol/L), 0.5 ml of phosphate buffer saline and 0.1 ml of extract. Then the reaction mixture was incubated at 25°C for 150 min. After incubation, 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) was added to 0.50 ml of the reaction mixture. After 5 min in order to complete diazotization, 1 ml of naphthyl ethylenediamine dihydrochloride was added, mixed and allowed to stand for 30 min at room temperature. A pink colored chromophore was formed in diffused light. Finally, 2 ml of distilled water was added to all samples. The absorbance of the solution was recorded at 540 nm against the corresponding black solutions.

$$\text{Nitric oxide radical scavenging \%} = \frac{\text{A blank} - \text{A sample}}{\text{A sample}} \times 1000$$

Extraction and hydrolysis for HPLC

For HPLC analysis, 0.5 g of dried and powdered plant material was extracted with 50% methanol/ water for 2 h at room temperature. The plant extract was hydrolyzed with 1.2 M HCl by refluxing in a water bath for 1 h. All samples were filtered through a 0.45 μm pore size syringe- driven filter before injection (Hertog et al.1992).

Chromatographic separation of phenolic compounds by HPLC

A 20 μl aliquot of sample solution was separated using high performance liquid chromatography system (Knuer, Germany) equipped with UV- Vis detector and a eurospher 100- 5 C- 18 column (25 cm $\times$ 4.6 cm; 5 μm). The mobile phase consisted of solvent A (water/ acetic acid, 98/ 2, V/ V) and solvent B (methanol). Solvent gradient was used as followed: 0- 5 min, 5% B; 5- 15 min, 10% B; 15- 26 min, a linear gradient of 10% to 100% B. After termination of the cycle, 20 min of column equilibrating was allowed prior next injection. The flow rate was 0.8 ml/ min, and the temperature was set at 25 °C. UV- detection was performed at 280 nm and identified phenolic compounds were quantified by comparison with curve constructed with solutions of each pure commercial compound (Figure S1).

Statistical analysis

For all the experiments three samples of each Bene genotype were analysed and all the assays was carried out in triplicate. Data analyses were performed using SPSS software version 16. Results were analysed using one- way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Correlation between various parameters was also investigated. Significance was determined at $p < 0.05$ and the results were expressed as mean values and standard error (SE) of the means.

Figure S1. Typical HPLC chromatogram of the standard curve (A) and shell extracts (B) recorded at 280 nm.

Table S1. Total phenolic and flavonoid contents (mg/g d.m.), FRAP assay (mg/ml), Nitric oxide radical scavenging (%), DPPH radical scavenging (%) and EC₅₀ (mg/ml) in different parts of *P.khinjuk* fruit (hull, shell and kernel). Results are mean of three replicates with standard errors (Means $\pm$ S.E, n=3). In each column different letters mean significant differences $p < 0.05$. d.m= dry mass..

Part	Phenolic contents	Flavonoid contents	FRAP assay	Nitric oxide scavenging	DPPH radical scavenging	EC ₅₀
Shell	^b 4.1 $\pm$ 0.10	^b 2.7 $\pm$ 0.09	^b 0.55 $\pm$ 0.01	^b 56.4 $\pm$ 0.17	^b 87.3 $\pm$ 0.27	^b 0.881 $\pm$ 0.028
Hull	^a 25.9 $\pm$ 0.94	^a 12.2 $\pm$ 0.36	^a 3.58 $\pm$ 0.09	^a 61.8 $\pm$ 0.06	^a 94.2 $\pm$ 0.35	^c 0.151 $\pm$ 0.014
Kernel	^b 3.1 $\pm$ 0.03	^c 1.4 $\pm$ 0.03	^b 0.58 $\pm$ 0.01	^c 46.6 $\pm$ 0.17	^b 84.9 $\pm$ 0.88	^a 1.282 $\pm$ 0.027
BHA	-	-	0.63 $\pm$ 0.01	-	97.7 $\pm$ 1.89	0.05 $\pm$ 0.002

Table S2. The correlation between total phenolic (mg GAEs/g d.m.) and flavonoid (mg CEs/g d.m.) contents, FRAP assay (mg AEAC/g extract), nitric oxide scavenging (%), DPPH radical scavenging (%) and EC₅₀ (mg/ml) in different parts of *P.Khinjuk* fruit (hull, shell and kernel). d.m.: dry mass.

	Phenolic contents	Flavonoid contents	FRAP assay	Nitric oxide scavenging	DPPH radical scavenging	EC ₅₀
Phenolic contents	1	0.99**	0.99**	0.79*	0.95**	- 0.94**
Flavonoid contents		1	0.98**	0.83**	0.96**	- 0.96**
FRAP assay			1	0.76*	0.94**	- 0.92**
Nitric oxide scavenging				1	0.88**	- 0.94**
DPPH radical scavenging					1	- 0.95**
EC ₅₀						1

* Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Table S3. Concentrations of phenolic compounds (expressed as μ g/g d.m.) in different parts of *P.Khinjuk* fruit (hull, shell and kernel). d.m.: dry mass. (1) Ascorbic acid; (2) Gallic acid; (3) Rutin; (4) Caffeic acid; (5) *p*-hydroxybenzoic acid; (6) Vanillic acid; (7) *p*-coumaric acid; (8) Syringic acid; (9) Ferulic acid; (10) Sinapic acid. Results are mean of three replicates with standard errors (Means $\pm$ S.E, n=3), $p < 0.05$. N. D.: not determined. r.t: retention time. In each column different letters mean significant differences $p < 0.05$.

	r.t (min)	Shell	Hull	Kernel
1	4.03	^b 7.42 $\pm$ 0.36	^a 14.85 $\pm$ 0.44	^c 2.33 $\pm$ 0.16
2	6.20	^b 10.6 $\pm$ 0.78	^a 15.1 $\pm$ 1.07	^c 2.81 $\pm$ 0.21
3	6.66	^b N.D.	^a 1.98 $\pm$ 0.24	^b N.D.
4	7.06	^b 95.4 $\pm$ 5.81	^a 230.1 $\pm$ 21.2	^c 17.3 $\pm$ 0.76
5	9.33	^a 21.4 $\pm$ 0.89	^a 20.6 $\pm$ 1.49	^b N.D.
6	18.10	^a 5.66 $\pm$ 0.38	^b 3.14 $\pm$ 0.28	^c N.D.
7	20.35	^b N.D.	^b N.D.	^a 13.2 $\pm$ 0.46
8	21.25	^c N.D.	^a 11.7 $\pm$ 0.79	^b 7.34 $\pm$ 0.25
9	23.18	^b 27.2 $\pm$ 0.81	^a 42.1 $\pm$ 1.08	^c N.D.
10	23.66	^b 16.7 $\pm$ 0.68	^a 44.2 $\pm$ 1.57	^c 11.4 $\pm$ 0.49
Total (μ g/g d.m.)		^b 184.4 $\pm$ 9.71	^a 383.7 $\pm$ 28.2	^c 54.3 $\pm$ 2.33

Table S4. Correlation matrix between antioxidant activity assays (FRAP, nitric oxide scavenging and DPPH radical scavenging) and the concentration of each phenolic compound. The highest correlations observed, belonging to the main contributors to antioxidant activity, are in bold.

Compound	FRAP assay	Nitric oxide scavenging	DPPH radical scavenging
Ascorbic acid	0.961**	0.907**	0.963**
Gallic acid	0.765*	0.990**	0.882**
Rutin	0.985**	0.767*	0.936**
Caffeic acid	0.918**	0.943**	0.956**
P- hydroxybenzoic acid	0.457	0.920**	0.659
Vanillic acid	0.54	0.680*	0.299
P- coumaric acid	-0.491	-0.936**	-0.681*
Syringic acid	0.785*	0.210	0.686
Ferulic acid	0.762*	0.999**	0.884**
Sinapic acid	0.983**	0.856**	0.972**

*Correlation is significant at the 0.05 level (2- tailed).

**Correlation is significant at the 0.01 level (2- tailed).

References

- Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Anal Biochem.* 239:70–76.
- Garrat DC. 1964. The quantitative analysis of drug. Japan: Chapman and Hall.
- Hertog MGL, Hollman PCH, Venema DP. 1992. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *J Agric Food Chem.* 40:1591–1598.
- Iris FF, Benzie IF, Strain JJ. 1999. Ferric reducing Antioxidant Power assay. *Method Enzymol.* 299:15–27.
- Lenucci MS, Cadinu D, Taurino M, Piro G, Dalessandro G. 2006. Antioxidant composition in cherry and high-pigment tomato cultivars. *J Agric Food Chem.* 54:2606–2613.
- Nilsson J, Pillai D, Onning G, Persson C, Nilsson A, Akesson B. 2005. Comparison of the 2,2'-azinobis-3-ethylbenzotiazo-line-6-sulfonic acid (ABTS) and ferric reducing anti-oxidant power (FRAP) methods to asses the total antioxidant capacity in extracts of fruit and vegetables. *Mol Nutr Food Res.* 49:239–246.
- Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods Enzymol.* 299:152–178.
- Tsantili E, Shin Y, Nock JF, Watkins CB. 2010. Antioxidant concentrations during chilling injury development in peaches. *Postharvest Biol Technol.* 57:27–34.
- Wijeratne SSK, Abou- Zaid MM, Shahidi F. 2006. Antioxidant polyphenols in almond and its coproducts. *J Agric Food Chem.* 54:312–318.
- Wu HC, Chen HM, Shiau CY. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Res Int.* 36:949– 957.