Total phenolic content and antioxidant activity of fruit and leaf of Bene (*Pistacia atlantica* subsp. Kurdica) in Ilam province

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Abstract

The objective of this study was to evaluate total phenolic content and antioxidant activity of leaf and different parts of fruit (hull, shell, and kernel) of Bene in Ilam province. Total phenolic content was determined with the Folin- Ciocalteu Reagent (FCR); antioxidant activity of methanolic extracts were examined by DPPH (2,2-diphenyl- 1- picrylhydrazyl), FRAP (ferric reducing antioxidant power) and nitric oxide radical scavenging methods. Among all the samples tested, leaf contained the highest total phenolic content followed by hull, kernel, and shell. There was a positive correlation between total phenol content and FRAP, Nitric oxide radical scavenging activity, and DPPH radical scavenging activity (r= 0.993, r= 0.972, and r= 0.969, respectively). The results indicated that antioxidant activity of leaf and hull extracts were significantly higher than kernel and shell extracts and this is attributed to their higher total phenolic and flavonoid contents. The FRAP, nitric oxide radical scavenging activity, and DPPH radical scavenging activity assay showed that leaf and hull extracts with potent activity can be introduced as a source of natural antioxidant. Compared with BHA, the extracts of leaf and hull revealed a remarkable DPPH free radical scavenging activity but shell and kernel extracts with higher EC_{50} had a weak activity.

Keywords: phenolic content; antioxidant activity; leaf; fruit; Bene

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Introduction

Bene (*Pistacia atlantica* subsp. Kurdica) belongs to the genus *Pistacia* from Ancardiaceae family. The genus *Pistacia* contains 11 species, which are shrubs or trees (Zohary, 1952). In addition, some of these species have edible nuts and are commercially and pharmaceutically valuable. The leaves and fruits of Bene are used in folk medicine for the treatment of throat infections, stomach, heart, and respiratory system disorders. The fruits of Bene are used by local people after grinding and mixing with other ingredients as food and the unripe fruit is used to make jam. Also hull and kernel oil is used as frying oil by natives. Oleoresin obtained from Bene is used to make chewing gum in Iran (Hatamnia et al., 2014; Hatamnia et al., 2015).

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Antioxidant compounds in plants play an important role as health protecting factors. Scientific evidence suggests that consumption of nuts has been related to reduction in the risk of cardiovascular heart disease (CHD) and cancer, and also induction of lower total and LDL cholesterol levels (Fraser et al., 1992; Albert et al., 2002; Hu and Willett, 2002).

There have been few studies on Bene regarding to the fatty acids and triglycerides, chemical composition of essential oils, and flavonoids (Kawashty et al., 2000; Pietta, 2000; Tzakou et al., 2007; Benhassaini et al., 2008; Farhoosh et al., 2008; Mercherara- Idjeri et al., 2008; Gourine et al., 2010; Farhoosh et al., 2011). However, in recent years focusing on natural antioxidant and phenolic compounds studies in the nuts has increased all over the world. On the other hand, due to lack of knowledge about total phenolic and flavonoid contents as well as antioxidant activity of Bene fruit and leaf extracts, the aims of this study were to determine the total phenolic and flavonoid contents, as well as to evaluate antioxidant capacity of extracts by different assays (DPPH radical scavenging activity assay, FRAP assay, and nitric oxide radical inhibition assay).

Materials and Methods

Plant material

The ripe fruit and leaf of Bene were collected from Ilam province during August-September 2011. Bene fruit and leaf were picked up by hand in Kol Kol village of Ilam province (Altitude: 1519 m, latitude (E): 33°53′, longitude (N): 46°23′).

Preparation of methanolic extract

Hulls, shells, and kernels of fruit samples were manually separated and then 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 defatted with n-Hexane and then 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 a filter paper.

Determination of total phenolics and flavonoids

Phenolic content of all extracts was determined using the Folin–Ciocalteu colorimetric method (Slinkard and Singleton, 1977) with some modification. Basically, 0.25 ml of extract was added to distilled water (11.5 ml) and 0.25 ml of Folin– Ciocalteu Reagent (FCR) and mixed thoroughly. The reaction was neutralized with 0.75 ml of sodium carbonate (2%). The absorbance at 760 nm was recorded after 2 h 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 (GEs) per 100 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 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 100 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 and 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 $Fe_2(SO_4)_3.7H_2O$. 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 $Fe_2(SO_4)_3.7H_2O$ (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.

Nitric oxide radical inhibition assay

Nitric oxide can be determined using Griess Illosvoy reaction according to the method of Garrat (1964). In this investigation, Griess Illosvoy reaction was modified using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of 1- napthylamine (5%). The reaction mixture was prepared by mixing 2 ml of sodium nitroprusside (10 mM/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 that, 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.

Nitric oxide radical scavenging % = $A_{blank} - A_{sample} \times 1000 / A_{sample}$

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 mM 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:

$RAS\% = (A_0 - A_1) / A_0 \times 100$

where A_0 is the absorbance of control reaction (containing all reagents except the extract) and A_1 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.

Statistical analysis

For all experiments three samples of each part of fruit were analyzed and all assays were carried out in triplicate. Data analyses were performed using SPSS software version 16. Results were analyzed 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 $P \leq 0.05$ and the results were expressed as mean values and standard error (SE) of the means.

Results

Table 1 presents total phenolic and flavonoid content in leaf, hull, shell, and kernel extract. Significant differences were found in total phenolic content between leaf, hull, shell and kernel extract, so that among all the sample tested, leaf contained the highest total phenolic content (51/41 mg/g) followed by hull, kernel, and shell in that order(Table 1). There was an approximately 15/6- fold differences in total phenolic content between leaf and shell extract, the highest, and the lowest total phenolic content, respectively.

The flavonoid content of leaf, hull, shell, and kernel extracts are shown in Table 1. The results showed that among all extracts, hull extract contained the highest flavonoid content (7.66 mg/g) followed by kernel leaf and shell. The flavonoid content in hull extract was 1.9-, 2.4-, and 15- fold, higher than that of kernel leaf and shell.

The FRAP assays of extracts expressed as AEAC (ascorbic acid equivalents) are summarized in Table 2. Among all the extracts analyzed, leaf extract had the greatest antioxidant activity (8.99 mg/ml) followed by hull (5.66 mg/ml), kernel (0.61 mg/ml), and shell (0.44 mg/ml) extracts. The results indicates a high positive correlation coefficient between FRAP assay and total phenolic content (r= 0.993), but no positive correlation coefficient were found between FRAP assay and flavonoid content (r= 0.401) (Table 3).

Nitric oxide radical scavenging activity in leaf, hull, shell, and kernel extracts is shown in Table 2. The results show that hull extract exhibited powerful nitric oxide radical scavenging activity (94.32%) followed by leaf, kernel, and shell extract (93.67%, 72.18%, and 69.32%, respectively). The results indicate that there was a positive correlation coefficient between nitric oxide radical scavenging activity and total phenol (r= 0.972) and flavonoid (r= 0.659) content (Table 3).

The results of the DPPH radical scavenging activity and the values of EC_{50} obtained from extracts and BHA (as a reference compound) is given in Table 2. The highest DPPH radical scavenging activity was observed in leaf extract (96.67%) followed by hull, kernel, and shell extract (96.49%, 73.45%, and 69.44%, respectively). Table 2 reports the value of EC_{50} in leaf, hull, shell, and kernel extracts. It ranged from 0.059 mg/ml (mean value of leaf extract) to 1.83 mg/ml (mean value of shell extract). There was a positive correlation coefficient between DPPH radical scavenging activity and total phenol and flavonoid content (r= 0.969 and r= 0.658, respectively). Also, there was a negative correlation coefficient between EC_{50} and total phenol (r= -0/951) and flavonoid content (r= -0.702) (Table 3).

There were significant and strong relations between FRAP and DPPH radical scavenging assays (r = 0/945), and between FRAP and nitric oxide radical scavenging assays (r = 0/944). Also a high correlation was observed

Table 1
Total phenols and flavonoids contents in leaf and different parts of fruit (hull, shell, and kernel); results are mean of three replicates with standard errors (Means ± S.E, n=3). In each column different letters mean significant differences p <0.05.

	Phenolic contents (mg/g)	Flavonoid contents (mg/g)
Leaf	^{a}51.41±1.40	^{b}3.11±0.25
Hull	^{b}39.69±0.52	^{a}7.66±0.43
Shell	^{c}3.30±0.12	^{c}0.51±0.03
Kernel	^{c}3.47±0.34	^{b}3.96±0.11
Mean	24.46±0.59	3.81±0.21

Table 2
FRAP assay, nitric oxide scavenging (%), DPPH radical scavenging (%), and EC50 (mg/ml) in leaf and different parts of fruit (hull, shell and kernel); results are mean of three replicates with standard errors (Means ± S.E, n=3). In each row different letters mean significant differences p <0.05.

	Leaf	Hull	Shell	Kernel	BHA
FRAP assay AEAC (mg/ml)	^{a}8.99± 0.38	^{b}5.66± 0.05	^{c}0.44± 0.01	^{c}0.61± 0.06	0.63 ±0.0098
Nitric oxide scavenging (%)	^{a}93.67± 0.18	^{a}94.32± 0.27	^{c}69.32± 0.21	^{b}72.18± 0.26	-
DPPH Radical scavenging(%)	^{a}96.67± 0.87	^{a}96.49± 0.55	^{b}69.44± 1.05	^{b}73.45±1.13	97.69 ± 1.89
EC50 (mg/ml)	^{c}0.059 ± 0.0075	^{c}0.068 ± 0.0025	^{a}1.83 ± 0.039	^{b}1.30 ± 0.078	0.048 ±0.0024

between DPPH radical scavenging and nitric oxide radical scavenging assays (r= 0/993).

Discussion

In general, plant phenolics are highly effective antioxidant activity and free radical scavengers. The results showed that leaf and hull extracts could be effective in the radical scavenging activity. The antioxidant of phenolic compounds can be attributed to their redox properties, which allow them to act as hydrogen-atom donors or reducing agents, their ability to chelate metals, inhibit lipoxygenase, and scavenge free radicals (Decker, 1997). Previous reports suggested that phenols and flavonoids have a great potential benefits because of their antioxidant activity (Alañón et al., 2011; Akbari et al., 2012; Hatamnia et al., 2015).

For assessing reduction ability of antiradical and antioxidant power of extracts, we used the ferric reducing antioxidant power (FRAP) assay that is estimated by transformation of ferric to ferrous ion (Prior and Cao, 1999). The antioxidant activity of leaf and hull extracts was almost 14 and 9 times more than BHA (a reference compound). This finding is consistent with a report by Gourine et al. (2010), that FRAP values of leaf extracts of *Pistacia atlantica* Desf were notably higher than that determined for BHA. Some authors have suggested the fact that the FRAP assay is one of the major assays for evaluating antioxidant capacity (Gourine et al. 2010; Farhoosh et al. 2011; Hatamnia et al. 2014).

Nitric oxide is generated from decomposition of sodium nitroprusside to form nitrite. Suppression of nitric oxide production may be partially related to direct nitric oxide radical scavenging by extracts, so that there was a positive correlation coefficient between nitric oxide radical scavenging and total phenol and flavonoid content (Table 3). The results previously reported by other authors that suggest that the scavenging of nitric oxide by extracts was in a phenolic and flavonoid contents-dependent manner (Akbari et al., 2012; Hatamnia et al., 2014).

The results indicated that DPPH radical scavenging activity of fruit and leaf of Bene extracts was phenol and flavonoid contents dependent, so that there was a positive correlation coefficient between DPPH radical scavenging activity and phenolic (r = 0/969) and flavonoid (r = 0/658) contents (Table 3). A positive relation between phenolic compounds as potent hydrogen donors and DPPH radical scavenging activity has been reported by pervious investigations (Gourine et al., 2010; Akbari et al., 2012; Hatamnia et al., 2014). Maqsood and Benjakul (2010) reported that phenolic compounds with higher number of hydroxyl

Table 3
Pearson's coefficient for the correlation between phenolic (mg GAEs/g extract)and flavonoid contents(mg CEs/g extract), FRAP assay(mg AEAC/ml extract), nitric oxide scavenging (%), DPPH radical scavenging (%) and EC 50 (mg/ml)in leaf and different parts of fruit (hull, shell and kernel)..* Correlation is significant at the 0.05 level (2- tailed).**Correlation is significant at the 0.01 level (2- tailed).

	Phenolic contents	Flavonoid contents	FRAP assay	Nitric oxide scavenging	DPPH radical scavenging	EC_{50}
Phenolic contents	1	0.479	0.993**	0.972**	0.969**	- 0.951**
Flavonoid contents		1	0.401	0.659*	0.658*	- 0.702*
FRAP assay			1	0.944**	0.945**	- 0.928**
Nitric oxide scavenging				1	0.993**	- 0.987**
DPPH radical scavenging					1	- 0.985**
EC_{50}						1

groups may be related to increased DPPH radical scavenging activity. The leaf and hull extracts with EC_{50} close to 0.048 mg/ml (EC_{50} of BHA) revealed good antioxidant activity, while the kernel and shell extracts presented higher EC_{50} value.

A positive correlation coefficient between total phenol and flavonoid content with different assay for measurement of antioxidant activity (FRAP, nitric oxide radical scavenging activity and DPPH radical scavenging activity assay) suggested that total phenol and flavonoid content can be responsible for their potent antioxidant activity, and this results has been previously demonstrated for a variety of species (Velioglu et al., 1998; Yang et al., 2004; Barros et al., 2007; Ferreira et al., 2007; Sousa et al., 2008; Gourine et al., 2010).

In order to prove if three different assays (FRAP, nitric oxide radical scavenging activity, and DPPH radical scavenging activity assays) yield comparable results, the correlation coefficient between different assays was calculated and high correlation between different assays indicated that the three assays provided comparable values when used for evaluating antioxidant activity. This finding is consistent with reports by other authors who analyzed the correlation between different assays (Alañón et al., 2011; Hatamnia et al., 2015).

After an overview of results we can observe that the antioxidant activity of all extracts was as follows: leaf >hull > shell > kernel. The antioxidant activity of hull extract is very close to activity of leaf extract so that there was no significant difference in antioxidant activity between leaf and hull extracts. Finally, FRAP, nitric oxide radical scavenging activity, and DPPH radical scavenging activity assay showed that leaf and hull extracts with potent activity can be introduced as a source of natural antioxidant.

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