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Full Length Research Paper

Antioxidant activity and phenolic contents of Persian walnut (*Juglans regia L.*) green husk extract

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Walnut (Juglans regia L.) green husk is a by-product of the walnut production that has many phytochemicals, but there is little information about its antioxidative capacity. In this study Antioxidant activity and protective effects in stabilizing sunflower oil of methanolic extract of Persian walnut (*Juglans regia L.*) green husk were studied. Total flavonoids and phenolics also were determined by using aluminum nitrate and Folin–Ciocalteu colorimetric methods and their amount were 144.65±2.1 mg quercetin and 3428.11±135.80 mg gallic acid equivalent per 100 gram of dry sample respectively. The antioxidant capacity of sample was assessed through reducing power assay, DPPH-scavenging effect, FRAP assay and oven test in sunflower oil. EC50 values of extract in reducing power and DPPH assays were 0.19 and 0.18 mg/mL respectively. The FRAP values of extract and Trolox at concentration of 100 µg/mL didn't show any significant difference (P>0.05). The 400 ppm extract was as effective as 200 ppm BHA in retarding sunflower oil deterioration at 60^{0}C. The results indicated that Persian walnut green husk as a noticeable source of antioxidant compounds can usefully add to food.

Keywords: Persian walnut green husks; Total phenolics; Total flavonoids; Antioxidant power; sunflower oil.

INTRODUCTION

Food products, especially lipids and lipid-containing foods, are susceptible to oxidation. Edible oils with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation (Zhang et al., 2009; Assimopoulou et al., 2005). There are some precautions that can be taken to prevent or retard lipids from being oxidized in foods. The use of antioxidants is the most preferred way to inhibit lipid oxidation (Erkan et al., 2009).

Recently, some negative side effects of the commonly used synthetic antioxidants have been established. Reports revealed that these compounds may be implicated in many health risks, including cancer and carcinogenesis (Bran, 1975; Assimopoulou et al., 2005; Goli et al., 2005; Zhang et al., 2009).

Hence, there is a tendency towards the use of natural antioxidants of plant origin to replace these synthetic antioxidants.

Numerous types of natural antioxidants with various activities have been identified but a lot of attention has recently been drawn to the addition of polyphenols to foods and biological systems, due to their known abilities to scavenge free radicals (Goli et al., 2005; Thaipong et al., 2006; Ahmadi et al., 2007; Zhang et al., 2009). The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play important roles in neutralizing free radicals, chelating transitional metals, and quenching singlet and triplet oxygen molecules through delocalizing or decomposing peroxides. These properties are linked to beneficial health functionality of phenolic antioxidants due to their inhibitory effects against development of many oxidative-stress related diseases, such as cardiovascular, inflammatory bowel syndrome and Alzheimer's disease (Ahmadi et al., 2007; Mohammadzade et al., 2007; Oliveira et al., 2008)

Walnut (*Juglans regia L.*) is a valuable crop being the nut very popular and largely consumed. Not only dry fruits (nuts) but also green walnuts, shells, kernels, barks, green walnut husks (epicarp) and leaves have been used in both cosmetic and pharmaceutical industries (Stampar et al., 2006). Walnut's green husk is a by-product of

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walnut production, having scarce use. Thus, using husk as a source of phytochemicals will increase the value of the walnut production, as well as offer utilization for a by-product, which is produced in a large quantity. Different works demonstrated the potential antioxidant of walnut products, especially fruits, leaves and liqueurs which produced by green fruits (Pereira et al., 2007; Pereira et al., 2008; Stampar et al., 2006).

Stampar et al. (2006) identified thirteen phenolic compounds in walnut husks: chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myricetin, and juglone. Oliveira et al. (2008) determined that walnut green husks can be used as an easily accessible source of compounds with health protective potential and antimicrobial activity.

The aim of this study was to evaluate the antioxidant potential of Persian walnut green husk and stabilization of sunflower oil in accelerated oxidation systems, compared with synthetic antioxidants, including BHA and TBHQ.

MATERIALS AND METHODS

Chemicals and Reagent

BHA (2-tert-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), α-tocopherol, DPPH (2,2-Diphenyl-1-picrylhydrazyl), Trolox (6-Hydroxy-2,5,7-tetramethyl chroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were analytical grade and obtained from Merck (Darmstadt, Germany). Methanol was obtained from Dr Mojalali Company (Tehran, Iran), Sunflower oil was obtained from Behshahr Company (Tehran, iran).

Samples

Persian walnuts (*Juglans regia L.*) (Accession 1) were collected on June 2008 from research botanic garden of faculty of agriculture in Karaj. The trees were ten years old and 1 meter far from each other, being pruned when necessary. No phytosanitary treatments were applied. Approximately 10 kg of healthy fruits were handpicked, put in plastic bags and transferred to the laboratory immediately.

The green husks of walnuts were removed, dried in room temperature in a dark place and then were ground to give 20-mesh size powder.

Extraction of phenolics

Ground dry material (1 g) was weighed into a small flask. A total of 20 mL of 60% aqueous methanol was added and microwave-assisted extraction was done for 4 min after the pre-leaching (90 min) (Pan et al., 2003). The extract was filtered through S&S no. 589[1] paper. The residue was re-extracted with the same solvent. The combined solution was evaporated under reduced pressure at 40^0C to remove the solvent, redissolved in methanol

Determination of total phenolics

Total phenolics in the obtained extracts were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after that the absorbance was read at 725 nm using a spectrophotometer (BioQuest, CE 2505). Gallic acid was used for constructing the standard curve (0 - 0.075 mg/mL). The results were expressed as mg of gallic acid equivalents/100g of dried sample.

Estimation of total flavonoids content

The total flavonoids were determined using a colorimetric method as described by Mohammadzade et al., (2007). Briefly 0.1 mL of the phytochemical extract was diluted with 0.9 mL of methanol. Aliquots of diluted extracts (0.5 mL) were added to test tubes and mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate and 4.3 mL of methanol. After standing for 40 min at room temperature, the absorbance of the reaction mixture was measured at 415 nm. Quercetin was used as a standard compound in the range of 0–100 µg/mL concentration to construct a standard curve. The results were expressed as milligrams of quercetin equivalents per 100 gram of dried sample.

Antioxidant Activity

Radical-scavenging activity assay

The capacity of scavenging the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to the method that was reported previously(Hatano et al.1998; Oliveira et al., 2008). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5}mol/L). The mixture was shacked vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH _ AS)/ ADPPH] × 100, where AS was the absorbance of the solution when the sample extract had been added at a particular level and ADPPH was the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC50), was calculated from the graph of scavenging effect percentage against extract concentration. BHA and α-tocopherol were used as reference compounds.

Reducing power assay

The reducing power was determined according to a described procedure (Ferreira et al., 2007; oliveria et al., 2008). Various concentrations of sample extracts (2.5 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ^{0}C for 20 min. After incubation 2.5 mL of 10% tricloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a centrifuge (UNIVERSAL 320), for 8 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the graph of absorbance registered at 700 nm against the corresponding extract concentration. BHA and α-tocopherol were used as reference compounds.

The ferric reducing antioxidant power (FRAP) assay

Reagent preparation

Reagents were prepared according to the method of Benzie and Strains (1996). Working FRAP reagent was prepared as required by mixing 25 mL of 300 mM acetate buffer, pH 3.6 (3.1 g sodium

acetate and 16 mL glacial acetic acid per liter of buffer solution) with 2.5 mL of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution (0.031 g of TPTZ in 10 mL of 40 mM HCl) and 2.5 mL of 20 mM FeCl3.6H2O solution (3.24 g of ferric chloride in 1 l of distilled water). Freshly prepared reagent was warmed at 37 ^{0}C.

FRAP assay

Aliquots (100 µl) of each diluted methanolic extracts were mixed with 3 mL of freshly prepared FRAP reagent. The FRAP values were obtained by comparing the absorbance changes of blue colored ferrous- tripyridyltriazine complex at 593 nm in diluted methanolic extracts of samples with those containing ferrous ions in known concentrations(Benzie and Strains,1996). Aqueous solutions of known ferrous sulphate concentrations in the range of 100 – 1000 µM were used for calibration. In order to make comparison, Trolox was also tested under the same conditions as a standard antioxidant compound.

Oxidative stability determination according to the oven storage test

Samples included green husk extract (400 ppm), BHA (200 ppm), TBHQ (100 ppm) and control (no antioxidant) were transferred to equal beaker (O.D 5.5cm) in equal amounts.
The oven test method was conducted at 60 ^{0}C ± 1 ^{0}C (AOCS 1997). The samples were analyzed for peroxide values (PV) (AOCS, 1997) and odor at 3-days interval for 21 days. All the experiments were carried out in triplicate and results were averaged.

Statistical analysis

All data were expressed as mean ± standard deviation of three independent replicates. The results were statistically analyzed by analysis of variance (ANOVA) and significant differences among means from triplicate analyses at ($P < 0.05$) were determined by Duncan's multiple range test (DMRT) using the Statistical Analysis System (SAS 9.1).

RESULTS AND DISCUSSION

Extract yield

The amount of extractable components (extract yield) obtained from samples, was 499± 2.1 mg/g of dry plant material (49.9% ± 0.21).

Amount of total phenolics and flavonoids

Phenols are very important plant constituents; because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, phenolics of plants may contribute directly to their antioxidant action (Othman et al., 2007; Kubola, & Siriamornpun, 2008). Total phenolics and flavonoids of Persian walnut green husk amounted to 3428.11 ± 135.8 mg gallic acid equivalents 100^{-1}g and 144.65±2.1 mg quercetin equivalents 100^{-1}g respectively in dry sample (table 1).
A few studies were carried out on the total phenolics of walnut green husks. Stampar et al. (2006) reported that the amounts of total phenolics in walnut green husk were 1526 ± 111mg/100 g of dry weight. On the other hand oliveria et al. (2008) reported the total phenolics in walnut green husks ranging from 32.61 to 74.08 mg gallic acid in aqueous extracts with the extraction yield ranging 31.63 to 33.69%. These results indicated the higher phenolics and yield extracts in our samples. These differences may be due to the differences in genetic factors, environmental conditions, sampling date and methods of phenolics extraction. Results also indicated that while Persian walnut green husk amounted high phenolics, only 4 percent of these compounds were flavonoids, and the highest amount of phenolics were nonflavonoids.

Scavenging effect on DPPH radicals

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. DPPH is a free radical compound that has been currently used to determine the radical-scavenging ability of various compounds. It is a stable free radical which dissolves in methanol, has purple colour and a characteristic absorption at 517 nm. As antioxidants donate protons to this radical, the purple colour from the DPPH assay solution becomes light yellow resulting in a decreases in absorbance. The decrease in absorbance is taken as a measure of the extent of radical scavenging (Ferreira et al., 2007; Othman et al., 2007; Kubola et al., 2008).
In this assay, results were expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wave length. Fig. 1 shows the DPPH radical-scavenging capacity of extracted samples along with the reference compounds BHA and TBHQ. The sample curve demonstrated a concentration-dependent scavenging activity in lower concentrations and in a critical level of phenolics the curve became flat, i.e. the higher concentrations didn't increase antioxidant activity.
The results showed that TBHQ is an excellent DPPH radical-scavenger, with about 95.6% of DPPH scavenged at the level of 100 ppm and 91.6% radical scavenged at the level of 50 ppm. BHA and sample extract also showed 91.8% of radical scavenging, but at the levels of 200 and 400 ppm respectively. The radical scavenging activity of sample, BHA and TBHQ were equal, but at the levels of 400, 200 and 50 ppm respectively.
On DPPH assay, EC50 values were obtained for samples, BHA and TBHQ (table 2). A lower value of EC50 indicates a higher antioxidant activity. These results agreed with the previous study that was reported by Oliveria et al (2008). Although in both studies the EC50 values of walnut green husk extract are lower than 1 mg/mL, our results showed less EC50 (stronger scavenging effect). As explained before, this difference

Table 1. Amounts of total flavonoid and total phenolic compounds in Persian walnut green husk samples (mg/100g dried matters)

Sample	Total phenolic compounds [a]	Total flavonoid content [b]
Walnut green husk	3428.11 ± 135.80	144.65 ± 2.1

Data expressed as means ± SE of three samples analyzed separately.
a gallic acid equivalents
b quercetin equivalents

Table 2. EC50 values (mg/mL)

Sample	DPPH (EC50)	Reducing power (EC50)
Green husk extract	0.18 ± 0.004	0.19 ± 0.01
BHA	0.034 ± 0.008	0.022 ± 0.005
TBHQ	0.014 ± 0.002	ND
α-tocopherol	ND	0.07 ± 0.009

Data are given as means ± SE, n = 3.
ND= not detected

Table 3. FRAP value (µM) for Persian walnut husk methanolic extracts and Trolox.

Concentration (ppm)	Frap value (µmol)/l walnut	Trolox
100	120.2 ± 4.02[a]	125.25 ± 5.74[a]
1000	1223.33 ± 31.79	2437 ± 51.28

Data expressed as means ± SE of three samples analyzed separately.
a There is no significant differences between sample extract with Trolox(P>0.05).

may be due to walnut's genetic factors, environmental conditions, sampling date and methods of phenolics extraction.

Reducing power

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Ahmadi et al., 2007; Oliveria et al., 2008; Zhang et al., 2009). In the reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each extract. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe3+/ferricyanide complex to the ferrous form (Fe2+) monitored at 700 nm (Pereira et al., 2007; Pereira et al., 2008).In this assay, increased absorbance indicated increased reducing power. The reducing power of sample increased in a concentration-dependent manner (Fig. 2). EC50 values were calculated from the graph of absorbance registered at 700 nm against the corresponding concentrations (table 3). Oliveria et al. (2008) explained that walnut green husk extract revealed a strong reducing power.Our study showed the less EC50 value (stronger reducing power) and the reason of this difference explained before.

FRAP assay

FRAP assay, measures the reducing potential of an antioxidant reacting with a ferric 2,4,6-tripyridyltriazine (Fe^{3+}–TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}–TPTZ)) (Benzie and Strain, 1996).Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Katalinic et al., 2005). According to Benzie and Strain (1996), the reduction of Fe^{3+}–TPTZ complex to blue-coloured Fe^{2+}–TPTZ occurs at low pH with an absorption maximum at 593 nm. The FRAP assay reagents are inexpensive and simple to prepare, results are fast and reproducible and the equipment required is of a type that commonly found in biochemical laboratories.
Thaipong et al. (2006) expressed that among different methods in assessment of the antioxidant activity (ABTS,

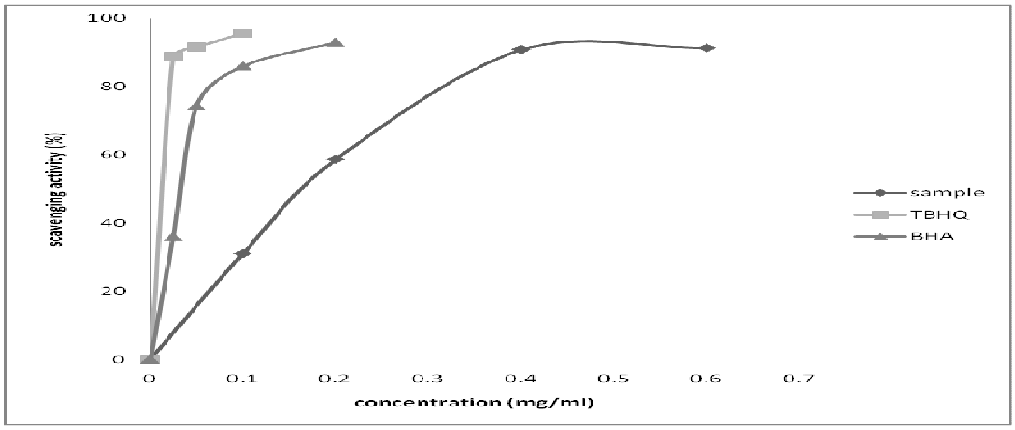

Figure 1. Radical-scavenging activities of walnut green husk extract, TBHQ and BHA by DPPH method at different concentrations (mg/mL).

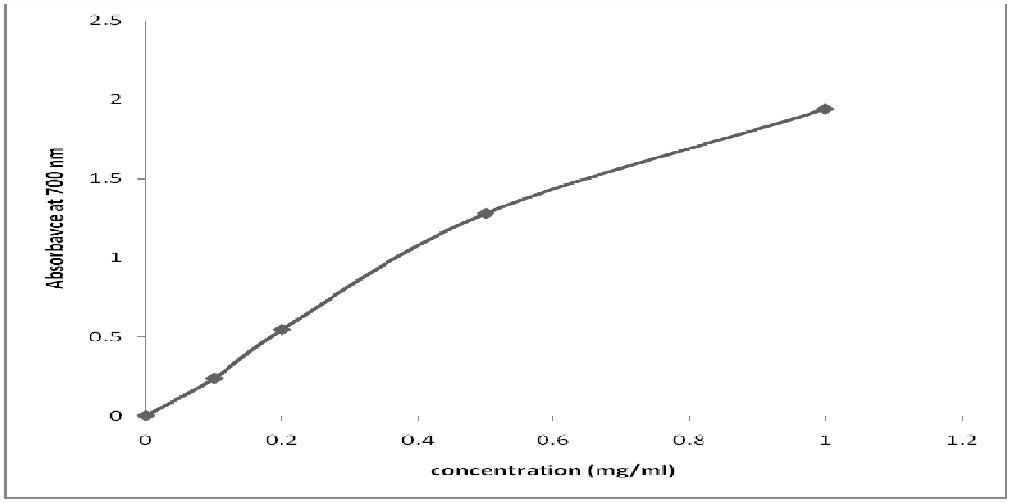

Figure 2. Methanolic extract reducing power values of Persian walnut green husk.

DPPH, FRAP, and ORAC assays), FRAP technique showed higher correlation with total phenolics.

The FRAP values have been calculated by comparing the absorbance changes in 593 nm in test samples with those containing ferrous ions in known concentrations ranging from 100 to 1000 µM. The antioxidant power of samples was compared with Trolox as a reference antioxidant (table 3). The results showed that the FRAP value of sample extract was comparable to Trolox at concentration of 100 µg/mL ($P>0.05$).

Oven test

At the 60^0C temperature oven test, undesirable alterations of the studied antioxidants such as decomposition, evaporation, etc. are less probable (Kovatcheva et al.,2001). The sample was used at level of 400 ppm (0.04 % w/w oil) because radical scavenging assay indicated that this level had high radical scavenging activity which didn't increased significantly by increasing the concentration. BHA and TBHQ were added at 200 and 100 ppm respectively, because oil industries used these compounds at these levels. The results were presented in Fig. 3. Production of off odor was coinciding with the decrease in PV, because of hydroperoxide decomposition, and the assay didn't continue afterward.

Figure. 1, 2 and 3 shows that the positive trend of peroxide value became slow by addition of the Persian walnut green husk extract to the oil sample which was

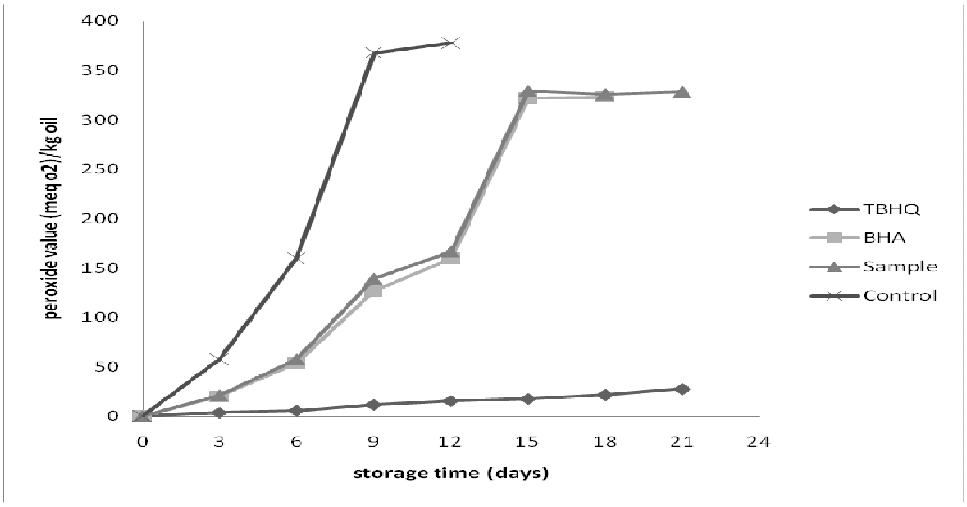

Figure 3. Effect of adding antioxidant on sunflower oil oxidation expressed as peroxide values (meq O2 kg^{-1} oil).

comparable to BHA without any significant difference between them ($p>0.05$).

In conclusion, the results of the present study apparently indicated that Persian walnut green husk may constitute a suitable source of phenolics and could be used as alternative natural antioxidants in food industries.

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