

Full Length Research Paper

Influence of environmental factors on antioxidant activity, phenol and flavonoids contents of walnut (*Juglans regia* L.) green husks

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Accepted 20 September, 2010

Antioxidant activities of methanol extract of walnut green husks (*Juglans regia* L.) from 11 regions of Iran with the different geographical and climatic conditions were measured. The phenolic and flavonoid contents of extracts were also evaluated. The antioxidant activities were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, nitric oxide scavenging and reducing power ability. Antioxidant activity has been compared with BHA, quercetin and vitamin C. All extracts were weaker than standards. The Chogholondi sample was found to have the better antioxidant activities in scavenging DPPH radicals however; Chino sample has better nitric oxide scavenging activity than others. The good correlation coefficient was existed between the phenolic and flavonoid contents with collection altitudes. Total phenolic contents were ranged from 15.15 to 108.11 mg gallic acid equivalents g⁻¹ of extract and the flavonoid contents varied from 3.59 to 22.91 mg quercetin equivalents g⁻¹ of extract. Among all extracts analyzed, Abali sample exhibited a significantly higher phenolic and flavonoid content than samples (p < 0.05).

Key words: *Juglans regia* L., antioxidant activity, phenol, flavonoid contents, walnut, green husks, 1,1-diphenyl-2-picrylhydrazyl.

INTRODUCTION

Recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease (Hou et al., 2003). Among the various medicinal and culinary plants, edible species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et

al., 2002; Dehpour et al., 2009). Additionally, animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as flavonoids in edible plants, exhibit potent antioxidant activities. So, recently interest has considerably increased in finding naturally occurring antioxidant to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis (Zhou et al., 2000). *Juglans regia* L. (Persian walnut) is a tree from *Juglandaceae* family. The walnut fruits consume extensively as a food, which are rich unsaturated fatty acids and its leaves has been widely used in traditional medicine for the treatment of skin

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Table 1. Collection data of the investigated *J. regia* L. green husks.

| Samples no. | Collection place | Latitude (Northern) | longitude (Eastern) | Altitude (m) | Annual raining average (mm) | Daily temperature average (°C) | Comparative humidity average (%) |
|-------------|--------------------------|---------------------|---------------------|--------------|-----------------------------|--------------------------------|----------------------------------|
| 1 | Ab-ali | 45° 35' | 51° 53' | 2465 | 525.3 | 8.6 | 50 |
| 2 | Afratakhte | 36° 50' | 55° 56' | 1500 | 593.8 | 16.5 | 60 |
| 3 | Esfahan | 32° 37' | 51° 40' | 1550 | 121.4 | 16.2 | 40 |
| 4 | Khoramabad | 32° 22' | 48° 22' | 1125 | 509 | 17.3 | 46 |
| 5 | Chino | 36° 55' | 54° 59' | 750 | 512.7 | 18.4 | 50 |
| 6 | Galikesh | 37° 14' | 55° 47' | 750 | 388.2 | 17.3 | 57 |
| 7 | Chogholondi | 33° 23' | 54° 59' | 1350 | 515.1 | 16.8 | 47 |
| 8 | Imam reza park (kordkoy) | 36° 52' | 54° 6' | 250 | 804.7 | 17.8 | 70 |
| 9 | Ghapan (kalale) | 37° 31' | 55° 49' | 850 | 421.8 | 18.6 | 60 |
| 10 | Alashtar | 33° 52' | 48° 15' | 1600 | 518.3 | 13.1 | 48 |
| 11 | Cheshme jozi | 36° 50' | 55° 7' | 1200 | 486.5 | 17.4 | 52 |

inflammations, hyperhidrosis and ulcers and for its anti-diarrheic, antihelmintic, antiseptic and astringent properties (Almeida et al., 2008). Anti-inflammatory, antinociceptive and antidiabetic activity of walnut leaves have been reported recently (Erdemoglu et al., 2003; Asgary et al., 2008; Said et al., 2008). Antioxidant effects of isolated tannin obtained from walnuts seeds and radical scavenging of kernel and leaves of *J. regia* have been recently reported (Fukuda et al., 2003; Almeida et al., 2008). Not only dry fruit (nuts) are used but also green walnuts, shells, kernels, bark, green walnut husks (epicarp) and leaves have been used in both cosmetic and pharmaceutical industry (Stampar et al., 2006; Oliveira et al., 2008). In the present study the effects of altitude, geographical and climatic conditions on antioxidant activities, phenol and flavonoids contents of methanol extract of *J. regia* green husks from 11 regions in Iran were evaluated. For determination of antioxidant activities, reducing power ability, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and nitric oxide radical scavenging were done. The results may be helpful to understanding of geographical and climate condition on biological activity of this useful plant.

MATERIALS AND METHODS

Plants material

J. regia L. green husks were collected from 11 different areas in Iran, in October 2008. Samples identified By Dr. Bahman Eslami. Voucher specimens are deposited with the faculty of pharmacy herbarium (No Ecc 24- Ecc 35). Altitudes, geographical and climatic conditions of gathering area are summarized in Table 1.

Preparation of extracts

Samples were dried at room temperature and coarsely ground

before extraction. A known amount of samples was extracted at room temperature by percolation method using absolute methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained. The yields were 18 to 23%.

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Nabavi et al., 2008; Ghasemi et al., 2009). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagents for 5 min and 2.0 ml of 75 g l⁻¹ sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using our recently published papers (Ebrahimzadeh et al., 2009). Briefly, 0.5 ml solution of each extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

DPPH radical-scavenging activity

The stable DPPH radical was used for determination of free radical scavenging activity of the extracts (Ebrahimzadeh et al., 2010a). Different concentrations of each extracts were added, at an equal volume, to methanol solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power

The Fe³⁺-reducing power of the extract was determined by the method of Yen and Chen with a slight modification. Different concentrations (50 - 800 µg/ml) of the extract (1 ml) were mixed

with 1 ml phosphate buffer (0.2 M, pH 6.6) and 1 ml potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 1 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.2 ml FeCl₃ solution (0.1%) was added. The absorbance was measured at 700 nm against an appropriate blank solution. All tests were performed triplicate (Ebrahimzadeh et al., 2010b).

Assay of nitric oxide-scavenging activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of medlar extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (Ebrahimzadeh et al., 2010c).

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range test. The IC₅₀ values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

Total phenol and flavonoid contents

Total phenol compounds are reported as gallic acid equivalents by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$) and flavonoids contents, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). The yield percent and total phenol and flavonoids contents are shown in Table 2. The maximum polyphenol and flavonoid contents were recorded in sample No. 1 (collected from Abali from highest altitude and lowest daily temperature average) and the lowest ones were recorded in Choghloni (No. 7) and Galikesh (No.6). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from natural sources, and they have been shown to possess significant antioxidant activities (Nabavi et al., 2010). In this study, the regression analyses indicated no correlations between collection place altitudes and phenolic contents (Figure 1, $r^2 = 0.21$) but a weak correlation found with flavonoid contents (Figure 1, $r^2 = 0.45$). Also, a weak correlation found between temperature and total phenolic contents (Figure 1, $r^2 = 0.40$). A better correlation found with flavonoid contents

(Figure 1, $r^2 = 0.58$).

DPPH radical-scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Nabavi et al., 2009). It was found that the radical-scavenging activities of all the extracts increased with increasing concentration. IC₅₀ for DPPH radical-scavenging activity exist in Table 2. Among these samples tested in this study, the Choghloni (No. 7) sample had the strongest radical scavenging activity (IC₅₀ $122 \pm 4.5 \mu\text{g ml}^{-1}$) while the Imam Reza Park sample (No. 8) exhibited the lowest radical scavenging activity (IC₅₀ $302 \pm 13 \mu\text{g ml}^{-1}$).

Reducing power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim et al., 2001). In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Nabavi et al., 2010). Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 2 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all the extracts also increased with the increase of their concentrations. There were no significant differences ($p > 0.05$) among extracts in reducing power. Vitamin C showed better activity ($p < 0.05$). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several extracts (Ebrahimzadeh et al., 2010 d, e).

Assay of nitric oxide-scavenging activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The percentage of inhibitions was increased with increasing concentration of the extracts. Results exist in Table 2. Chino collected sample extract exhibit better

Table 2. Antioxidant activity, total phenol and flavonoids contents of *J. regia* L. green husks. For compounds 1-11 refer to Table 1.

| Sample no. | Total phenol content ^a | Flavonoid content ^b | Nitric oxide scavenging (IC ₅₀ µg ml ⁻¹) ^c | DPPH free radical scavenging (IC ₅₀ µg ml ⁻¹) ^d |
|------------|-----------------------------------|--------------------------------|--|---|
| 1 | 108.11 ± 4.6 | 22.91 ± 1.1 | 2890 ± 121 | 186 ± 8.1 |
| 2 | 37.24 ± 1.2 | 12.94 ± 0.5 | 303 ± 13 | 166 ± 6.1 |
| 3 | 56.02 ± 2.3 | 9.67 ± 0.4 | 161 ± 4.7 | 140 ± 6.5 |
| 4 | 57.29 ± 2.4 | 7.88 ± 0.2 | 146 ± 6.1 | 140 ± 5.3 |
| 5 | 70.39 ± 3.1 | 4.56 ± 0.1 | 141 ± 0.4 | 164 ± 7.1 |
| 6 | 16.88 ± 0.9 | 3.59 ± 0.1 | 209 ± 5.9 | 232 ± 9.3 |
| 7 | 15.15 ± 0.4 | 11.85 ± 0.4 | 161 ± 4.1 | 122 ± 4.5 |
| 8 | 20.12 ± 0.6 | 10.86 ± 0.3 | 646 ± 22 | 302 ± 13 |
| 9 | 46.08 ± 1.7 | 10.11 ± 0.3 | 357 ± 16 | 285 ± 11 |
| 10 | 50.97 ± 2.4 | 8.82 ± 0.2 | 1101 ± 49 | 189 ± 6.7 |
| 11 | 57.92 ± 2.1 | 9.92 ± 0.4 | 398 ± 16 | 142 ± 5.2 |

^a mg gallic acid equivalent/ g extract, ^b mg quercetin equivalent/ g extract, ^c IC₅₀ for quercetin was 17, ^d IC₅₀ for BHA, quercetin and vitamin C were 53.96 ± 3.2, 5.28 ± 0.3 and 5.05 ± 0.2 respectively, data presented as Mean ± SD.

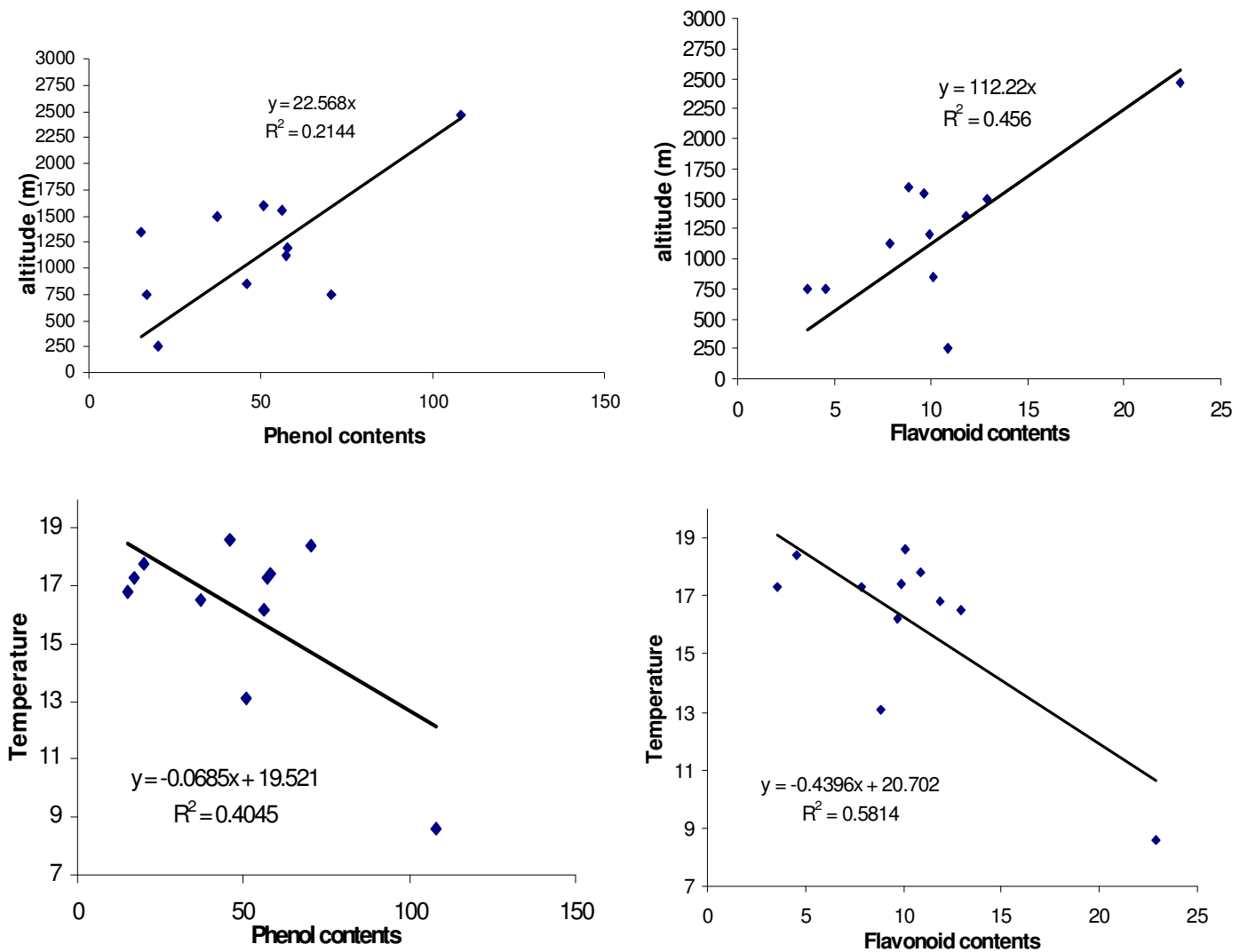


Figure 1. Correlation between phenol and flavonoids contents, collection place altitude and temperature in *J. regia* L. green husks.

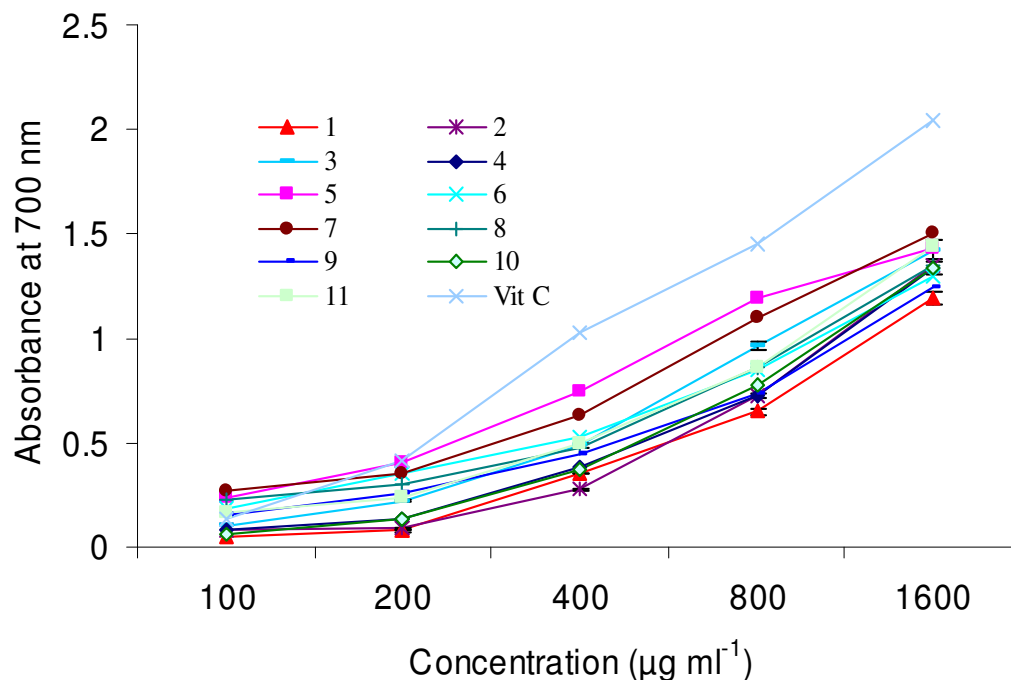


Figure 2. Reducing power of extracts. Vitamin C used as control. For compounds 1-11 refer to Table 1.

activity than the others ($IC_{50} = 141 \pm 0.4 \mu\text{g ml}^{-1}$). Sample No 1 showed lowest activity ($IC_{50} = 2.89 \pm 0.12 \text{ mg ml}^{-1}$). However the activity of quercetin was very more pronounced than extracts ($17 \pm 1.5 \mu\text{g ml}^{-1}$). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada et al., 1991).

Conclusion

There are significant differences between phenolic and flavonoids contents of samples. All tested extracts showed good antioxidant activity in some tests. The above results indicated that geographical and climatic condition in different region could lead to significant differences both in the content of bioactive compounds and their bioactivities. *J. regia L.* green husks can be a source of plant antioxidants, with a potential use in food, cosmetics and pharmaceutical fields. Further investigation of individual compounds, their *in vivo* antioxidant activities and in different antioxidant mechanisms is needed.

ACKNOWLEDGEMENT

This research was partially supported by a grant from the research council of Medical Sciences University of Mazandaran, Iran.

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