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Anti-radical activity of different parts of walnut

***Tehmeena Ahad, Jasia Nissar and Dr. A.H.Rather**

*Division of food science and technology Skuast –k Shalimar 190025

ABSTRACT

The objective of this work was to analyse phenolic compounds and antiradical capacity of different parts of walnut fruit among six genotypes of Juglans regia L. Therefore, total phenolic and flavonoid content were determined and methanolic extracts of walnut genotypes were considered by the reducing power, DPPH (2,2-diphenyl-1-picrylhydrazyl), superoxide anion and nitric oxide radical scavenging. Significant differences were found in phenolic content and radical scavenging capacity of different parts of fruits. High correlation coefficient (R = 0.81) was observed between phenol content and radical scavenging activity, but this was not always true (R2 = 0.01). These results demonstrated that walnut genotypes have different phenolic compounds and phenolic compounds have different radical scavenging power. The differences of phenolic compounds were confirmed by using high performance liquid chromatography (HPLC).

Key Words:- Walnuts, DPPH, Co-Relation, Genotype

I INTRODUCTION

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, Solar, Hudina, Veberic, & Colaric, 2006). Walnut's green husk is a by-product of 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 utilisation 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, 2008; Stampar et al., 2006).

Phenolic compounds are bioactive substances widely distributed in plants and are important constituents of the human diet. Plant phenolics comprise a great diversity of compounds, such as flavonoids (anthocyanins, flavonols, flavones, etc.) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes). Walnuts are particularly rich in total phenolic content. The total phenol content among nuts varies widely, with pecans, pistachios, and walnuts being the richest sources and Brazil nuts, macadamias, and pine nuts containing the lowest concentrations (Kornsteiner, Wagner, & Elmadfa, 2006). Antioxidant activity of phenolic compounds varies with the types and molecular structures (Maqsood & Benjakul, 2010). Research has shown that aflatoxin production by fungi isolated from tree nuts and figs is markedly decreased by the presence of natural

antioxidants that occur in nut trees, including walnut hydrolysable tannins, flavonoids and phenolic acids. In vitro testing of individual compounds showed that the anti-aflatoxinogenic effect correlates with the structure and concentration of such compounds in individual nut varieties and species (Molyneux, Mahoney, Kim, & Campbell, 2007). The structure– activity relationship evaluation of these phenolic compounds suggested that the number of hydroxyl groups was the most important factor in determining the antioxidant activities of the phenolic compounds (Zhang, Liao, Moore, Wu, & Wang, 2009). The levels of 10 particular phenolic compounds in walnut liquor highly depended on the cultivar and the picking time of walnut fruit (Jakopic et al., 2007). The results further support the fact that skins of walnuts, which are a rich source of phenolics, are responsible for effective scavenging of free radicals (Samaranayaka, John, & Shahidi, 2008). 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. Pereira et al. (2007) determined that walnut green husks can be used as an easily accessible source of compounds with health protective potential and antimicrobial activity.

Significant environmental effects on phenolic and antioxidant properties likely reflect the role of such factors in the synthesis of phenolic and other antioxidant compounds. Currently, there is a considerable amount of interest in dietary antioxidants as bioactive components of food. The extracts and pure phenolic compounds from J. regia L. might also be used as natural antioxidants and alternatives to synthetic antioxidants such as BHT (2,6-ditert-butyl-4-methylphenol) (Zhang et al., 2009). Their contents depend on many environmental conditions, as well as genotype of different cultivars (Mpofu, Sapirstein, & Beta, 2006). The aim of this study was to ascertain the influence of genotype factor on total phenolic content as well as on some potential antiradical activity in different genotypes of J. regia L. pellicles, hulls, shells and kernels.

II MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co.

Sample preparation

Materials were taken from six walnut genotypes for analyses, in Kolyaei region located in Kermanshah, Iran at the end of September 2009. Green walnut fruits at full maturity were picked by hand and each of six genotypes selected from those in Kanikareh (three genotypes, K1, K2, K3), Birgholi (two genotypes, B1, B2), and Gerdakaneh (one genotype G1).

Preparation of methanolic extracts

The fruits were collected and their hull, shell, pellicle (brown skin) and kernels were separated. Then they were dried at room temperature and reduced to fine powder. This powder (1.5 g) was extracted with pure methanol (25 ml) in a soxhlet apparatus at 60 C for 30 min (Wijeratne, Abou-Zaid, & Shahidi, 2006). The samples were

centrifuged for 20 min at 4000g. The supernatant was filtered through filter paper and stored at 4 C until analysis for one week.

Determination of total phenolic content

The concentrations of phenolic compounds in all walnuts were expressed as mg gallic acid equivalents (GAEs), determined with Folin–Ciocalteu Reagent (FCR) according to the method of Slinkard and Singleton (1997) with minor modifications. Briefly, some appropriate dilutions of the filtered extracts (0.25 ml) were added to distilled water (11.5 ml) and FCR (0.25 ml) and mixed thoroughly. After 3 min, the reaction mixture was neutralised with 2% sodium carbonate (0.75 ml) and shaken intermittently at room temperature for 2 h. The absorbance was read at 760 nm by using a spectrophotometer (Biowave, WPA S2100, UK). The concentrations of phenolic compounds were calculated according to the following equation that was obtained using gallic acid standard curve and stated as the mean [mg of gallic acid equivalents (GAEs) per 100 g of sample] ± SE for the triplicate extracts:

Concentration ¼ FðAbsorbanceÞ ¼ 64:25X 0:0015ðR^2 ¼ 0:99Þ; X ¼ Absorbance:

Total flavonoid content assay

The total flavonoid content of the all extracts was quantified using a modified colorimetric method (Yang, Liu, & Halim, 2009). Briefly, 1:10 diluted extracts (0.25 ml) was mixed with distilled water (1.25 ml) and subsequently with 5% sodium nitrite solution (0.07 ml) and allowed to react for 5 min. Then 10% aluminium chloride solution (0.15 ml) was added and allowed to react further for 6 min before addition of one molar sodium hydroxide (0.5 ml). Finally, distilled water was added to all samples in 1 ml portions. The absorbance of the mixture was immediately measured at 510 nm. The flavonoid content was determined by using a catechin standard curve and stated as the mean [mg catechin equivalents (CEs) per 100 g of sample] ± SE for the triplicate extracts:

Concentration ¼ FðAbsorbanceÞ ¼ 253:89X þ 0:0301ðR^2 ¼ 0:9902Þ; X ¼ Absorbance:

Reducing power assay

The reducing power of pellicle, hull, shell and kernel extracts were determined according to the method of Oyaizu (1986). The solution extracts in methanol (50 ll) were mixed in 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 C for 20 min. Afterwards 2.5 ml of TCA (10%) was added. Then, the mixture was centrifuged for 10 min at 1000g. Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% ferric chloride (0.5 ml), and the absorbance was read 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 (2-tert-butyl-4-methoxyphenol) was used as the reference compound.

DPPH radical scavenging activity assay

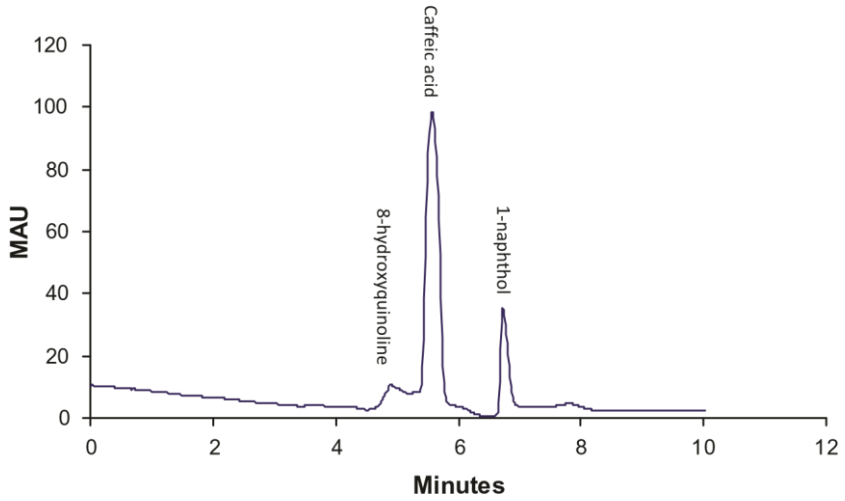
DPPH radical scavenging activity was determined as described by Wu, Chen, and Shiau (2003) with slight modification. Methanolic extracts (0.5 ll) were added to 0.15 mmol/l 2,2-diphenyl-1-picrylhydrazyl (DPPH) (1.5 ml) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 515 nm. The percentage of DPPH radical scavenging activity (RSA) by each sample extract was calculated using the following equation:

RSA% ¼ ðA$_0$ A$_1$Þ=A$_0$ 100:

Where A$_0$ is the absorbance of control, A$_1$ is the absorbance of the sample. The extract concentration providing 50% inhibition (EC50) was calculated from the graph of the scavenging effect percentage against the corresponding extract concentration. BHA was used as the reference compound.

Superoxide radical inhibition assay

For superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol autoxidation system (Jing & Zhao, 1995). A volume of 9 ml of Tris–HCl buffer solution (50 mmol/l, pH 8.2) was added into a test tube and incubated in a water bath at 25 C for 20 min. Then 50 ll of pyrogallol solution (45 mmol/l of pyrogallol in 10 mmol/l of HCl), which was also preincubated at 25 C, was injected to the above test tube with a microlitre syringe and were mixed up. The mixture was incubated at 25 C for 3 min. Finally, a drop of ascorbic acid (0.002 mol/l) was dripped into the mixture promptly to terminate the reaction. The absorbance at 420 nm marked as A$_0$ was measured 5 min later, and this A$_0$ denotes the speed of pyrogallol autoxidation. The A$_1$ autoxidation speed was obtained applying the above method and with the addition of 25 ll of extract into the Tris–HCl buffer solution. Simultaneously, a blank control of reagent was obtained as A$_2$.



Nitric oxide radical inhibition assay

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture containing sodium nitroprusside (10 mmol/l, 2 ml), phosphate buffer saline (0.5 ml) and walnut pellicle, hull, shell and kernel extracts (50 ll) was incubated at 25 C for 150 min. After incubation, the reaction mixture (0.5 ml) was mixed with sulfanilic acid reagent (0.33% in 20% glacial acetic acid) (1 ml) and allowed to stand for 5 min for completing diazotization. Then, naphthyl ethylenediamine dihydrochloride (1 ml) was added, mixed and allowed to stand for 30 min at 25 C. A pink coloured chromophore was formed in diffused light. Finally, 2 ml of distilled water were added to all samples. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

Nitric oxide radical scavenging% ¼ A_{blank} A_{sample} 100=A_{blank}

Extraction and hydrolysis for HPLC

For HPLC analysis, 500 mg of dried and powdered plant material was extracted with 50% methanol/water for 2 h at room temperature. The plant extract was hydrolysed with 1.2 M HCl by refluxing in a water bath for 1 h. All samples were filtered though a 0.45 lm pore size syringe-driven filter before injection (Hertog, Hollman, & Venema, 1992).

Chromatographic separation of phenolic compounds

A 20 ll aliquot of sample solution was separated using a HPLC system (Knuer, Germany) equipped with UV–Vis detector and a eurospher 100–5 C-18 column (25 cm 4.6 mm; 5 lm). The mobile phase consisted of purified water with 2% acetic acid (A) and acetonitrile (B). Solvent gradient was used as follows: from 0 to 5 min isocratic 85% A flow, from 5 to 19 min (14 min) a linear gradient of 85% A to 100% B. After termination of the cycle, 15 min of column equilibration (85% A) were allowed prior next injection.

Phenolic compounds were eluted under the following conditions: 0.5 ml/min flow rate, and the temperature was set at 25 C. Phenolic compounds were detected at a wavelength of 254 nm and identified by comparing their relative retention times and UV spectra with authentic compounds and were detected using an external standard method. Typical HPLC chromatogram of the walnut hull extract of the cultivar K3 recorded at 254 nm (Fig. 1).

Statistical analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) of the mean. Data analyses were performed using SPSS software version 17 and the means were compared using Duncan's multiple range (DMRT) test at $p < 0.05$ following analysis of variance (ANOVA).

III RESULTS AND DISCUSSION

Total phenolic and flavonoid content

The amount of total phenolics varied widely in plant materials and ranged from 112 (kernel of genotype K1) to 6059 (pellicles of genotype B2) mg GAE/100 g (Table 1). There were significant differences among phenolic contents in pellicle, hull and shell but not in kernel. The highest level of phenolic content was observed in pellicle (6059). Furthermore, the results obtained from evaluation of total flavonoid content also indicated great variations (Table 1). In the pellicles, the content of flavonoid compounds as catechin equivalents were notably higher (966 to 1495 mg CE/100 g) than in kernels (61 to 124 mg CE/100 g); significant differences ($p < 0.05$) were also observed between all sections in all genotypes tested. The decrease of total phenolic and flavonoid contents is most probably caused by the increase of sulphur-containing compounds and terpenoid substances present in the essential oil of kernels. Measurement of total phenolic content approximates the amount of phenolic compounds present without distinguishing between phenolic structures. The genotype differences reported in total phenolic content of walnut or walnut fractions likely indicate that genotype has a significant influence on the biosynthesis and accumulation of one or more of these phenolic compounds.

Reducing power

Reducing power is to measure the reductive ability of antiradical and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of sample extracts. Higher absorbance of the reaction mixture indicates greater reducing power. The reducing power of methanolic plant extracts are summarised in Table 2. EC50 values were obtained for samples and BHA (Table 2). In compare with BHA, the extracts from pellicles had higher reducing power than those obtained from the other parts (Table 2), but higher correlation coefficient was observed in shell extracts ($R^2 = 0.96$) (Table 3). In addition, significant differences ($p < 0.05$) were observed among all sections in all genotypes tested. However, reducing power of

methanolic extract was lower in the kernel of genotypes. The reducing properties are generally associated with the presence of reductants which have been shown to exert antiradical action by donating a hydrogen atom and breaking the free radical chain.

Table 1

Total phenolic [gallic acid equivalents (GAEs)] and flavonoid contents [catechin equivalents (CE)] in six genotypes of J. regia L. pellicles, hulls, shells and kernels: 1: K1, 2: G1, 3: B1, 4: K2, 5: K3, 6: B2.

Data are means of three replicates with standard errors (Mean ± S.E, n = 3), $p < 0.05$. Values in the same column with different letters present significant differences $p < 0.05$.

Table 2

DPPH, superoxide, nitric oxide radical scavenging (%) and reducing power in six genotypes of J. regia L. pellicles, hulls, shells and kernels: 1: K1, 2: G1, 3: B1, 4: K2, 5: K3, 6: B2.

Genotype	DPPH		Superoxide scavenging (%)	Nitric oxide scavenging (%)	Reducing power	
	Radical scavenging (%)	EC50 (mg/ml)			(700 nm)	EC50 (mg/ml)
1	ae 78.9 ± 0.64	0.14 ± 0.01	a 136.28 ± 3.97	a 87.53 ± 0.87	acd 1.32 ± 0.01	0.009 ± 0.002
2	a 73.77 ± 0.82	0.15 ± 0.002	bc 123.39 ± 0.90	b 68.26 ± 1.33	a 1.27 ± 0.04	0.03 ± 0.01
3	c 53.83 ± 2.83	0.20 ± 0.04	b 120.98 ± 1.88	c 40.60 ± 3.38	b 1.09 ± 0.00	0.06 ± 0.01
4	ed 86.24 ± 0.42	0.12 ± 0.021	ac 134.42 ± 1.55	b 75.35 ± 1.41	d 1.38 ± 0.01	0.006 ± 0.003
5	c 56.87 ± 1.98	0.18 ± 0.04	bc 123.46 ± 2.80	e 52.66 ± 3.86	b 1.11 ± 0.01	0.05 ± 0.02
6	d 90.38 ± 0.4	0.09 ± 0.011	ac 132.32 ± 1.62	a 86.58 ± 0.97	ad 1.37 ± 0.03	0.008 ± 0.001
Mean	73.33 ± 1.18	0.15 ± 0.021	128.48 ± 2.12	68.50 ± 1.97	1.25 ± 0.02	0.03 ± 0.008
Hull 1	a 17.27 ± 0.08	0.25 ± 0.005	ac 238.87 ± 2.32	a 81.67 ± 0.73	a 0.88 ±	0.08 ± 0.02

					0.02		
2	b 14.35 ± 0.11	0.33 ± 0.018	ab 246.18 ± 1.83	bc 67.15 ± 2.27	b 0.61 ± 0.01	0.11 0.04	±
3	c 7.99 ± 0.06	0.40 ± 0.02	c 236.32 ± 2.71	b 71.75 ± 1.97	c 0.37 ± 0.01	0.25 0.08	±
4	d 8.96 ± 0.04	0.37 ± 0.063	ab 246.95 ± 1.36	bc 65.82 ± 1.69	c 0.46 ± 0.02	0.14 0.07	±
5	e 11.97 ± 0.1	0.39 ± 0.028	b 253.82 ± 0.19	bc 65.82 ± 1.69	c 0.43 ± 0.03	0.20 0.05	±
6	f 16.43 ± 0.12	0.31 ± 0.045	ab 246.62 ± 0.67	c 62.85 ± 1.09	b 0.63 ± 0.01	0.10 0.04	±
Mean	12.83 ± 0.08	0.34 ± 0.03	244.76 ± 1.52	69.18 ± 1.57	0.56 ± 0.02	0.15 0.05	±
Shell 1	a 8.37 ± 0.09	0.39 ± 0.001	a 105.15 ± 2.23	a 73.78 ± 3.23	a 0.47 ± 0.01	0.20 0.05	±
2	a 8.95 ± 0.05	0.27 ± 0.034	a 111.83 ± 2.98	a 67.22 ± 3.80	b 1.02 ± 0.03	0.09 0.04	±
3	a 8.77 ± 0.04	0.44 ± 0.071	a 108.33 ± 0.85	a 70.44 ± 0.62	c 0.32 ± 0.01	0.30 0.08	±
4	b 4.7 ± 0.00	0.48 ± 0.014	a 108.45 ± 2.02	a 68.22 ± 0.97	d 0.17 ± 0.00	0.38 0.06	±
5	bc 5.25 ± 0.28	0.47 ± 0.024	a 109.01 ± 2.51	a 65.11 ± 3.57	d 0.22 ± 0.01	0.34 0.04	±
6	d 7.08 ± 0.2	0.41 ±	a	a	a	0.28	±

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		0.003	105.94 ± 1.65	76.78 ± 0.99	0.45 ± 0.01	0.09
Mean	7.19 ± 0.11	0.41 ± 0.024	108.12 ± 2.04	70.26 ± 2.19	0.44 ± 0.01	0.26 ± 0.06
Kernel 1	a 0.47 ± 0.04	0.95 ± 0.028	a 73.27 ± 0.20	a 18.04 ± 1.69	a 0.14 ± 0.0	0.87 ± 0.24
2	bcd 1.31 ± 0.1	0.90 ± 0.014	b 52.14 ± 1.70	b 5.33 ± 1.55	be 0.12 ± 0.0	0.81 ± 0.18
3	ab 0.78 ± 0.17	0.75 ± 0.007	a 73.74 ± 1.37	ab 8.37 ± 0.50	c 0.11 ± 0.0	0.80 ± 0.27
4	c 1.93 ± 0.19	0.69 ± 0.061	c 65.81 ± 0.86	ab 8.96 ± 0.78	ab 0.13 ± 0.0	0.65 ± 0.1
5	abi 1.03 ± 0.08	0.67 ± 0.036	cd 61.46 ± 0.94	ab 6.58 ± 0.84	ce 0.12 ± 0.0	0.61 ± 0.06
6	cdi 1.7 ± 0.05	0.65 ± 0.004	bd 56.95 ± 0.90	ab 11.96 ± 5.55	a 0.14 ± 0.0	0.52 ± 0.01
Mean	1.2 ± 0.10	0.77 ± 0.025	63.89 ± 0.100	9.87 ± 1.82	0.13 ± 0.0	0.71 ± 0.14
BHA	94.07 ± 0.1	0.054 ± 0.011	–	–	1.52 ± 0.28	0.005 ± 0.001

Data are means of three replicates with standard errors (Mean ± S.E, n = 3), $p < 0.05$. Values in the same column with different letters present significant differences $p < 0.05$.

Genotype	Pellicle	Hull	Shell	Kernel
Phenolic contents (mg/100 g) 1	ac 5396 ± 129	a 3610 ± 55	a 1968 ± 80	a 112 ±.00
2	abd 5068 ± 110	b 2533 ± 82	b 3211 ± 5	a 115 ± 12
3	b 4365 ± 46	c 1961 ± 59	c 1689 ± 52	a 140 ± 16
4	cd 5725 ± 372	c 2174 ± 1	d 939 ± 35	a 166 ± 19
5	ab 4615 ± 23	c 1970 ± 17	d 1108 ± 49	a 178 ± 15
6	c 6059 ± 83	b 2561 ± 41	ac 1911 ± 28	a 161 ± 11
Mean	5205 ± 127.61	2468 ± 42.84	1804 ± 42.02	145 ± 12.58
Flavonoid contents (mg/100 g) 1	a 1495 ± 29	a 826 ± 32	a 591 ± 26	a 61 ± 12
2	b 1064 ± 81	b 1064 ± 81	b 811 ± 41	a 70 ± 11
3	c 810 ± 34	b 617 ± 40	ac 500 ± 66	ab 96 ± 3
4	b 1178 ± 34	b 631 ± 50	cd 314 ± 46	ab 85 ± 5
5	bc 966 ± 59	b 537 ± 15	d 301 ± 19	b 124 ± 5
6	b 1064 ± 14	b 534 ± 6	ad 403 ± 22	b 119 ± 8
Mean	1096 ± 42.27	609 ± 28.12	486 ± 37.22	93.07 ±

				7.86

DPPH radical scavenging activity

Table 3

Correlations among the total phenol content, flavonoid content, DPPH radical scavenging, superoxide scavenging, nitric oxide scavenging and reducing power in six genotypes of J. regia L. pellicles, hulls, shells and kernels.

	Phenolic contents (mg GAEs/g extract)	Flavonoid contents (mg CEs/g extract)	DPPH radical scavenging (%)	Superoxide scavenging (%)	Nitric oxide scavenging (%)	Reducing power (700 nm)
Pellicle Phenolic contents	1	0.221	0.813	0.369	0.645	0.743
Flavonoid contents		1	0.298	0.527	0.560	0.386
DPPH radical scavenging			1	0.547	0.824	0.89
Superoxide scavenging				1	0.637	0.589
Nitric oxide scavenging					1	0.785
Reducing power						1
Hull Phenolic contents	1	0.437	0.635	0.110	0.433	0.945
Flavonoid contents		1	0.039	0.24	0.524	0.313
DPPH radical scavenging			1	0.003	0.055	0.763
Superoxide scavenging				1	0.456	0.043
Nitric oxide scavenging					1	0.276
Reducing power						1
Shell Phenolic contents	1	0.793	0.625	0.056	0.018	0.957
Flavonoid contents		1	0.636	0.055	0.000	0.765
DPPH radical scavenging			1	0.002	0.035	0.46
Superoxide scavenging				1	0.133	0.08
Nitric oxide scavenging					1	0.001

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Reducing power						1	
Kernel Phenolic contents	1	0.393	0.099	0.013	0.083	0.003	
Flavonoid contents		1	0.068	0.057	0.071	0.025	
DPPH radical scavenging			1	0.226	0.096	0.037	
Superoxide scavenging				1	0.168	0.012	
Nitric oxide scavenging					1	0.334	
Reducing power						1	

The ability of the investigated walnut extracts to act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its reduced form of DPPH-H was investigated in DPPH assay. All the assessed extracts revealed a reduction in stability, purplecoloured radical DPPH into the yellow-coloured DPPH-H. The values were as follows: 53.83% to 90.38% for extract of pellicles (1), 7.99% to 17.27% for extract of hulls (2), 4.7% to 8.95% for extract of shells (3), 0.47% to 1.93% for extract of kernels (4) and 94.07 for BHA as a reference compound (Table 2). On DPPH assay, EC50 values were obtained for samples and BHA. A lower value of EC50 indicates a higher antiradical activity (Table 2). In compare with BHA, the extracts from pellicles had higher scavenging capacity than those obtained from the other parts and this activity was phenol content-dependent (R^2 = 0.81) (Table 3). In addition, significant differences were observed among all sections in genotypes tested ($p < 0.05$). Using the same volume, the descending order of DPPH radical scavenging activity of the methanolic extracts of each genotype was as follows: pellicle > hull > shell > kernel. Low correlation coefficient was observed between phenol content and radical scavenging activity in kernel extract (R^2 = 0.1) (Table 3). The carboxyl group is an electron-withdrawing group, which does not benefit the radical scavenging activity of the compound (Thiago Inacio, Roberta, Nidia, & Neli, 2008), inversely the presence of higher numbers of hydroxyl groups in phenolic compounds most likely associated with the increased DPPH radical scavenging activity (Maqsood & Benjakul, 2010). Thus, the chemical nature and content of phenolics present in each walnut fraction may have a profound effect on their antiradical activity. The earlier published data (Samaranayaka et al., 2008) also indicated great scavenging capacity for pellicle (tan/brown-coloured pellicle), but there were differences in results of scavenging activities of walnut extracts, their relationship to the content of different phenolic compounds.

Also it is mentioned that extracts fractions depended upon the extraction solvents employed (Alasalvar et al., 2009). The results further support the fact that pellicles of walnuts, which are a rich source of phenolics, are responsible for effective scavenging of free radicals.

Superoxide radical scavenging activity

The scavenging capacity of walnut pellicle, hull, shell and kernel extracts towards superoxide anion radicals was evaluated by using a pyrogallol autoxidation system. There were significant differences in the inhibition effects of walnut pellicle, hull, shell and kernel extracts on the autoxidation of pyrogallol in studied genotypes (Table 2).

The scavenging capacity was in the decreasing order of: pellicle > hull > shell > kernel, but in relation with total phenolic content and scavenging capacity of superoxide it is noteworthy that although the difference between phenolic content in genotypes for parts (pellicle, hull and shell) is mostly significant ($p < 0.05$) (Table 1), but not in the O_2 scavenging capacity (shell parts) (Table 2). In previous studies, it was proven that these effects do not always correlate with the presence of large quantities of phenolics (Bozin, Mimica-Dukic, Samojlik, Goran, & Igic, 2008). These results suggest that phenolic compounds with different capacity for scavenging radicals were present in all genotypes. Also, ESR measurements of the phenolic acids (Zhou, Yin, & Yu, 2006), showed that syringic acid, as the 3,5-dimethoxy derivative of 4-OH benzoic acid had strongest O_2 scavenging activity among all tested phenolic acids, followed by ferulic and 4-coumaric acids indicating that an additional O-methoxy (OCH_3) group on the phenyl ring may enhance the O_2 scavenging activity of either benzoic or cinnamic acid derivatives. These results clearly suggested that the antiradical activity of perusal walnut genotypes was related to molecular structures of phenolic compounds, in addition phenolic content.

Nitrite oxide radical scavenging activity

Nitric oxide generated from nitroprusside reacts with oxygen to form nitrite. Scavengers of nitric oxide compete with oxygen leading to reduction of nitrite production (Marcocci, Packer, Droy-Lefai, Sekaki, & Gardes-Albert, 1994). The inhibitory effects of walnut

Table 4
Phenolic content (mg/100 g) of walnut pellicle and hull in different cultivars.

	Pellicle						Hull					
Genotype	1	2	3	4	5	6	1	2	3	4	5	6
Kl	18.0 ± 1.39	11.7 ± 1.01	10.5 ± 1.04	20.1 ± 1.4	–	–	–	–	–	70.5 ± 8.75	–	–
Gl	–	–	–	–	–	–	–	–	–	47.0 ± 2.26	–	–
Bl	–	111.8 ± 3.23	24.3 ± 1.8	392.4 ± 17.47	7.82 ± 0.75	–	111.5 ± 2.54	–	110 ± 3.37	–	2.7 ± 0	12–
K2	–	–	16.0 ± 1.44	114.9 ± 3.32	–	254.2 ± 8.8S	120.4 ± 4.19	–	9.0 ± 0.7	–	1.9 ± 0	13–
K3	1429.7 ± 95.4	–	–	–	–	–	–	35.2 ± 2.01	–	383 ± 15.04	16.5 ± 1.41	± –
B2	63.4 ± 2.63	–	–	11.1 ± 1.5	–	–	–	213.9 ± 3.05	44.5 ± 2.45	–	–	–

(1) Tannic acid; (2) 8-hydroxyquinoline; (3) Salicylic acid; (4) Caffeic acid; (5) 1-naphthol; (6) Vanillic acid.
Data are means of three replicates with standard errors (Mean ± S.E, n = 3), $p < 0.05$.

pellicle, hull, shell and kernel extracts on nitric oxide radical are shown in Table 2. Significant differences ($p < 0.05$) were exhibited between the whole part of walnut other than shell extracts ($p < 0.05$) for all genotypes tested.

In a previous study Jahanban Sfahlan, Mahmoodzadeh, Hasanzadeh, Heidari, and Jamei (2009) observed a high positive correlation between nitric oxide radical scavenging and phenolic content, but in our study this event was not seen in shell extract ($R^2 = 0.02$) (Table 3). These results indicate that various parts of walnut have different phenolic compounds, which have different functions in competition with NO to react with O_2, thus have different inhibition effects for NO.

Phenolic compounds analysis by HPLC

Vanillic acid, 1-naphthol, caffeic acid, salicylic acid, 8-hydroxyquinoline, and tannic acid were investigated and determined as phenolic compounds in fruits. The individual phenolic compounds were identified by comparing their UV–vis spectra with those obtained from standards in combination with retention times as well as by the addition of standard solutions. In this study, cultivar variations in phenolics contents were observed among them in walnut hulls as well as in walnut pellicles (Table 4). In pellicle extracts, among the identified phenolic compounds, the highest and lowest amount are tannic acid (K3 genotype) and salicylic acid (K1 genotype) respectively. In addition, none of considered compounds were found in G1 genotype. But in hull extracts, among the identified phenolic compounds, the highest and lowest amount are caffeic acid (K3 genotype) and 1-naphthol (K2 genotype), respectively. Also, vanillic acid was found in none of investigated genotypes.

Results of HPLC confirm the antiradical activities as previously discussed. Thus, there is not always a direct relationship between the ability of radical collecting, with the amount of phenolic compound, so one genotype may contain a high level of phenols, but those phenols have a reduced activation of collecting which have descended the capacity of antiradical activity and vice versa ($R^2 = 0.01$) (Table 3).

It has been obvious that, different genotypes and even different parts contain phenolic compounds with diverse structure in gained analysis.

IV CONCLUSIONS

In selecting plants for the extraction of phenol compounds, in addition to the high content of phenolic compounds, type of phenol should be considered, because the antiradical activity of phenolic compounds vary with different types and molecular structures ($R^2 = 0.02$ and $R^2 = 0.06$ for shell parts). The differences can be emanated from ecological, genetical, nutritial factors. Results of this investigation showed that walnut fruit by-products phenolic extract possess antiradical activity that this phenolic extract helpful in preventing or slowing the progress of various oxidative stressrelated diseases that could be used as alternative natural antioxidants in food industries.

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