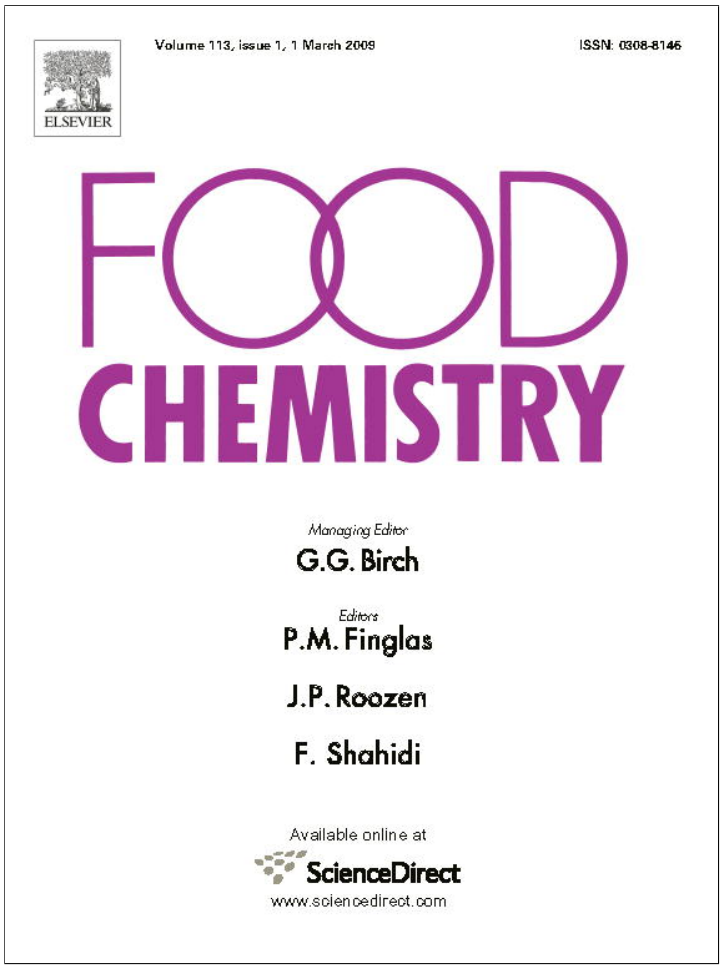

Food Chemistry 113 (2009) 160–165

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant phenolic compounds from walnut kernels (*Juglans regia* L.)

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ARTICLE INFO

Article history:
Received 29 January 2008
Received in revised form 18 July 2008
Accepted 22 July 2008

Keywords:
Juglans regia
Antioxidant
Radical scavenging activity
DPPH
Phenolic compounds

ABSTRACT

An activity-directed fractionation and purification process was used to isolate 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) scavenging components from *Juglans regia* kernels. Ethyl acetate and *n*-butanol fractions showed greater DPPH· scavenging activities compared to those of water and petroleum ether fractions. The ethyl acetate fraction was subjected to purification using column chromatography. Seven phenolic compounds, pyrogallol (1), *p*-hydroxybenzoic acid (2), vanillic acid (3), ethyl gallate (4), protocatechuic acid (5), gallic acid (6) and 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one (7), containing significant antioxidant activities were isolated and identified in *J. regia* by spectroscopic methods for the first time in this study. The relative order of DPPH· scavenging capacity for these compounds was 7 > 6 ⩾ 4 ⩾ 1 > Trolox ⩾ 5 > 3 > 2. The results of this study suggested that the antioxidant activities of these phenolic compounds may be influenced by the number of hydroxyls in their aromatic rings.

1. Introduction

There is an increasing interest in the role of free radical-mediated damage in the aetiology of human diseases. Free radicals formed during oxidation process occurring in various products and biological systems are known to be responsible for oxidative deterioration, health damage and accelerated aging (Aruoma, 1998). Consequently, antioxidants have become an essential part of preservation technology and contemporary health care. The potential toxicity of some synthetic antioxidants, however, has intensified research efforts to discover and utilise antioxidants from natural sources such as fruits and vegetables (Madsen & Bertelsen, 1995; Nieto et al., 1993; Shahidi & Wanasundara, 1992; Thamavit et al., 1985). Several nuts such as walnuts and peanuts are among these dietary plants known to have significant antioxidant contents (Amarowicz, Troszynska, & Shahidi, 2005; Blomhoff, Carlsen, Andersen, & Jacobs, 2006; Isanga & Zhang, 2007; Miraliakbari & Shahidi, 2008).

The stable radical species 1,1-diphenyl-2-picrylhydrazyl (DPPH·), has been widely used for antioxidant capacity screening and estimation due to its clear reaction mechanism, solvent compatibility and the technical simplicity of its assays which requires no special equipment (Chen, Wang, Rosen, & Ho, 1999; Cheng, Moore, & Yu, 2006; Maisuthisakul, Pongsawatmanit, & Gordon, 2007). The purple coloured DPPH· has a strong characteristic absorption at 515 nm and can undergo reactions with hydrogen donating antioxidant compounds to yield the stable yellow DPPH–H molecule easily monitored with UV spectroscopy (Prior, Wu, & Schaich, 2005; Roginsky & Lissi, 2005). The DPPH· scavenging capacity assay was therefore used in this study to evaluate the free radical scavenging capacity of the extracts from walnut kernels and pure isolated compounds.

Walnut species are important sources of nuts and timbers in the temperate zones across the world. In China, *Juglans regia* L. (Juglandaceae) is not only an agricultural commodity, but its leaves, barks, stems, pericarps, fruits, flowers and ligneus membranes are all applied for different medicinal uses. Semen Juglandis (English Walnut Seed), the dried ripe seed of *J. regia* is a nutrient rich food containing abundant phospholipids, proteins, tocopherols and unsaturated fatty acids (Cannella & Dernini, 2005; Li, Tsao, Yang, Kramer, & Hernandez, 2007). In addition, Semen Juglandis is traditionally used to tonify the kidney, warm the lung and relax the bowels in China. To the best of our knowledge, previous phytochemical studies of Semen Juglandis have focused on its oil

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doi:10.1016/j.foodchem.2008.07.061

composition (Savage, McNeil, & Dutta, 1998; Tsamouris, Hatziantoniou, & Demetzos, 2002; Wang, Zhang, Li, Zhao, & Zhao, 2004). Recent studies on walnut seed have found it to contain several groups of polyphenoilcs identified mainly as tannins (Fukuda, Ito, & Yoshida, 2003; Ito, Okuda, Fukuda, Hatano, & Yoshida, 2007), most of which were demonstrated to contribute the overall antioxidant activity of the walnut extract with different *in vitro* and *in vivo* antioxidant estimation models (Anderson et al., 2001; Fukuda, Ito, & Yoshida, 2004; Fukuda et al., 2003; Ito et al., 2007; Li et al., 2006). In addition, some monomeric phenolics such as gallic acid and ellagic acid have been reported in the walnut extracts and shown to inhibit plasma and LDL oxidation *in vitro* (Anderson et al., 2001). This implies that, in addition to tannins, some low molecular weight phenolics should be present in walnuts. However, little research has been done on the nature of these types of phenolics and their contribution to the total antioxidant of walnuts. Therefore, to promote the utilisation of walnut kernels for optimal human health, and to search for new active free radical scavengers from natural resources, the objectives of this study were (i) to isolate and identify the middle-polar antioxidant constituents from walnut kernels using DPPH· scavenging activity-directed isolation and (ii) to evaluate and compare the DPPH· scavenging capacity of the isolated pure phenolic compounds.

2. Materials and methods

2.1. General procedures and reagents

^{1}H and ^{13}C NMR spectra were obtained using a Bruker-AMX 500 instrument using deuterated dimethyl sulfoxide (DMSO-d_6) or methanol (CD_3OD) as solvents. Electrospray ionisation (ESI) mass spectra were acquired in the negative ion mode on a LCQ DECA XP instrument (Thermo Finnigan, San Jose, CA, USA) equipped with an ion trap mass analyzer. Column chromatography was carried out on silica gel (200–300 mesh, Qingdao Marine Chemistry Company, Qingdao, China) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) were used for TLC bioautography analysis. Optical density measurements were made with a SPECTRA MAX190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH·) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were of analytical reagent grade and used without any further purification.

2.2. Plant materials

Kernels of *Juglans regia* L. were collected from Xinjiang Uygur Autonomous Region, China, in October 2006, and authenticated by Dr. Lihong Wu, Shanghai R&D Centre for Standardization of Chinese Medicines. A voucher specimen of this collection (No. HTR-00001) has been deposited at the Herbarium of the Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine.

2.3. Extraction and isolation of bioactive compounds

Walnut kernel antioxidants were extracted and fractionated according to their polarity as shown in Fig. 1. Briefly, the powdered kernels of *J. regia* (19 kg) were sonicated three times with 3 L of petroleum ether (60–90 °C) for 1 h. The resulting supernatants were collected and filtered through absorbent cotton, followed by evaporation of the solvent at 50 °C under reduced pressure. The resulting liquid residue was labeled as the petroleum ether fraction (PEF). The defatted material remaining from petroleum ether extraction was re-extracted 5 times with 15 L of 80% ethanol for 3 h under reflux. This 80% ethanol extract was then filtered through absorbent gauze, and the filtrate was concentrated under reduced pressure to remove ethanol. The resulting suspension was successively partitioned with ethyl acetate and *n*-butanol. The ethyl acetate and *n*-butanol extracts were separately combined and evaporated to dryness under reduced pressure, while the aqueous layer was lyophilised to dryness. These three fractions were designated as EEF (132 g), BUF (338 g) and AF (600 g), respectively. The DPPH· scavenging activities of the four fractions including EEF, BUF, AF and PEF were determined using a spectrophotometric method. Greater antioxidant activities were found in the EEF and BUF fractions compared with PEF and aqueous fractions.

The DPPH·-active EEF fraction was subjected to column chromatography (CC) over silica gel eluted with increasing polarities of a mixture of petroleum ether and ethyl acetate resulting in 16

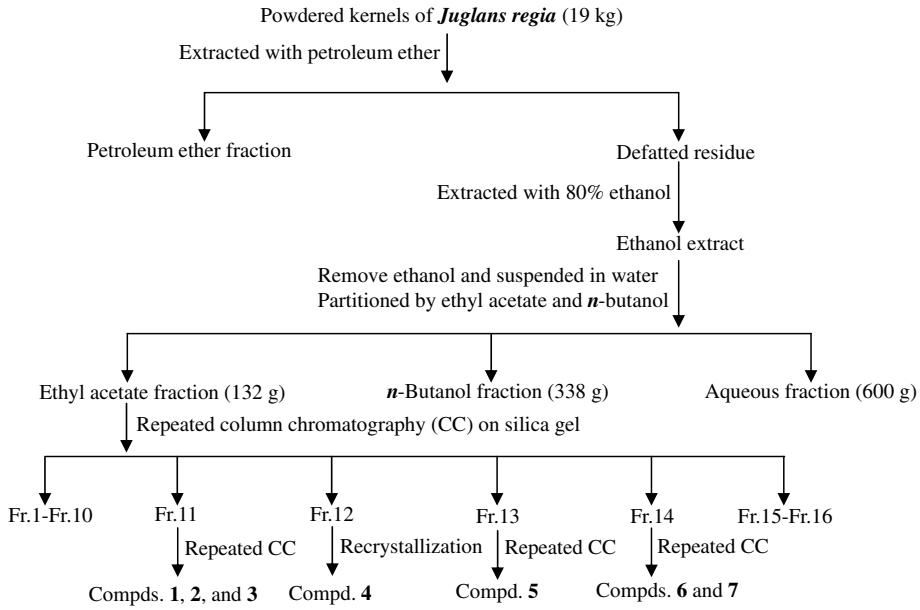


Fig. 1. Extraction, fractionation and column chromatography separation of *Juglans regia* kernels.

fractions (Fr.1–Fr.16). Fr.11 was further purified by repeated CC on Sephadex LH-20 (MeOH), followed by silica gel CC using $CHCl_3$–acetone (40:1, v/v) as eluting solvents to yield compounds 1 (5 mg), 2 (10 mg) and 3 (10 mg). Compound 4 (100 mg) was obtained from Fr.12 by recrystallization from methanol. Fr.13 yielded compound 5 (10 mg) after CC with Sephadex LH-20 (CH_2Cl_2–EtOH, 1:1, v/v) and further purification with silica gel CC ($CHCl_3$–MeOH, 20:1). From Fr.14, compounds 6 (100 mg) and 7 (10 mg) were separated by silica gel CC and eluted with a mixture of $CHCl_3$–MeOH.

2.4. DPPH· scavenging capacity

The DPPH· scavenging capacity were measured using a method described by Masuda et al. (1999) and Brand-Williams, Cuvelier, and Berset (1995) with modifications. Briefly, aliquots of sample extracts (125 μL) in acetone or individual pure compounds in water at different concentrations were added to 125 μL of 0.2 mM DPPH· methanol solution. After gentle mixing and 40 min of standing at room temperature, the absorbance of the resulting solutions was measured at 515 nm.

The percent DPPH· scavenged by each sample extract was calculated using the following equation:

$$\%\text{DPPH·quenched} = [A_0 - (A_1 - A_s)]/A_0 \times 100 \quad (1)$$

where A_0, A_1, and A_s represent the absorbance of the control, antioxidant extracts, and blank reaction at 40 min.

The percent DPPH· scavenged by each pure compound was calculated using the following equation:

$$\%\text{DPPH·quenched} = (A_0 - A_1)/A_0 \times 100 \quad (2)$$

where A_0 and A_1 stand for the absorbance of the control, and antioxidant compound reaction at 40 min.

IC_{50} values were calculated using nonlinear regression and expressed in mg dried material equivalents/mL for sample extracts or in mM for pure compounds. IC_{50} values express the concentration of antioxidant samples necessary to quench 50% radicals in the reaction mixture. The nonlinear regression analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Trolox was used as a positive control.

All the antioxidant measurements were performed in duplicate, and the data were expressed as average ± standard deviations (SD).

3. Results and discussion

3.1. Isolation of antioxidant compounds

Kernels of *Juglans regia* were air dried, ground to a powder and extracted with petroleum ether. The defatted leftover was then re-extracted with 80% aqueous ethanol. The ethanol extract was further fractionated by being partitioned with ethyl acetate and *n*-butanol. All the fractions including PEF, EEF, BUF and AF were evaluated for their DPPH· scavenging capacities using a conventional spectrophotometric assay. Among the four fractions, EEF and BUF showed the greatest DPPH· scavenging activities with IC_{50} values of 0.83 and 0.88 mg dried raw material equivalents/mL, respectively (Table 1). In the present study, further experiments were carried out on the EEF fraction to separate its antioxidant components, while the BUF fraction was reserved for further investigation. Repeated silica gel column chromatography was conducted throughout the purification, in which the DPPH·-active compounds were monitored by a TLC bioautography method (Cimpoiu, 2006). In this method, the eluants were spotted and developed on TLC plates. After air drying, the TLC plates were sprayed with DPPH· solution and examined under visible light to screen for potential antioxidant components visually observed as white yellow spots on a purple background. Seven pure compounds with potential antioxidant activity were isolated from EEF, as shown in Fig. 1. While all 7 compounds have been reported in the literature, this is the first study to isolate them from *J. regia*, and the first study to report of the presence of compounds 1, 4, and 7 in the genus *Juglans*.

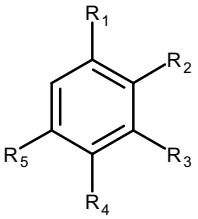
Table 1
The DPPH· scavenging activities of the extracts and the isolated phenolic compounds of *Juglans regia*[a]

Test materials	IC_{50}
PEF	20.00 ± 2.20
EEF	0.83 ± 0.25
BUF	0.88 ± 0.01
AF	1.70 ± 0.08
Pyrogallol (1)	0.015 ± 0.000
p-Hydroxybenzoic acid (2)	0.350 ± 0.017
Vanillic acid (3)	0.090 ± 0.004
Ethyl gallate (4)	0.013 ± 0.002
Protocatechuic acid (5)	0.032 ± 0.000
Gallic acid (6)	0.011 ± 0.001
3,4,8,9,10-Pentahydroxydibenzo-[*b*,*d*]pyran-6-one (7)	0.007 ± 0.000
Trolox	0.026 ± 0.001

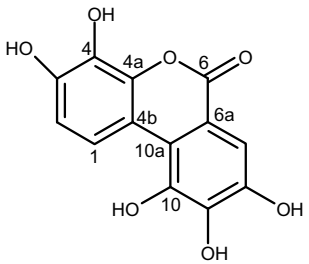
[a] PEF, EEF, BUF, and AF represent the petroleum ether fraction, ethyl acetate fraction, *n*-butanol and aqueous fraction of kernels of *J. regia*, respectively. The IC_{50} values of PEF, EEF, BUF, and AF are expressed as mg dried material equivalents/mL. The IC_{50} values of compounds 1–7 and Trolox are expressed in mM.

3.2. Structure elucidation for isolated compounds

The structures of the isolated compounds were identified on the basis of spectroscopic analyses including ^1H, ^{13}C NMR spectros-



Compound	R_1	R_2	R_3	R_4	R_5
1	-OH	-OH	-OH	-H	-H
2	-COOH	-H	-H	-OH	-H
3	-COOH	-H	$-OCH_3$	-OH	-H
4	$-COOCH_2CH_3$	-H	-OH	-OH	-OH
5	-COOH	-H	-OH	-OH	-H
6	-COOH	-H	-OH	-OH	-OH



Compound 7

Fig. 2. Chemical structures of compounds 1–7.

copy, and electrospray ionisation mass spectrometry (ESI MS), the chemical structures of which are shown in Fig. 2.

Compound 1 was obtained as white colourless needles from methanol, m.p. 133–134 °C. The molecular formula was established as $C_6H_6O_3$ by ESI MS (*m*/*z* 125.2 $[M-H]^-$) and NMR spectroscopic analysis. Its ^1H and ^{13}C NMR data, listed in Tables 2 and 3, agreed well with the data reported (Wang, Kadota, Liu, & Liu, 2005). Thus, compound 1 was identified as pyrogallol.

Compound 2 was obtained as white amorphous powder, and its molecular formula was established as $C_7H_6O_3$ by a combinational analysis of ESI MS (*m*/*z* 137.2 $[M-H]^-$), and ^1H and ^{13}C NMR spectral data. The detailed NMR data (Tables 2 and 3) agreed well with the reported compound, *p*-hydroxybenzoic acid (Wang et al., 2005; Wu, Chen, Wu, Chen, & Tu, 2007).

Compound 3 was obtained as white amorphous powder. The quasi-molecular ion peak $[M-H]^-$ at *m*/*z* 167.3 in ESI MS was consistent with the molecular formula $C_8H_8O_4$ established by NMR spectra analysis. Its detailed ^1H and ^{13}C NMR data listed in Tables 2 and 3 agreed well with the reported compound, vanillic acid (Zou & Yang, 2005).

Compound 4 was recrystallized from methanol as white needles, m.p. 160–162 °C, ESI MS: *m*/*z* 197.3 $[M-H]^-$. Its detailed ^1H and ^{13}C NMR spectral data (Tables 2 and 3) were in good agreement with the reported compound, ethyl 3,4,5-trihydroxybenzoate, named ethyl gallate (Zhang, Tan, & Chen, 2003).

Compound 5 was isolated as white amorphous powder with the molecular formula of $C_7H_6O_4$ established by ESI MS (*m*/*z* 153.3 $[M-H]^-$) and NMR spectroscopic analysis. The ^1H and ^{13}C NMR data of 5 were listed in Tables 2 and 3, respectively, which agreed well with the reported compound, protocatechuic acid (Wang et al., 2005; Wu et al., 2007).

Compound 6 was recrystallized from methanol as while needles with a m.p. of 252–255 °C. The ESI MS showed a quasi-molecular ion at *m*/*z* 169.3 $[M-H]^-$, corresponding to the molecular formula of $C_7H_6O_5$ deduced from its NMR data (Tables 2 and 3). The chemical shift assignments for this compound were also listed in Tables 2 and 3, consistent with the data reported (Wang et al., 2005; Zhang et al., 2003). Thus, compound 6 was identified as gallic acid.

Compound 7 was obtained as pale yellow needles after crystallization from methanol with a m.p. of 252–255 °C. The molecular formula of $C_{13}H_8O_7$ was deduced from ESI MS (*m*/*z* 275.5 $[M-H]^-$) and NMR data. The NMR assignments were listed in Table 4, which agreed well with the data reported (Nawwar, Hussein, & Merfort, 1994; Nawwar & Souleman, 1984; Yan & Yang, 2005). Compound 7 was identified as 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one.

This is the first study to report these compounds from *J. regia* and the first report of 1, 4 and 7 from the genus *Juglans*. In 2003, Fukuda and co-workers isolated and identified 15 ellagitannins from the *n*-butanol fraction of the 80% aqueous ethanol extract of walnut (Fukuda et al., 2003). In contrast to that study, the present study focused on the isolation of phenolics of the middle-polar fraction (ethyl acetate fraction) of 70% aqueous ethanol extract to better understand the comprehensive phenolic profile of walnut.

3.3. DPPH· scavenging capacities

The DPPH· scavenging capacity of all fractions and isolated pure phenolic compounds were determined and expressed as IC_{50} values. Among the fractions, the EEF and BUF fractions were found to be the most potent DPPH radical scavengers with IC_{50} values of 0.83 and 0.88 mg dried raw material equivalents/mL, respectively, while the PEF was the least active scavenger (Table 1).

The seven phenolic compounds isolated from the DPPH·-active EEF fraction as well as the positive control, Trolox, were also evaluated and compared for their DPPH· scavenging capacities. As shown in Table 1, compounds 3,4,8,9,10-pentahydroxydibenzo-[*b*,*d*]pyran-6-one (7), gallic acid (6), ethyl gallate (4), and pyrogallol (1) showed significant free radical scavenging capacities with IC_{50} values of 0.007, 0.011, 0.013 and 0.015 mM, respectively, all of which were much more active than Trolox. The relative order of DPPH· scavenging capacity for the isolated phenolic compounds was found to be: 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one (7) > gallic acid (6) ⩾ ethyl gallate (4) ⩾ pyrogallol (1) > Trolox ⩾ protocatechuic acid (5) > vanillic acid (3) > *p*-hydroxybenzoic

Table 2
^1H NMR data of compounds 1–6 isolated from *Juglans regia* (DMSO-d6, 500 MHz)

No.	1	2	3[a]	4	5	6[a]
2		7.89 (d, 8.5)	7.43 (d, 1.8)	6.95 (s)	7.33 (d, 2.0)	7.05 (s)
3		6.82 (d, 8.5)				
4	6.25 (s)					
5	6.42 (s)	6.82 (d, 8.5)	6.78 (d, 8.2)		6.78 (d, 8.2)	
6	6.25 (s)	7.89 (d, 8.5)	7.46 (dd, 1.8, 8.2)	6.95 (s)	7.29 (dd, 2.0, 8.2)	7.05 (s)
$-CH_2$				4.27 (q, 7.1)		
$-CH_3$			3.77 (s)	1.34 (t, 7.1)		
3-OH	8.56 (brs)			9.21 (s)	9.47 (brs)	
4-OH	8.56 (brs)	10.18 (brs)		8.87 (s)	9.47 (brs)	
5-OH	8.56 (brs)			9.21 (s)		
–COOH		12.35 (brs)			12.04 (brs)	

[a] NMR measurement was performed by using CD_3OD as solvents. Values in parentheses are coupling constants (Hz).

Table 3
^{13}C NMR data of compounds 1–6 isolated from *Juglans regia* (DMSO-d_6, 125 MHz)

Carbon	1	2	3[a]	4	5	6[a]
1	146.2	121.3	121.7	119.6	121.1	122.4
2	133.1	131.4	114.4	108.4	116.5	110.7
3	146.2	115.1	147.3	145.5	144.8	146.7
4	107.1	161.5	151.3	138.3	149.9	139.9
5	118.4	115.1	112.4	145.5	115.1	146.7
6	107.1	131.4	123.9	108.4	121.7	110.7
7		167.1	168.6	166.8	167.2	170.7
8			55.0	59.9		
9				14.2		

[a] NMR measurement was performed by using CD_3OD as solvents.

Table 4
NMR data of compound 7 isolated from *Juglans regia* (CD_3OD)[a]

Carbon	^{13}C NMR	1H NMR
1	117.7	8.46 (d, 9.0)
2	111.4	6.77 (d, 9.0)
3	139.6	
4	131.9	
4a	145.4	
4b	111.0	
6	162.4	
6a	110.7	
7	106.6	7.39 (s)
8	145.0	
9	140.4	
10	142.6	
10a	117.1	

[a] Values in parentheses are coupling constants (Hz).

acid (2). The significant activity differences between these benzoic acid derivatives were likely due to the number of hydroxyls present in the aromatic ring (Sroka & Cisowski, 2003), though the series of compounds tested was limited. For example, 3,4,8,9,10-pentahydroxydibenzo[*b*,*d*]pyran-6-one (7) was found to be the most active free radical scavenger because of its five hydroxyl groups. The compounds including gallic acid (6), ethyl gallate (4) and pyrogallol (1) with three hydroxyls showed similar antioxidant activities, all of which were more active than those with one or two hydroxyl(s) such as protocachuic acid (5) and *p*-hydroxybenzoic acid (2). However, other substitutions such as carbonyl and methoxyl on the aromatic ring contributed less antioxidant activity than hydroxyls. For example, pyrogallol (1) and gallic acid (6) with the same number of hydroxyls and different number of carbonyl demonstrated similar antioxidant activities. In addition, it is noteworthy that protocachuic acid (5) showed a higher antioxidant activity compared to vanillic acid (3), indicating that an adjacent substituted methoxyl group of a hydroxyl group in the aromatic ring reduced its free radical scavenging capacity. A possible explanation for this result is the difficulty of quinone formation involved in the reaction mechanism between phenolics and free radicals (Han, Weng, & Bi, 2008). The results from this study support the notion that *ortho*-hydroxyl structures are crucial for the enhanced antioxidant activity because the *ortho*-quinone was easily formed (Choi et al., 2002; Han et al., 2008). This study also suggests that reported protective effects of polyphenols present in fruits may be attributed to their antioxidant activities.

4. Conclusions

Seven antioxidant compounds were isolated and identified from kernels of *J. regia* by an activity-guided isolation and were found to have significant DPPH· scavenging activity. The structure-activity relationship evaluation of these phenolic compounds suggested that the number of hydroxyls was the most important factor in determining the antioxidant activities of the phenolic compounds. Among the seven compounds, four demonstrated higher antioxidant activities than the positive control, Trolox. In addition, this investigation of antioxidant phenolic compounds suggested that the potency of these compounds could provide a chemical basis for some of the health benefits claimed for *J. regia* in foods and folk medicine. The results also indicated that the extracts and pure phenolic compounds from *J. regia* might be used as natural antioxidants and alternatives to synthetic antioxidants such as BHT. Further studies are necessary to assess the chemical profile of the DPPH·-active *n*-butanol fraction which in this study had a free radical scavenging capacity similar to the ethyl acetate fraction.

Acknowledgements

This study was financially supported in part by a grant from Ministry of Education awarded to Prof. Z. T. Wang (No. 706025) and a grant from Shanghai Rising Star Project of Shanghai Science and Technology Committee (No. 06QA14047 awarded to Dr. T. Wu).

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