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Antioxidant Effect of *Juglans mandshurica* Bark Gallotannins

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ABSTRACT : The bark of *Juglans mandshurica* Maxim. was collected, extracted with acetone-H_2O (7:3, v/v), fractioned with *n*-hexane, CH_2Cl_2 and EtOAc, then each fraction was freeze-dried to give some powders. A portion of the EtOAc (28.4 g) fraction was chromatographed on a Sephadex LH-20 column eluting with a series of MeOH-H_2O and EtOH-hexane mixture. Four gallotannins, gallic acid (**1**), ellagic acid (**2**), 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (**3**) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**4**), have been isolated from the EtOAc fraction Their structures were elucidated on the basis of chemical evidence and spectrometric analysis such as NMR and MS. The antioxidant activities on each fraction and the isolated gallotannins were evaluated by DPPH radical scavenging test.

Keywords : *Juglans mandshurica* Maxim., Bark, Gallotannin, Antioxidant activity, DPPH

INTRODUCTION

Free radicals have been complicated in various diseases due to the oxidative damage to DNA, lipids, and proteins and which can result in failure of cellular functions (Chen *et al*., 2006; Halliwell and Gutteridge, 1999). However, dietary intake of antioxidant compounds will reduce oxidative damage (Ames *et al*., 1993). Some synthetic antioxidant compounds show side effects, for example butylated hydroxyanisole (BHA) has been reported to increase incidences of neoplasias (Ito *et al*., 1983). Therefore, in recent years, considerable effort has been directed at identifying safe and natural antioxidants that can protect against oxidative stress.

Juglans mandshurica Maxim. (Juglandaceae), a fast growing deciduous tree, is widely distributed in China, Siberia and Korean peninsula. The tree has been used as a folk medicinal plant for treatment of esophageal, gastric, cardiac and lung cancer (Kim, 1994). Other scientists reported that chemicals volatilized from the species inhibit the growth of the neighboring plants and can be developed for chemurgy. Several naphthoquinones, naphthalenyl glycosides, α-tertalonyl glucopyranosides, flavonoids and diarylheptanoyl glucopyranosides have been isolated from this plant (Li *et al*., 2003; Min *et al*., 2003). In a previous paper, we isolated 11 antioxidant flavonoids and flavonoid glycosides from *J. mandshurica* bark (Kim *et al*., 2006). As a part of a systematic study of this tree, we report the isolation of 4 gallotannins from the EtOAc fraction and their antioxidant activities including crude fractions, in this paper.

MATERIALS AND METHODS

Plant materials

The stem bark of *J. mandshurica* was collected in the experimental forest of Kangwon National University, Korea in April, 2005 and air-dried for 2 weeks at room temperature before grinding.

Equipments

Column chromatography was performed using Sephadex

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LH-20 (Sigma, v/v/v). Eluents were collected with a fraction collector (Gilson FC 204). TLC analyses were performed on DC-Plastikfolien Cellulose F (Merck) plates and developed with *t*-BuOH-HOAc-H_2O (3 : 1 : 1, v/v, solvent A) and HOAc-H_2O (3 : 47, v/v, solvent B). Visualization was by UV light (254 and 365 nm) or by spraying with 1% ethanolic $FeCl_3$ solution followed by heating.

The ^{1}H NMR, ^{13}C NMR, DEPT and correlation NMR spectra such as HMBC, HMQC and TOCSY were recorded in MeOH-d_4 with TMS as an internal standard using a Bruker Avance DPX 400 spectrometer at the operating frequency of 400 MHz (^{1}H) and 100 MHz (^{13}C) in Central Laboratory of Kangwon National University. EI-MS spectroscopy was performed with a Micromass Autospec M363 spectrometer and MALDI-TOF-MS spectroscopy was done on a Model Voyager-DE STR spectrometer.

Extraction and isolation

The air-dried, ground bark (3.2 kg) was extracted in aqueous acetone (70%) overnight at room temperature. The extract was filtered with Advantec No. 2 filter paper and concentrated to give an aqueous residue, then successively fractionationed and freeze-dried to give *n*-hexane, CH_2Cl_2, EtOAc and H_2O soluble fractions.

A portion of the EtOAc fraction (28.4 g) was applied to a Sephadex LH-20 column eluting with MeOH-H_2O (3 : 1, v/v) to give 3 fractions: E_1 (6.9 g), E_2 (16.1 g) and E_3 (5.3 g). Fraction E_2 was reapplied to a Sephadex column using MeOH-H_2O (1 : 1, v/v) for further purification to yield 3 subfractions and the second subfraction was stepwisely retreated with MeOH-H_2O (1:3 and 1:5, v/v) and EtOH- hexane (3 : 1 and 1 : 1, v/v) to give compounds **1** (387 mg), **2** (92 mg) and **3** (204 mg). When treated with MeOH, compound **4** (589 mg) was isolated in E_3 fraction as a precipitate.

Compound 1

Yellowish amorphous powder; R_f: 0.54 (solvent A) and 0.42 (solvent B); EI-MS: *m/z* $[M]^+$ 170; ^{1}H-NMR (400 MHz, δ, MeOH-d_4): 7.09 (2H, *s*, H-2,6); ^{13}C-NMR (100 MHz, δ, MeOH-d_4): 109.85 (C-2,6), 122.36 (C-1), 138.42 (C-4), 145.69 (C-3,5), 170.67 (C-7).

Compound 2

Yellowish amorphous powder; R_f: 0.12 (solvent A) and 0.02 (solvent B); EI-MS: *m/z* $[M]^+$ 302; ^{1}H-NMR (400 MHz, δ, MeOH-d_4): 7.47 (2H, *s*, H-5,5′); ^{13}C-NMR (100 MHz, δ, MeOH-d_4): 107.53 (C-1,1′), 110.11 (C-5,5′), 112.22 (C-6,6′), 136.26 (C-2,2′), 139.48 (C-3,3′), 148.00 (C-4,4′), 159.05 (C-7,7′).

Compound 3

Yellowish amorphous powder; R_f: 0.48 (solvent A) and 0.51 (solvent B); MALDI-TOF-MS: *m/z* $[M+Na]^+$ 811, $[M+K]^+$ 827; ^{1}H-NMR (400 MHz, δ, MeOH-d_4): 7.12, 7.03, 7.02, 6.93 (8H, *s*, 4×galloyl-H-2,6), 6.09 (1H, *d*, *J*= 8.2 Hz, glu. H-1), 3,98~5.58 (6H, *m*, glu. H-2~5); ^{13}C-NMR (100 MHz, δ, MeOH-d_4): 64.42 (glu. C-6), 70.05 (glu. C-4), 72.79 (glu. C-2), 76.88 (glu. C-3), 77.02 (glu. C-5), 94.33 (glu. C-1), 110.67, 110. 75, 110.82, 110.85 (4×galloyl C-2′,6′), 120.25, 120.80, 121.38, 121.63 (4×galloyl C-1′), 140.40, 140.43, 140.65, 141.12 (4×galloyl C-4′), 146.77, 146.82, 146.91, 146.94 (4×galloyl C-4′), 166.77, 167.65, 168.18, 168.64 (4×galloyl C-7′).

Compound 4

Yellowish amorphous powder; R_f: 0.22 (solvent A) and 0 (solvent B); MALDI-TOF-MS: *m/z* $[M+Na]^+$ 963, $[M+K]^+$ 879; ^{1}H-NMR (400 MHz, δ, MeOH-d_4): 7.11, 7.06, 6.98, 6.96, 6.91 (10H, *s*, 5×galloyl-H-2,6), 6.24 (1H, *d*, *J*=8.4 Hz, glu. H-1), 4.15~5.90 (6H, m, glu. H-2~5); ^{13}C-NMR (100 MHz, δ, MeOH-d_4): 63.54 (glu. C-6), 70.21 (glu. C-4), 72.61 (glu. C-2), 74.53 (glu. C-3), 74.83 (glu. C-5), 94.24 (glu. C-1), 110.75, 110.80, 110.82, 111.04, 111.26 (5×galloyl C-2′,6′), 120.13, 120.61, 120.65, 120.77, 121.45 (5×galloyl C-1′), 140.43, 140.56, 140.73, 140.78, 141.19 (5×galloyl C-3′, 5′), 146.70, 146.80, 146.85, 146.88,146.97 (5×galloyl C-4′),

166.65, 167.35, 167.45, 167.73, 168.36 (5×galloyl C-7′).

Antioxidant activity assay

The antioxidant activity was determined on the basis of the scavenging activity of the stable DPPH free radical method introduced by Yoshida *et al*. (1989) with slight modification. MeOH solutions (4 ml) of samples at different concentrations (2~40 μg/ml) were added to a solution of DPPH (1.5×10^{-4} M, 1 ml) in MeOH. After mixing gently and standing at room temperature for 30 min, the optical density was measured at 517 nm with a UV-visible spectrophotometer (Libra S32, Biochrom LTD). The results were calculated by taking the mean of all triplicated values. IC_{50} values were obtained through extrapolation from concentration of sample necessary to scavenge 50% of the DPPH free radicals. BHT and α-tochopherol were controls.

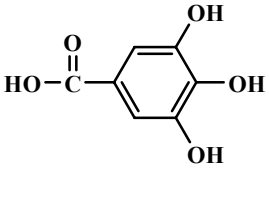
RESULTS AND DISCUSSION

Identification of the isolated gallotannins

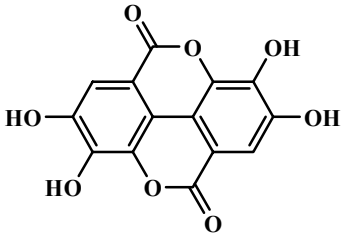
The column separation of EtOAc soluble fraction of *J. mandshurica* bark led to the isolation of 4 gallotannins and their structures were elucidated as gallic acid (**1**), ellagic acid (**2**), 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**) and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**) (Fig. 1) by a combination of spectroscopic methods and comparison with the literature data (Matthew *et al*., 2007; Min *et al*., 2000; Hatano *et al*., 1989; Kashiwada *et al*., 1984). Compounds **1**, **3** and **4** are hydrolysable tannins while compound **2** is an ellagitannin. To our knowledge, this was the first time isolating compounds **2** and **4** from this species.

Antioxidant activity

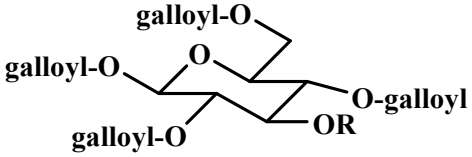
The antioxidant activities of the crude extracts, 4 fractions and 4 gallotannins were determined by DDPH assay. The results were summarized in Table 1. Among these, crude extracts, EtOAc and H_2O fractions and 3 hydrolysable tannins indicated positive antioxidant activities compared the controls. While the ellagitannin (Ellagic acid, **2**) and *n*-hexane and CH_2Cl_2 fractions showed weak activities. The IC_{50} values for **1**, **3** and **4** were 7.40, 7.02 and 6.70 μg/ml, respectively, which suggested that the galloyl moiety plays a critical role in the antioxidant function of hydrolysable tannins. The results also suggested that these hydrolysable tannins could be mainly responsible for the positive antioxidant effect of the EtOAc fraction of the bark. This fact indicated that the extracts of the bark has high antioxidant potential and can be used as a good source for the antioxidants.



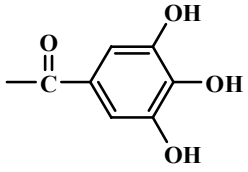
Gallic acid (**1**)



Ellagic acid (**2**)



R = H: 1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose (**3**)
R = galloyl: 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**4**)



(Galloyl)

Fig. 1. Gallotannins from *J. mandshurica* bark.

Table 1. Antioxidant activities (IC_{50} values) of fractions and gallotannins from *J. mandshurica* bark

Samples		IC_{50} (μg/ml)
Controls	*α*-tocopherol	12.1
	BHT	13.8
Fractions	Crude extracts	11.7
	n-Hexane soluble fraction	47.0
	CH_2Cl_2 soluble fraction	44.5
	EtOAc soluble fraction	9.16
	H_2O soluble fraction	10.2
Isolates	Gallic acid (1)	7.40
	Ellagic acid (2)	14.9
	1,2,4,6-tetra-*O*-galloyl-*β*-D-glucose (3)	7.02
	1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (4)	6.70

CONCLUSION

Four gallotannins were isolated by column chromatography using Sephadex LH-20 from *J. mandshurica* barks and elucidated by spectral and chemical evidences. Among these, compounds **2** and **4** have not previously been reported in this tree.

The antioxidant test showed that crude extract, EtOAc and H_2O fractions, and hydrolysable tannins were significantly positive potential compared the controls.

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