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Free Radical Scavenging and Hepatoprotective Constituents from the Leaves of *Juglans sinensis*

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In the course of searching for hepatoprotective agents from natural products, six compounds were isolated from the MeOH extract of the leaves of *Juglans sinensis*, as guided by their DPPH free radical scavenging activity. The structures were determined as juglanoside B (**1**), quercetin 3-O-α-L-arabinofuranoside (avicularin, **2**), quercetin 3-*O*-α-L-arabinopyranoside (guaijaverin, **3**), quercetin 3-O-α-L-rhamnopyranoside (quercitrin, **4**), (+)-catechin (**5**) and quercetin 3-O-β-D-galactopyranoside (hyperin, **6**). Compounds **2-6** showed significant DPPH free radical scavenging effects. An evaluation for the hepatoprotective activity of the isolated compounds on drug-induced cytotoxicity was conducted, and compounds **1**, **2**, and **5** showed protective effects against nitrofurantoin-induced cytotoxicity, and compound **5** also exhibited a moderate protective effect on amiodarone-induced cytotoxicity in Hep G2 cells.

Key words: *Juglans sinensis*, Juglandaceae, Hepatoprotective, Hep G2 Cells, Free radical scavenging

INTRODUCTION

In the search for hepatoprotective agents from natural sources, it is important to employ the appropriate use of an assay system to human liver toxicosis in order to provide more therapeutically relevant agents. For a long time, liver toxicity induced by the chemicals and drugs has been recognized as a toxicological problem. Especially, certain drugs given for a prolonged period of time and in high doses are known to lead to serious clinical concern. However, there are few reported studies on the development of hepatoprotective agents using assay systems with great relevance to human liver toxicosis, such as some liver toxic drugs. Amiodarone [2-butyl-3-{3, 5-diiodo-4-(β-diethylaminoethoxy)benzoyl}benzofuran] (Singhal *et al.*, 2003), nitrofurantoin [1-(5-nitro-2-furfurylideneamino)-hydantoin] (Edoute *et al.*, 2001), and tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine hydrochloride) (Watkins *et al.*, 1994) belong to such categories of drugs. Therefore, studies on natural constituents, with protective effect on drug-induced hepatotoxicity, would be valuable in providing preparations of potential therapeutic use.

In addition, there is now increasing evidence that free radicals and active oxygen species are involved in a variety of pathological events (Halliwell, 1994). Free radical-mediated cell damage and free radical attack on polyunsaturated fatty acids result in the formation of lipid radicals. These lipid radicals react readily with molecular oxygen to produce peroxy radicals that are responsible for initiating lipid peroxidation. The peroxidation of cellular membrane lipid can lead to cell necrosis, which has been implicated in a number of pathophysiological conditions, as well as in the toxicity of many xenobiotics (Kappus, 1985).

As part of the search for hepatoprotective agents against hepatotoxic drugs, studies on the natural products, with protective effects on tacrine-induced cytotoxicity, have been conducted in recent years (Song *et al.*, 2001; Oh *et al.*, 2002a, 2002b, 2004). In the course of our continuing search for hepatoprotective agents from medicinal plants, we employed the DPPH free radical scavenging assay as a primary screening system, as the antioxidative effect has been established as being closely related to hepatoprotective activity (Oh *et al.*, 2004). The MeOH extract of *Juglans sinensis* Dode (Juglandaceae) leaves was found to exhibit a promising free radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The leaves of *J. sinensis* (Juglandaceae) have been used in Korean

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folk medicine for the treatment of leucorrhea, scabies and elephantiasis (Bae, 2000). This paper deals with the isolation and identification of the free radical scavenging constituents of *J. sinensis* leaves, and their protective effects on drug-induced cytotoxicity in Hep G2 cells.

MATERIALS AND METHODS

General experimental procedure

NMR spectra were obtained on a JEOL JNM-ECP 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer, and ESI-MS spectra on an API-2000 spectrometer. TLC was carried out on precoated silica gel 60 F_{254} and RP-18 F_{254} plates (0.25 mm, Merck, Germany). Column chromatography was performed on silica gel 60 (230-400 mesh, Merck) and Sephadex LH-20 (Pharmacia).

Plant materials

The leaves of *J. sinensis* were collected from Iksan city, Korea, during July 2002. A voucher specimen (WP 525) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

Extraction and isolation

Dried and pulverized leaves of *J. sinensis* (458 g) were extracted with hot MeOH (2 L×2) for 2 h. The MeOH extract (35.75 g) was suspended in H_2O, and successively partitioned with CH_2Cl_2 and *n*-BuOH (each 800 mL×3). The *n*-BuOH soluble fraction (7.58 g) was separated by silica gel column chromatography (CH_2Cl_2:MeOH, 4:1) to give three fractions (A-C). Fraction B (from 400 to 700 mL, 2.18 g) was subjected to chromatography on a Sephadex LH-20 column, with MeOH as eluent, to give two subfractions (B1 & B2). Fraction B1 (from 20 to 50 mL, 675 mg) was purified by Sephadex LH-20 column chromatography, with CH_2Cl_2:MeOH (6:1) as eluent, to afford compound **1** (149.7 mg, 0.033 w/w %). Fraction B2 (from 70 to 120 mL, 1.26 g) was separated by Sephadex LH-20 column chromatography (CH_2Cl_2:MeOH, 2:1) to afford six subfractions (B21-B26). Fraction B22 (from 50 to 60 mL) was recrystallized from MeOH to yield compound **2** (101.2 mg, 0.022 w/w %). Fraction B21 (from 40 to 50 mL, 510 mg) was purified by Sephadex LH-20 column chromatography (CH_2Cl_2:MeOH, 10:1) to yield compound **3** (17.6 mg, 0.0038 w/w %). Fraction B24 (from 60 to 70 mL, 228 mg) was purified with Sephadex LH-20 column chromatography (CH_2Cl_2:MeOH, 4:1) to give two subfractions (B241 & B242). Fraction B242 (from 70 to 110 mL, 118 mg) was further purified with Sephadex LH-20 column chromatography (CH_2Cl_2:MeOH, 12:1) to give compound **4** (52.3 mg, 0.011 w/w %). Fraction B26 (from 85 to 100 mL, 192 mg) was subjected to chromatography on a Sephadex LH-20 column, with CH_2Cl_2:MeOH (6:1) as eluent, to give compound **5** (160 mg, 0.035 w/w %). Fraction C (from 750 to 1,400 mL, 1.3 g) was subjected to chromatography on a silica gel column, with $CHCl_3$:MeOH:H_2O (4:1:0.1) as eluent, to give compound **6** (89.1 mg, 0.019 w/w %).

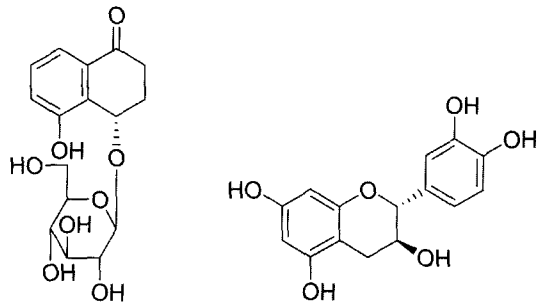
The isolated compounds were identified as juglanoside B (**1**) (Liu *et al.*, 2004), quercetin 3-O-α-L-arabinofuranoside (avicularin, **2**, Agrawal, 1989; Lee *et al.*, 2002), quercetin 3-O-α-L-arabinopyranoside (guaijaverin, **3**, Agrawal, 1989; Sanbongi *et al.*, 1998), quercetin 3-O-α-L-rhamnopyranoside (quercitrin, **4**) (Jung *et al.*, 1999), (+)-catechin (**5**, Kim and Shin, 1998) and quercetin 3-O-β-D-galactopyranoside (hyperin, **6**, Jung *et al.*, 1999), on the basis of the physico-chemical evidence and by comparison of the spectral data with those in the previously reported literature.

In vitro free radical scavenging and hepatoprotective assays

The DPPH free radical scavenging and *in vitro* hepatoprotective assays used have been described elsewhere (Song *et al.*, 2001). Briefly, human hepatoma Hep G2 cells, from the American Type Culture Collection, were maintained at 2×10^5 cells/well in complete medium, consisting of RPMI supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL) and streptomycin (100 μg/mL), and incubated at 37°C in a humidified 5% CO_2 and 95% air atmosphere. The cytotoxicity was assessed after a 2 h incubation period in media containing either amiodarone (0.25 mM), tacrine (1.2 mM) or nitrofurantoin (1.7 mM), or without drugs (control), and evaluated using the MTT assay. Each sample was tested in triplicate at three different concentrations (10, 50, and 100 μg/mL). All experimental data were calculated from the protective percentages of viability vs. control cells, and expressed as the mean ± S.D. of three independent experiments. A one-way ANOVA test was applied to detect any significant difference. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In the DPPH free radical scavenging test on the MeOH extract of *J. sinensis* leaves and its fractions, the extract showed a promising effect, with an IC_{50} value of 94.3 mg/mL, but the *n*-BuOH soluble fraction (IC_{50} = 46.7 μg/mL) exhibited the greatest effect among the samples tested. The subsequent DPPH free radical scavenging activity-guided fractionation of the *n*-BuOH soluble fraction afforded four flavonol glycosides; quercetin 3-O-α-L-arabinofuranoside (avicularin, **2**), quercetin 3-O-α-L-arabinopyranoside (guaijaverin, **3**), quercetin 3-O-α-L-rhamnopyranoside (quercitrin, **4**) and quercetin 3-O-β-D-galactopyranoside



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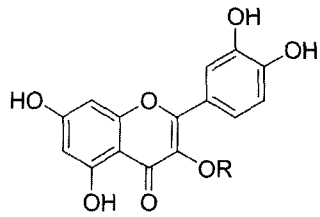

2 R = α-L-Ara(f)
3 R = α-L-Ara(p)
4 R = α-L-Rha(p)
6 R = β-D-Gal(p)

Fig. 1. Chemical structures of compounds **1-6** isolated from *J. sinensis*

(hyperin, **6**), together with (+)-catechin (**5**) and α-tetralonyl glucoside (juglanoside B, **1**). The free radical scavenging effects of the isolated compounds were tested on DPPH radicals. With the except of compound **1**, four flavonol glycosides (**2-4** and **6**) and (+)-catechin (**5**) showed significant DPPH free radical scavenging effects (Table I).

Tacrine is an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, but reversible hepatotoxicity has been reported as a side effect of this pharmaceutical reagent (Watkins *et al.*, 1994). Oxidative stress has also been suggested as one of the mechanisms involved in tacrine cytotoxicity (Osseeni *et al.*, 1999). Amiodarone is useful for the treatment of ventricular and supraventricular arrhythmias, but has been associated with hepatic toxicity (Singhal *et al.*, 2003). Nitrofurantoin is a synthetic nitrofuran commonly used for the treatment and prophylaxis of urinary tract infections. It has been reported that liver cirrhosis and fatal liver necrosis are associated with the use of nitrofurantoin (Edoute *et al.*, 2001). Although the mechanisms of amiodarone- and nitrofurantoin-induced liver toxicity have not been fully established, the involvement of increased lipid peroxidation (Sarma *et al.*, 1997) and oxidative stress (Klee *et al.*, 1994), respectively, have been reported. These suggest that antioxidative compounds may have protective effects against drug-induced cytotoxicity. Therefore, the isolated antioxidative compounds were investigated to test whether the hepatoprotective effects were mediated through their antioxidative effects.

Although the relative hepatoprotective activities of these compounds did not precisely coincide with their relative free radical scavenging activities, compounds **1**, **2**, and **5** exhibited moderate protective effects against nitrofurantoin-induced cytotoxicity in Hep G2 cells, with effective protection ratios of 14.2 ± 1.1, 15.1 ± 0.9, and 25.2 ± 2.3%, respectively, at a concentration of 100 μg/mL (Table II). In comparison, the protective effects of quercetin 3-*O*-monoglycosides (compounds **2**, **3**, **4**, and **6**), compound **2** showed

Table I. DPPH free radical scavenging effects of compounds **1-6**

Samples	IC_{50}(μM)[a]
1	96.29
2	10.90
3	14.30
4	9.88
5	16.20
6	13.10
L-Ascorbic acid	50.30

[a]Inhibitory effect was expressed as the mean of the 50% inhibitory concentration of triplicate determinations, obtained by interpolation of the concentration-inhibition curve.

Table II. Protective effects of compounds **1-6** on amiodarone- (**A**) and nitrofurantoin-(**B**) induced cytotoxicities in Hep G2 cells

Compound	Concentration (μg/mL)	Protection ratio (%)	
		A	**B**
1	10	1.6 ± 0.6	10.3 ± 1.2
	50	4.1 ± 0.5	10.8 ± 1.4
	100	18.1 ± 1.3	14.2 ± 1.1*
2	10	1.3 ± 0.2	3.2 ± 0.4
	50	4.0 ± 0.4	3.6 ± 0.7
	100	10.1 ± 0.2	15.1 ± 0.9*
3	10	7.0 ± 0.5	1.2 ± 0.5
	50	8.1 ± 0.2	3.0 ± 0.2
	100	6.6 ± 3.3	5.3 ± 1.3
4	10	1.4 ± 0.4	1.8 ± 0.5
	50	3.3 ± 0.6	5.4 ± 0.2
	100	9.9 ± 1.1	6.0 ± 1.3
5	10	3.8 ± 0.5	2.7 ± 0.5
	50	14.7 ± 0.4	15.8 ± 1.2
	100	31.1 ± 1.2*	25.2 ± 2.3*
6	10	7.1 ± 1.2	3.0 ± 0.5
	50	9.1 ± 1.4	6.1 ± 0.7
	100	12.5 ± 1.2	6.8 ± 2.3
Silybin	10	21.8 ± 3.3[a]	-
	50	-	11.1 ± 2.1[a]

Values represent the mean ± S.D. of three independent experiments.
*P<0.05 (one-way ANOVA test), significantly different from the control.
[a]The maximum protection ratio of the positive control (silybin) in each assay system.

a prominent protective effect in our bioassay system. Although it is not clear at this point, the above result could be due to the type of sugar moiety in the quercetin skeleton. Differences in the biological effects have also been reported in some quercetin glycoside derivatives (Arts *et al.*, 2004; Chin *et al.*, 2004; Yan *et al.*, 2002). Compound **5** also exhibited a moderate protective effect on amiodarone-induced cytotoxicity in Hep G2 cells. Not all of the isolated compounds showed protective effects on tacrine-induced cytotoxicity (data not shown). Silybin (Sigma Chemical Co.) is a well-known hepatoprotective agent commonly used as a positive control in liver-protective assay systems; however, it only showed low protective effects in our system. The hepatoprotective effect of **5** has been reported (Thabrew *et al.*, 1997), but there have been no previous published reports, to our best knowledge, on the hepatoprotective activity of compounds **2** and **5** on drug-induced cytotoxicity. This is the first report on the biological activity of compound **1**, which was recently isolated from the fruits of *Juglans mandshurica* (Liu *et al.*, 2004).

In conclusion, five phenolic compounds from *J. sinensis* showed significant free radical scavenging effects *in vitro*, and compounds **1**, **2**, and **5** exhibited moderate hepatoprotective effects on drug-induced cytotoxicity.

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