Arch Pharm Res Vol 28, No 7, 804-809, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Neuroprotective and Free Radical Scavenging Activities of Phenolic Compounds from *Hovenia dulcis*

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(Revised June 28, 2005)

The EtOAc-soluble fraction from a methanolic extract of *Hovenia dulcis* Thunb. exhibited neuroprotective activity against glutamate-induced neurotoxicity in mouse hippocampal HT22 cells. The neuroprotective activity-guided isolation resulted in 8 phenolic compounds (**1**-**8**), such as vanillic acid (**1**), ferulic acid (**2**), 3,5-dihydroxystilbene (**3**), (+)-aromadendrin (**4**), methyl vanillate (**5**), (-)-catechin (**6**), 2,3,4-trihydrobenzoic acid (**7**), and (+)-afzelechin (**8**). Among these, compounds **6** and **8** had a neuroprotective effect on the glutamate-induced neurotoxicity in HT22 cells. Furthermore, compound **6** had a DPPH free radical scavenging effect with an IC_{50} value of 57.7 μM, and a superoxide anion radical scavenging effect with an IC_{50} value of 8.0 μM. Both compounds **6** and **8** had ABTS cation radical scavenging effects with IC_{50} values of 7.8 μM and 23.7 μM, respectively. These results suggest that compounds **6** and **8** could be neuroprotectants owing to their free radical scavenging activities.

Key words: *Hovenia dulcis*, HT22 Cells, Neuroprotection, (-)-Catechin, (+)-Afzelechin, DPPH, ABTS, Superoxide

INTRODUCTION

Hovenia dulcis Thunb. (Rhamnaceae) is used in Korean and Chinese traditional medicine to treat liver diseases and as a detoxifying agent for alcoholic poisoning (Cho *et al.*, 2004). There are several papers demonstrating that the extracts of *H. dulcis* or its compounds hasten the detoxification of alcoholic poisoning (Kiyoshi, 1987; Okuma *et al.*, 1995; Yoshikawa *et al.*, 1997; Kim *et al.*, 2000; Ji *et al.*, 2001; Xu *et al.*, 2003) and protect the liver from hepatotoxic substances (Hase *et al.*, 1997a, 1997b; Kim, 2001). However, the sweetness-reducing (Saul *et al.*, 1985; Kolodny *et al.*, 1988; Kennedy *et al.*, 1988; Yoshikawa *et al.*, 1992, 1993), antioxidative and antimicrobial (Cho *et al.*, 2000), and anti-diabetic effects (Ji *et al.*, 2002) of the extracts or compounds isolated from *H. dulcis* have recently been reported. Nevertheless, research on the neuroprotective activity of this plant and its related active compounds has not been reported.

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This study examined the potential protective effect of the solvent fractions from *H. dulcis* stem bark on glutamate-induced neurotoxicity in mouse hippocampal HT22 cells, which are frequently used to study neurotoxicity related to oxidative stress (Braun *et al.*, 2000). In addition, their free radical scavenging activities were investigated, which was followed by the bioassay-guided isolation and identification of its neuroprotective compounds.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured using a JASCO P-1010 Routine Automatic Polarimeter (Tokyo, Japan). The NMR spectra were recorded on a Varian Unity Inova 300 MHz spectrometer. Samples were dissolved in chloroform-d_1 or CD_3OD and chemical shifts were reported in ppm downfield from TMS. Stationary phases for column chromatography (Silica gel 60, 70-230 and 270-400 mesh, and Lichroprep RP-18 gel, 40-63 μm, Merck) and TLC plates (Silica-gel 60 F_{254} and RP-18 F_{254}, Merck) were purchased from Merck (Darmstadt, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), vitamin E, and glutamate were purchased from Sigma

Chemicals (St. Louis, MO, U.S.A.). All other chemicals and solvents with analytical grade were used without further purification.

Plant materials

This study used the stem bark of *H. dulcis* collected in October 2003 in a mountainous area of Okcheon-gun, Chungcheongbuk-do, Korea, and dried at room temperature for two weeks. A voucher specimen (PB-3750) is preserved at the Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology in Daejeon, Korea.

Extraction and Isolation

The dried and powdered stem bark of *H. dulcis* (10.0 kg) were extracted twice with 100% MeOH (40 L) at room temperature. The MeOH solution was evaporated to dryness (0.8 kg) and the residue was suspended in H_2O (1 L) and extracted successively with hexane (3×1 L), $CHCl_3$ (3×1 L), EtOAc (3×1 L) and *n*-BuOH (3×1 L) to give the hexane (50 g), $CHCl_3$ (90 g), EtOAc (60 g) and *n*-BuOH-soluble fractions (250 g), respectively. The EtOAc-soluble fraction (60 g) was chromatographed on a silica gel column (230-400 mesh, 100×8.5 cm) with $CHCl_3$-MeOH-H_2O in a stepwise gradient mode. The fractions (500 mL in each flask) were combined on the basis of silica gel TLC and 16 fractions (F1-F16) were obtained. The fraction F7 (1.5 g) was rechromatographed over a reverse-phase column (40-63 μm, 50×4.5 cm) with CH_3OH-H_2O (gradient from 30% to 100% MeOH) in a stepwise gradient to give vanillic acid (**1**, 574.3 mg, white powder, Cho *et al.*, 2000), ferulic acid (**2**, 78.1 mg, white powder, Cho *et al.*, 2000) and 3,5-dihydroxystilbene (**3**, 6.2 mg, white powder, Wollenweber *et al.*, 2003). The fraction F8 (1.8 g) was rechromatographed over a reverse-phase column by the same method to give (+)-aromadendrin (**4**, 7.3 mg, colorless amorphous powder, $[\alpha]_D^{23}$ +44.6°, *c* 0.42, CH_3OH, Shen and Theander, 1985; Takahashi *et al.*, 1988) and methyl vanillate (**5**, 6.8 mg, white power, Quideau *et al.*, 2001). The fraction F11 (1.5 g) was also rechromatographed over a reverse-phase column (40–63 mm, 50×3.0 cm) with CH_3OH-H_2O in a stepwise gradient to give subfractions F11-1–F11-10. Fraction F11-1 was rechromatographed on a reverse phase column (60×2.0 cm, LiChroprep RP-18) with a MeOH-H_2O (gradient from 20% to 100% MeOH) eluent to afford (–)-catechin (**6**, 67.3 mg, yellowish amorphous powder, $[\alpha]_D^{23}$ -33.0°, *c* 0.64, MeOH, Watanabe, 1998) and 2,3,4-trihydrobenzoic acid (**7**, 34.6 mg). (+)-Afzelechin (**8**, 38.0 mg, yellowish amorphous powder, $[\alpha]_D^{23}$ +25.4°, *c* 0.26, MeOH, Wan and Chan, 2004; Drewes *et al.*, 1992) was isolated from the subfraction F11-4 (400 mg) by the same method (60×2.0 cm, LiChroprep RP-18, MeOH-H_2O gradient from 30% to 100% MeOH). Compound **7** was identified through the comparison with an authentic sample (Sigma).

Cell culture

The mouse hippocampal HT22 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in Dulbeccos Modified Eagle's Medium (DMEM) (GibcoBRL, Gaithersburg, MD, U.S.A.) supplemented with 10% FBS, 10 mM HEPES, 200 IU/mL penicillin and 200 μg/mL of streptomycin. The culture medium was replaced every other day. After attaining confluence, the cells were subcultured following trypsinization with 0.25% trypsin-EDTA solution.

Measurement of cell viability

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Cells were plated at density of 2.5×10^3 cells/100 μL medium in a 96-well plate and treated with 5 mM glutamate with or without of compounds for 24 hours. The medium was incubated with 10 μL of 5 mg/mL of MTT solution for 4 h at 37°C. Culture medium was removed, and 200 μL of DMSO was added to each well to dissolve formazan. Absorbance was measured at 540 nm using an ELISA reader (Molecular Devices, Versa MAX Sunnyvale, CA, U.S.A.). Cell viability was expressed as percent of the control culture (Mosmann, 1983; Kim-Han and Sun, 1998).

DPPH radical scavenging activity

The DPPH assay performed as described (Yun *et al.*, 2000). Each concentration of a test sample solution in DMSO (10 μL) was added to 190 μL of 150 μM DPPH–ethanol solution. After vortex mixing, the mixture was incubated for 10 minutes at room temperature and the absorbance values were measured at 517 nm. The differences in absorbance between a test sample and a control (DMSO) were taken and the IC_{50} values were determined as the concentration of the sample that gave a 50% decrease in the absorbance from a blank test.

Superoxide anion radical scavenging activity

Superoxide anion radical ($O_2^{\cdot-}$) scavenging activity was assayed by the modified irradiated riboflavin/EDTA/nitroblue tetrazolium (NBT) system (Kim *et al.*, 2003). The reaction mixture contained 150 μL of 0.03 mM riboflavin, 1 mM EDTA, 0.06 mM methionine and 0.03 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 μL of methanolic solutions of various concentrations of test compounds in a 96-well plate. The photoinduced reactions were performed in an aluminum foil-lined box

with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated at 25°C for 7 minutes. The photochemically reduced riboflavin generated $O_2^{\cdot-}$, which reduced NBT to form blue formazan. The unilluminated reaction mixture used as a blank. Reduction of NBT was measured at 570 nm before and after irradiation using a microplate reader. The effective relative concentration IC_{50} is the concentration of sample that inhibits 50% of NBT reduction.

ABTS cation radical scavenging activity

2,2-Azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) cation radical ($ABTS^{\cdot+}$) scavenging activity assay was carried out according to the method reported by Braca *et al.* (2003) with minor modifications. $ABTS^{\cdot+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate. The $ABTS^{\cdot+}$ solution was diluted with phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30°C. The reaction was initiated by the addition of 190 μL of diluted ABTS to 10 μL of each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to blank absorbance (DMSO).

RESULTS AND DISCUSSION

The MeOH extract of the stem bark of *H. dulcis* was suspended in H_2O and then partitioned subsequently with hexane, chloroform, EtOAc, and *n*-BuOH. Among the partitioned fractions, the EtOAc-soluble fraction exhibited significant neuroprotective activity (71.3 ± 8.1%) against the glutamate-induced neurotoxicity in HT22 cells at a concentration of 5 μg/mL, and showed obvious antioxidant activity against DPPH, ABTS, and superoxide radical-scavenging assay with IC_{50} values of 18.3, 4.9, and 4.0 μg/mL, respectively (Table I). This indicates that the EtOAc-soluble fraction from the methanolic extract of *H. dulcis* stem bark may be a neuroprotectant owing to its free radical scavenging activity. The EtOAc-soluble fraction was further chromatographed on a silica gel column and reverse-phase C18 column, which afforded eight phenolic compounds (**1-8**), such as vanillic acid (**1**, Cho *et al.*, 2000), ferulic acid (**2**, (ho *et al.*, 2000), 3,5-dihydroxystilbene (**3**, Wollenweber *et al.*, 2003), (+)-aromadendrin (**4**, Shen and Theander, 1985; Takahashi *et al.*, 1988), methyl vanillate (**5**, Quideau *et al.*, 2001), (-)-catechin (**6**, Watanabe, 1998), 2,3,4-trihydrobenzoic acid (**7**), and (+)-afzelechin (**8**, Wan and Chan, 2004; Drewes *et al.*, 1992). Their structures were identified by a comparison of the ^{1}H-NMR, ^{13}C-NMR, DEPT, MS data, and optical rotation with those reported in the literature.

Table I. The protective activities of the solvent fractions (5 μg/mL) on glutamate-induced neurotoxicity in HT22 cells and their free radical scavenging effects (IC_{50} values)

	Cell viability (% of controls)[f]	DPPH[c] (μg/mL)[g]	Superoxide[d] (μg/mL)	ABTS[e] (μg/mL)
Glutamate[a]	38.3±4.1			
MeOH extract	46.6±5.9	67.1	22.4	17.5
H_2O fraction	40.1±5.3	97.6	14.9	7.2
Hexane fraction	38.3±6.8	> 200.0	> 200.0	> 200.0
$CHCl_3$ fraction	35.4±4.9	124.3	15.6	29.0
EtOAc fraction	71.3±8.1*	18.3	4.0	4.9
n-BuOH fraction	36.3±7.2	27.4	5.1	5.7
Vitamin E[b]	89.9±5.9*	8.7	NA[h]	NA

[a] Glutamate (5 mM) neurotoxicity in HT22 cells
[b] positive control
[c] DPPH free radical
[d] Superoxide anion radical
[e] ABTS cation radical
[f] Data (n = 5) are presented as mean±SEM. *P<0.01, with versus the glutamate alone.
[g] The values are the means of triplicate data.
[h] NA: not applicable

Compounds **6**, **7**, and **8** from the genus *Hovenia* have not previously been reported.

Among these purified compounds, (-)-catechin (**6**) and (+)-afzelechin (**8**) had protective effects (78.0±6.0% and 73.0±7.8%, respectively) on the glutamate-induced neurotoxicity in HT22 cells at a concentration of 5.0 μg/mL, but compounds **1-5** and **7** were inactive (Table II).

The antioxidant activities of these isolated compounds (**1-8**) were examined to determine if the neuroprotective effects were mediated through the antioxidant effects of these compounds. As shown in Table II, two neuroprotective compounds, **6** and **8**, exhibited ABTS cation radical scavenging effects with IC_{50} values of 7.8 μM and 23.9 μM, respectively. In addition, compound **6** also had a superoxide anion radical scavenging effect with an IC_{50} value of 8.0 μM, and a DPPH free radical scavenging effect with an IC_{50} value of 57.7 μM. These observations suggested that the neuroprotective effects of compounds **6** and **8** on the glutamate-induced neurotoxicity in HT22 cells might be medicated *via* their free radical scavenging activities. However, compounds **6** and **8** exhibited less protective activity than vitamin E, which used as the positive control.

It was reported that the oral administration of (-)-catechin (**6**) protected the hippocampal CA1 pyramidal cells against neurotoxicity induced by transient focal brain ischemia in gerbils, and that this neuroprotective effect was accompanied by an increase in the superoxide scavenging activity (Inanami *et al.*, 1998). Both a co- and post-treatment with (+) racemer of catechin were capable

Table II. The protective activities of isolated compounds on glutamate-induced neurotoxicity in HT22 cells and their free radical scavenging effects (IC_{50} values)

	Cell viability (% of controls)[g]			DPPH[d] (μM)[h]	Superoxide[e] (μM)	ABTS[f] (μM)
	5 (μM)	1 (μM)	0.5 (μM)			
Glutamate[a]	37.9±4.5					
1	34.2±3.2	NA[i]	NA	>100.0	4.9	>100.0
2	39.5±3.9	NA	NA	>100.0	10.2	12.6
3	37.0±4.0	NA	NA	>100.0	17.9	18.0
4	35.1±3.8	NA	NA	>100.0	>100.0	>100.0
5	37.8±2.8	NA	NA	>100.0	35.1	>100.0
6	78.0±6.0*	63.0±9.1*	59.2±8.9*	57.7	8.0	7.8
7	38.6±3.2	NA	NA	>100.0	>100.0	>100.0
8	73.0±7.8*	64.5±2.7*	58.0±7.0*	>100.0	>100.0	23.9
Vitamin E[b]	90.0±5.9*	82.1±3.9*	77.6±8.4*	20.2	NA	NA
Trolox[c]	NA	NA	NA	NA	36.8	12.8

[a] Glutamate (5 mM) neurotoxicity in HT22 cells
[b] positive control
[c] positive control (Oh *et al.*, 2004)
[d] DPPH free radical
[e] Superoxide anion radical
[f] ABTS cation radical
[g] Data (n = 5) are presented as mean±SEM. *P<0.01, with versus the glutamate alone.
[h] The values are the means of triplicate data.
NA: not applicable

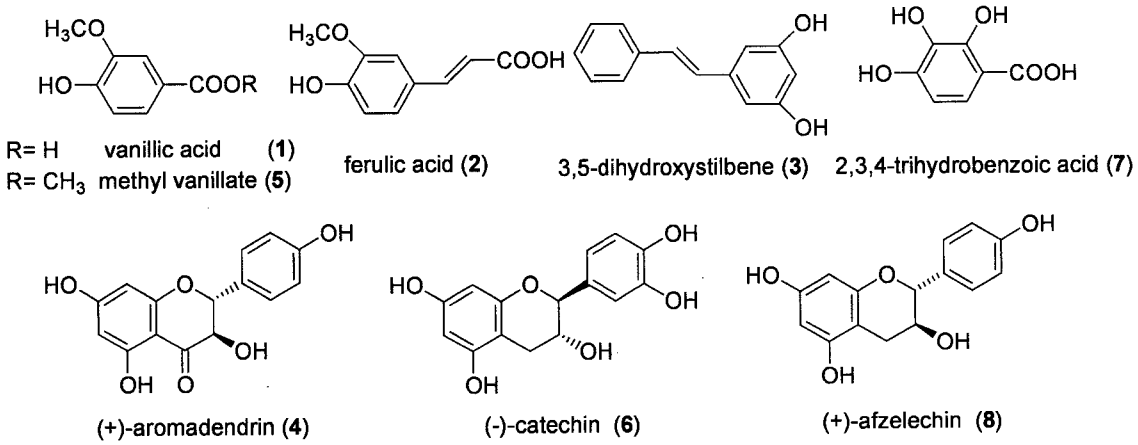

Fig. 1. Structures of compounds **1-8** isolated from the stem bark of *Hovenia dulcis*

of attenuating the hippocampal cultured cell death and the accumulation of intracellular reactive oxygen species produced by sodium nitroprusside (SNP, Bastianetto *et al.*, 2000). However, (+)-catechin could not protect the HT22 cells from glutamate toxicity (Ishige *et al.*, 2001), which had an opposite behavior to (-)-catechin (**6**). (-)-Epiafzelechin was reported to have selective inhibitory activity against cyclooxygenase-1 (COX-1) over COX-2 (Wan and Chan, 2004). However, the neuroprotective and free radical scavenging activities of (+)-afzelechin (**8**) has not been previously reported.

In conclusion, (-)-catechin (**6**) and (+)-afzelechin (**8**) from the stem bark of *H. dulcis* were isolated as the major compounds. These compounds had protective effects on the glutamate-induced neurotoxicity in HT22 cells, and this neuroprotection may be due to their free radical scavenging activities. These results suggest that (-)-catechin (**6**) and (+)-afzelechin (**8**) might be potent neuroprotectants.

ACKNOWLEDGEMENTS

This research was financially supported by a grant

(PF0300401-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology of the Republic of Korea and a grant of KRIBB Research Initiative Program (KGS0570412).

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