Neuroprotective Compounds from *Salix pseudo-lasiogyne* Twigs and Their Anti-Amnesic Effects on Scopolamine-Induced Memory Deficit in Mice

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Abstract

Bioassay-guided fractionation of an 80% methanolic extract of *Salix pseudo-lasiogyne* twigs has resulted in the isolation of two new compounds (**1**–**2**) along with ten known ones (**3**–**12**). The new compounds were determined to be 3′-*O*-acetylsalicin (**1**) and 2′,6′-*O*-acetylsalicortin (**2**) by using spectroscopic analyses. Compounds (**3**–**12**) were identified as salicin (**3**), 2′-*O*-acetylsalicin (**4**), salicortin (**5**), 2′-*O*-acetylsalicortin (**6**), 3′-*O*-acetylsalicortin (**7**), 6′-*O*-acetylsalicortin (**8**), 2′-*O*-(*E*)-*ρ*-coumaroylsalicortin (**9**), grandidentatin (**10**), isograndidentatin (**11**), and saligenin (**12**). Among the isolated compounds, compounds **2, 5, 6, 7**, and **8** bearing 1-hydroxy-6-oxo-2-cyclohexenecarboxylate moiety significantly inhibited lipopolysaccharide-induced nitric oxide production in BV2 microglial cells *in vitro*. Further, we studied anti-amnesic activities of the 80% methanolic extract, the EtOAc fraction, and compound **6** from *S. pseudo-lasiogyne*. They exerted a significant cognitive-enhancing effect on scopolamine-induced memory deficit in mice. In addition, they also significantly increased the reduced activities of glutathione reductase and superoxide dismutase and the glutathione content in the hippocampus and cortex of scopolamine-induced amnesic mice.

Key words

Salix pseudo-lasiogyne · Salicaceae · salicortin · 1-hydroxy-6-oxo-2-cyclohexenecarboxylate · BV2 microglial cell · passive avoidance test

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The genus *Salix* (Salicaceae) comprises approximately 400 species of deciduous trees distributed in cold and temperate regions of the Northern hemisphere. The willow tree is the most abundant among the *Salix* spp. and has been used to relieve pain and inflammation for thousands of years [1]. *Salix pseudo-lasiogyne* H. Lev., distributed over several Asian countries, has been used for the treatment of pain and fever in Korean traditional medicine [2]. While searching for anti-inflammatory natural products using BV2 microglial cells, which are widely employed in *in vitro* assay systems [3,4], it was found that an 80% methanolic extract of *S. pseudo-lasiogyne* significantly inhibited NO production induced by lipopolysaccharide (LPS) in BV2 microglial cells. Thus, we attempted to isolate active compounds from *S. pseudo-lasiogyne* and to evaluate those compounds' anti-inflammatory activity in BV2 microglia. It has also been reported that excessive NO-induced inflammation can increase oxidative stress in the brain, which consequently can induce neurodegenerative disorder accompanied by memory deficit [5]. Thus, we also examined the memory-enhancing effects of an extract of *S. pseudo-lasiogyne* and of a major compound from this plant that showed the most potent anti-inflammatory activity *in vitro* on scopolamine–induced amnesic mice using the passive avoidance test.

Dried and pulverized *S. pseudo-lasiogyne* twigs were extracted with 80% methanol by using an ultrasonic apparatus. The 80% methanolic extract was suspended in distilled water and successively partitioned with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc fraction, which showed the most potent inhibitory activity on NO production of LPS-induced BV2 cells, was subjected to repeated column chromatography (CC) and high-performance liquid chromatography (HPLC), resulting in the isolation of two new compounds, **1**–**2**, and ten known ones (**3**–**12**) (**Fig. 1**). Structures of these compounds were unequivocally determined by 1D, 2D NMR experiments, MS analyses, as well as by comparison with reference materials of known compounds.

Compound **1** was isolated as yellowish needles, $[\alpha]_D^{25}$ −60.1 (*c* 0.80, EtOH), and its molecular formula ($C_{15}H_{20}O_8$) was established by positive HRFABMS (*m/z* 329.1234 $[M + H]^+$, calcd. for 329.1236). In the ^{1}H NMR spectrum, the chemical shifts and coupling constants of the characteristic signals at δ_H 7.76 (1H, d, J = 6.9 Hz, H-3), 7.54 (1H, d, J = 10.9 Hz, H-6), 7.22 (1H, td-like, J = 8.5, 1.5 Hz, H-5), and 7.09 (1H, t, J = 7.3 Hz, H-4) indicated the presence of a 1, 2-disubstituted benzene moiety. Also, the signals at δ_H 5.52 (1H, d, J = 7.9 Hz, H-1′) suggested that compound **1** had an anomeric proton. In the HMBC spectrum of **1**, the anomeric proton at δ_H 5.52 and two methylene protons at δ_H 5.25 (1H, d, J = 13.8 Hz, H-7a) and 5.08 (1H, d, J = 13.8 Hz, H-7b) correlated with quaternary carbons at δ_C 156.3 (C-1) and at δ_C 133.0 (C-2), respectively (**Fig. 2**). Thus, a sugar residue and an oxygenated methylene moiety were present at C-1 and C-2. From the above information, compound **1** was deduced to be similar to a known compound, salicin (**3**), except for the signals at δ_H/δ_C 1.98 (3H, s, H-2″)/21.1 (C-2″) and at δ_C 170.7 (C-1″) [6,7]. HMBC spectrum correlation between the proton at δ_H 5.89 (1H, t, J = 9.5 Hz, H-3′) and a carbonyl carbon inacetyl group (δ_C 170.7) determined the position of the acetyl group at C-3′. It was previously reported that an acetyl moiety was located at C-2′ or C-6′ instead of at C-3′ in the structure of the same aglycone [8,9]. On the basis of the above-described information, compound **1** was determined to be 3′-*O*-acetylsalicin.

Compound **2** was isolated as a whitish, amorphous powder, $[\alpha]_D^{25}$ −130.1 (*c* 1.12, EtOH). The positive HRFABMS of **2** exhibited *m/z* 531.1481 $[M + Na]^+$ (calcd. for 531.1478) indicating $C_{24}H_{28}O_{12}$ as its molecular formula. The ^{1}H and ^{13}C NMR spectra and the 2D NMR analysis showed features similar to those of compound **3** except for characteristic peaks that indicated the structure of 1-hydroxy-6-oxo-2-cyclohexenecarboxylate and two acetyl moieties. In the HMBC spectrum, the position of 1-hydroxy-6-oxo-2-cyclohexenecarboxylate was determined by the cross-peaks from δ_H 5.13 (1H, m, H-7a) and 5.12 (1H, m, H-7b) to δ_C 172.2 (C-8) (**Fig. 2**). Correlation peaks from δ_H 4.99 (1H, t, J = 9.6 Hz, H-2′) to δ_C 172.7 (C-1″) and from δ_H 4.40 (1H, m, H-6′a) and 4.27 (1H, m, H-6′b) to δ_C 173.4 (C-1‴) confirmed that the position of the two acetyl moieties were at OH-1″ and OH-6″, respectively. Thus, compound **2** was identified as 2′,6′-*O*-acetylsalicortin. Although it was previously reported in an organic synthetic study, this is the first report of its occurrence in nature [10].

The ten known compounds were identified as salicin (**3**) [6,7], 2′-*O*-acetylsalicin (**4**) [8], salicortin (**5**) [7,11], 2′-*O*-acetylsalicortin

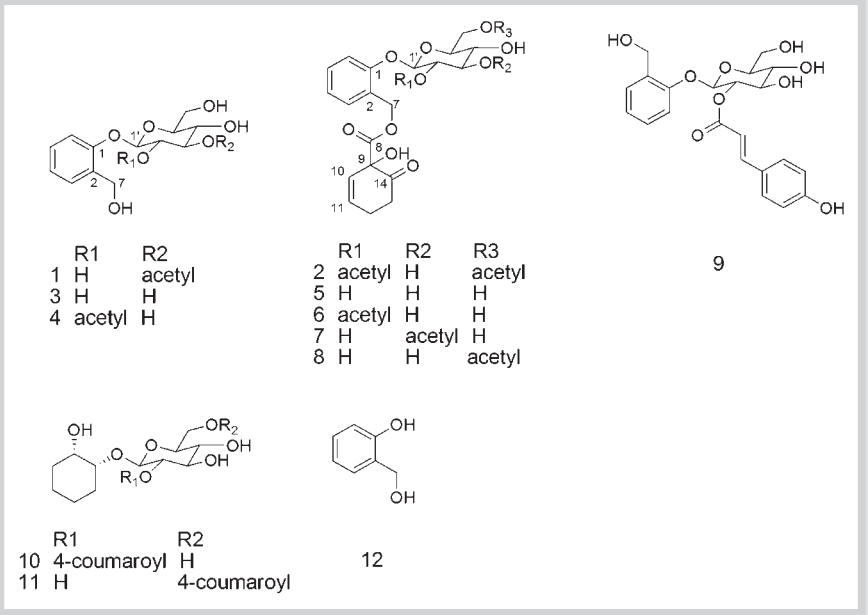

Fig. 1 Structures of compounds **1**–**12** isolated from *S. pseudo-lasiogyne* twigs.

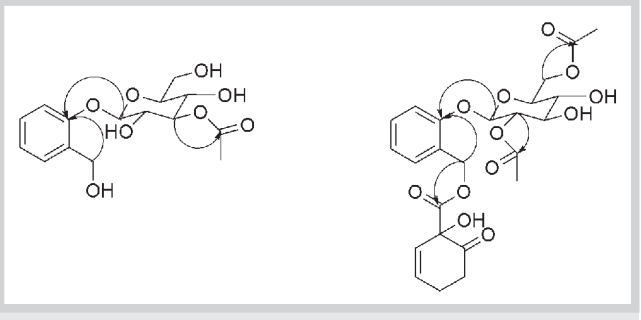

Fig. 2 Key correlations of HMBC spectral data of compounds **1** and **2**.

(**6**) [8], 3′-*O*-acetylsalicortin (**7**) [12], 6′-*O*-acetylsalicortin (**8**) [13], 2′-*O*-*(E)*-*ρ*-coumaroylsalicortin (**9**) [13], grandidentatin (**10**) [14], isograndidentatin (**11**) [14], and saligenin (**12**) [15] (**Fig. 1**).

We examined the inhibitory activity of an 80% methanolic extract of *S. pseudo-lasiogyne* and its *n*-hexane, EtOAc, and *n*-BuOH fractions on LPS-stimulated NO production in BV2 microglia. The EtOAc fraction which showed the most potent inhibitory activity (IC_{50} 17.6 mg/mL; data not shown) was subjected to repeated chromatographic techniques. The two new compounds **1**–**2** along with the ten known compounds **3**–**12** were isolated, and their inhibitory activity on LPS-stimulated NO production in BV2 cells was determined (**Table 1**). Compounds **2, 5, 6, 7**, and **8** exhibited more potent inhibitory activities than the other compounds. Compound **3** and its derivatives (**1** and **4**) showed weak inhibitory activity, while saligenin (**12**), a metabolite of **3**, showed no inhibitory activity. The key difference between the salicin- (**1, 3**, and **4**) and salicortin-type (**2, 5, 6, 7**, and **8**) compounds was the presence of a 1-hydroxy-6-oxo-2-cyclohexenecarboxylate moiety at OH-7. These data suggest that the moiety could be an important factor in elucidating the inhibitory activity of LPS-induced NO production in BV2 microglia.

Further, we aimed to determine whether the 80% methanolic extract, the EtOAc fraction, and a compound from *S. pseudo-lasiogyne*, which all had anti-inflammatory effects *in vitro*, had cognitive–enhancing activity in mice with memory deficits induced by scopolamine. Moreover, we attempted to preliminarily examine the action mechanisms *in vivo*. Since compound **6** was the most abundant among the obtained compounds **1**–**12** and had 1-hydroxy-6-oxo-2-cyclohexenecarboxylate moiety, which significantly inhibited LPS-induced NO production in BV2 microglial cells, we investigated the effect of compound **6** on attenuated memory deficits induced by scopolamine in mice. The cognitive-enhancing effect of the 80% methanolic extract, EtOAc fraction of *S. pseudo-lasiogyne*, and compound **6** was evaluated using the passive avoidance test (**Table 2**). The step-through latency of the scopolamine-treated mice (1 mg/kg body weight s.c.; 28.1 s) was significantly reduced compared to that of the 0.5% carboxylmethyl cellulose-treated control mice (176.1 s). However, the short step-through latency induced by scopolamine was significantly reversed by treatment with the 80% methanolic extract (100 mg/kg body weight p.o.), the EtOAc fraction (100 mg/kg body weight p.o.), and compound **6** (1 and 2 mg/kg body weight p.o.). Donepezil (2 mg/kg body weight p.o.), an acetylcholinesterase inhibitor and the most widely used treatment for Alzheimer's disease, was used as a positive control, and it restored step-through latency time by 119.7 s. In comparison with the cognitive-enhancing activity of donepezil, the 80% methanolic extract (109.3 s at 100 mg/kg body weight p.o.) and compound **6** (109.4 s at 2 mg/kg body weight p.o.) were able to significantly restore memory deficits induced by scopolamine in mice.

There has been a controversy over whether oxidative stress plays a primary role in, or is only a consequence of, the process of neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases [16]. Nevertheless, the suppression of elevated oxidative stress can be a therapeutic target when attempting to attenuate neurodegenerative disorders [17]. Thus, we studied whether the 80% methanolic extract, the EtOAc fraction, and compound **6** from *S. pseudo-lasiogyne* twigs, which exhibited anti-inflammatory activities in BV2 microglial cells, have the potential to repress oxidative stress on scopolamine-induced memory deficit in mice. In the passive avoidance test, treatments with the 80% methanolic extract (50 mg/kg body weight, p.o.) or the

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	> 100	**8**	11.4 ± 2.1
2	13.2 ± 1.3	**9**	> 100
3	> 100	**10**	> 100
4	> 100	**11**	> 100
5	15.3 ± 3.1	**12**	> 100
6	11.4 ± 2.2	L-NIL	65.1 ± 4.1
7	11.9 ± 0.5		

IC_{50} means the 50% inhibitory concentration (μM) on LPS-induced NO production in BV2 cells. The nitrite concentration in vehicle- and LPS-treated cells was 6.3 ± 0.2 and 54.1 ± 0.2 μM, respectively. L-NIL was used as the positive control

Table 1 Inhibitory effects of compounds **1**–**12** from *S. pseudo-lasiogyne* twigs on LPS-induced NO production in BV2 microglial cells.

Experimental treatment	Step-through latency (s) (% of control)
Control[a]	176.1 ± 3.7 (100%)
Sco[b]	28.1 ± 5.0 (16.0%)
Sco + 80% methanolic extract (50 mg/kg b.w.[c])	63.1 ± 13.1 (35.8%)
Sco + 80% methanolic extract (100 mg/kg b.w.)	109.3 ± 23.1 (62.1%)*
Sco + EtOAc fraction (50 mg/kg b.w.)	31.9 ± 3.8 (18.1%)
Sco + EtOAc fraction (100 mg/kg b.w.)	68.9 ± 14.9 (39.1%)
Sco + compound **6** (1 mg/kg b.w.)	39.8 ± 6.8 (22.6%)
Sco + compound **6** (2 mg/kg b.w.)	109.4 ± 19.9 (62.1%)*
Sco + donepezil (2 mg/kg b.w.)	119.7 ± 16.9 (68.0%)**

The values shown are the mean latency ± SEM. Results differ significantly from the value in the scopolamine-treated group (* $p < 0.05$ and ** $p < 0.01$). [a] Control indicates the 0.5% CMC and saline-treated group (10 mL/kg body weight, p.o.). [b] Scopolamine (Sco) indicates the 0.5% CMC and scopolamine-treated group (1 mg/kg body weight, s.c.). [c] b.w.: body weight

Table 2 The cognitive–enhancing effect of 80% methanolic extract, EtOAc fraction, and compound **6** of *S. pseudo-lasiogyne* twigs on scopolamine-induced amnesic mice in the passive avoidance test[a].

EtOAc fraction (50 mg/kg body weight p.o.) did not result in remarkable changes in step-though latency (**Table 2**). However, the mice treated with 80% methanolic extract or the EtOAc fraction at 100 mg/kg body weight p.o. significantly recovered their memory deficit. After the passive avoidance test, the cortex and hippocampus of the mice were removed for an antioxidant enzyme assay. The amnesic mice treated with the 80% methanolic extract or the EtOAc fraction had significantly restored the reduced levels of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as restored cellular glutathione content (**Table 3**). In addition, treatment with compound **6** resulted in a significant reversal in GR activity and glutathione content, which had been lowered by scopolamine in the mouse cortex and hippocampus; however the activities of SOD and GPx were virtually unchanged. In a redox cycle, GR reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) by using NADPH [18]. We suggest that compound **6** may selectively restore the activity of GR, after which there is an elevation in GSH content. In contrast to the results from the compound **6**-treated mice, 80% methanolic extract- and EtOAc fraction-treated mice substantially reversed all of the antioxidant markers, including SOD and GPx, as well as increased the activity of GR and GSH content. Several studies have reported that the synergistic effects of combined components in a natural products extract are more effective than the effects of isolated single compounds or of the sum of some of them [19–21]. Although a profound mechanism study of the synergic effect between compound **6** derivatives and other components in *S. pseudo-lasiogyne* twigs would be needed, it seems that processed extractor compounds from this plant might have potential as therapeutic agents in cognitive disorders.

Materials and Methods

CC was carried out on Kiesgel 60 silica gel (40–60 μm, 230–400 mesh; Merck), YMC-GEL ODS-A (5–150 μm; YMC), and Sephadex LH-20 (25–100 μM; Pharmacia). Thin-layer chromatography was carried out on Kiesgel 60 F_{254} coated normal silica gel and RP-18 F_{254} coated C_{18} silica gel. The1D and 2D spectral data were measured on a Bruker AMX 400 or 500 spectrometer. Solvent signals were used as internal standards. High-resolution and low-resolution FABMS results were obtained on a JEOL JMS-AX505WA. The FT-IR spectra were measured with a JASCO FT/IR-300 spectrophotometer. The HPLC system consisted of a G-321 pump (Gilson), a G-151 UV detector (Gilson), and an YMC-Pack Pro C_{18} column (250 mm × 10 mm i.d.; 5 μm). HPLC grade solvents (Fisher Scientific) were used in the MeOH-H_2O system.

The *S. pseudo-lasiogyne* twigs were collected at the Medicinal Plant Garden, Seoul National University, Goyang, Korea, in July 2009. Air-dried *S. pseudo-lasiogyne* twigs were identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University, Korea. A voucher specimen (SNUPH-1105) has been stored in the Herbarium of the Medicinal Plant Garden, Seoul National University, Korea.

The obtained *S. pseudo-lasiogyne* twigs (17 kg) were extracted with 80% MeOH (15 L × 3) three times in an ultrasonic apparatus (3 h × 3). The solvent was removed *in vacuo*, and an 80% MeOH extract (1.2 kg) was suspended in H_2O and successively partitioned into *n*-hexane (48 g), EtOAc (121 g), and *n*-BuOH (160 g) fractions. The EtOAc fraction was subjected to silica gel CC (20 × 60 cm) eluted with $CHCl_3$:MeOH of increasing polarity (50 : 1, 30 : 1, 10 : 1, 5 : 1, 3 : 1, 0 : 1; 20 l) to give eight fractions (SXE1–8). SXE7 was subjected to normal silica gel CC (2 × 60 cm) to afford three fractions (SXE7A–C). By reverse-phase (RP) C_{18}HPLC with MeOH : H_2O (7 : 3, 2 mL/min), compound **2** (28 mg)

Table 3 Antioxidative effects of 80% methanolic extract, EtOAc fraction, and compound **6** of *S. pseudo-lasiogyne* twigs on the activities of antioxidant enzymes and glutathione level within the cortex and hippocampus of scopolamine-induced amnesic mice in the passive avoidance test.

Groups	SOD (U mg-1 protein)		GPx (μmol NADPH oxidized/min/mg protein)		GR (μmol NADPH oxidized/min/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Control	15.411 ± 0.605	28.402 ± 2.194	0.105 ± 0.005	0.173 ± 0.023	46.857 ± 5.939	52.019 ± 3.086
Sco	10.381 ± 0.171$^{\#}$	17.122 ± 0.117$^{\#\#}$	0.084 ± 0.009$^{\#\#}$	0.106 ± 0.012$^{\#\#}$	32.273 ± 0.823$^{\#\#}$	40.207 ± 2.757$^{\#\#}$
Total	14.907 ± 0.761*	27.122 ± 0.778**	0.102 ± 0.007	0.125 ± 0.007	43.157 ± 1.834**	43.157 ± 1.834
EtOAc	15.875 ± 0.688**	24.867 ± 1.503**	0.100 ± 0.010*	0.178 ± 0.008**	45.786 ± 2.377**	49.443 ± 6.685**
Compound **6** (1 mg/kg)	12.592 ± 0.213*	16.825 ± 0.948	0.083 ± 0.007	0.114 ± 0.002	39.675 ± 1.713*	37.413 ± 1.687
Compound **6** (2 mg/kg)	11.407 ± 0.975	17.567 ± 2.759	0.086 ± 0.008	0.143 ± 0.006**	41.851 ± 0.554*	41.737 ± 3.830
Donepezil	15.093 ± 0.358**	24.704 ± 3.343**	0.094 ± 0.004**	0.129 ± 0.019*	45.193 ± 0.387**	50.122 ± 3.848**
Groups	**Total GSH (nmol/mg protein)**		**GSH (nmol/mg protein)**		**GSSG/total GSH**	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Control	19.958 ± 0.578	22.616 ± 0.545	13.165 ± 0.341	14.926 ± 1.127	0.346 ± 0.047	0.328 ± 0.030
Sco	17.669 ± 1.008	14.615 ± 0.533$^{\#\#}$	10.451 ± 0.289	9.240 ± 1.013$^{\#\#}$	0.381 ± 0.027	0.364 ± 0.037
Total	25.779 ± 0.650**	22.171 ± 1.414**	19.262 ± 0.485	15.001 ± 0.592**	0.272 ± 0.015	0.291 ± 0.034
EtOAc	24.559 ± 0.550**	25.871 ± 2.268**	19.351 ± 0.441**	18.654 ± 0.545**	0.227 ± 0.022	0.280 ± 0.031
Compound **6** (1 mg/kg)	16.909 ± 0.581	16.473 ± 0.958	12.411 ± 0.578*	11.750 ± 0.470*	0.285 ± 0.071	0.262 ± 0.025
Compound **6** (2 mg/kg)	20.657 ± 0.858*	20.420 ± 0.914**	15.596 ± 0.552**	14.671 ± 0.829*	0.256 ± 0.024	0.241 ± 0.039
Donepezil	20.049 ± 1.282	18.609 ± 1.115*	14.426 ± 0.515**	13.362 ± 0.347*	0.271 ± 0.046	0.290 ± 0.024

Each value represents the mean ± SD. Sco: scopolamine. Results differ significantly from the value in the scopolamine-treated group (* $p < 0.05$ and ** $p < 0.01$) and from the value of normal control group: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$

was obtained from SXE7A3 which was separated from ODS silica gel CC (MeOH : H_2O 2 : 8, 8 : 2, 2 mL/min). SXE7B was separated into four fractions (SXE7B1–4) by normal silica gel CC (2 × 60 cm) with $CHCl_3$: MeOH (15 : 1, 0 : 1; 1 l). SXE7B4 was chromatographed by RP C_{18} HPLC (MeOH : H_2O = 8 : 2, 2 mL/min) to give compound **1** (9 mg).

3′-O-*Acetylsalicin* (**1**): yellowish needles; $[\alpha]_D^{25}$ −60.1 (*c* 0.80, EtOH); ^{1}HNMR (500 MHz, pyridine-d_5): δ 7.76 (1H, d, *J* = 6.9 Hz, H-3), 7.54 (1H, d, *J* = 10.9 Hz, H-6), 7.22 (1H, td, *J* = 8.5, 1.5 Hz, H-5), 7.09 (1H, t, *J* = 7.3 Hz, H-4), 5.89 (1H, t, *J* = 9.5 Hz, H-3′), 5.52 (1H, d, *J* = 7.9 Hz, H-1′), 5.25 (1H, d, *J* = 13.8 Hz, H-7a), 5.08 (1H, d, *J* = 13.7 Hz, H-7b), 4.48 (1H, dd, *J* = 9.7, 2.0 Hz, H-6′a), 4.38 (2H, m, H-4′ and H-6′b), 4.28 (1H, t, *J* = 8.0 Hz, H-2′), 4.06 (1H, m, H-5′), 1.98 (3H, s, H-2″); δ 170.7 (C-1″), 156.3 (C-1), 133.0 (C-2), 128.6 (C-3 and C-5), 122.9 (C-4), 116.3 (C-6), 103.3 (C-1′), 79.3 (C-3′), 78.6 (C-5′), 73.0 (C-2′), 68.9 (C-4′), 61.8 (C-6′), 60.1 (C-7), 21.1 (C-2″); FABMS *m/z* 329 $[M + H]^+$; HRFABMS *m/z* 329.1234 $[M + H]^+$ (calcd. for 329.1236).

2′,6′-O-*Acetylsalicortin* (**2**): colorless oil; $[\alpha]_D^{25}$ −130.1 (*c* 1.12, EtOH); ^{1}HNMR (500 MHz, CD_3OD): δ 7.29 (1H, m, H-3), 7.28 (1H, m, H-5), 7.13 (1H, d, *J* = 8.1 Hz, H-6), 7.04 (1H, t, *J* = 7.5 Hz, H-4), 6.15 (1H, m, H-11), 5.77 (1H, d, *J* = 9.8 Hz, H-10), 5.13 (2H, m, H-7), 5.07 (1H, m, H-1′), 4.99 (1H, t, *J* = 9.6 Hz, H-2′), 4.40 (2H, m, H-6′a), 4.27 (1H, m, H-6′b), 3.68 (1H, m, H-5′), 3.63 (1H, m, H-3′), 3.47 (1H, m, H-4′), 2.88 (1H, m, H-13a), 2.66 (1H, m, H-12a), 2.53 (1H, m, H-13b), 2.48 (1H, m, H-12b), 2.12 (3H, s, H-2″), 2.02 (3H, s, H-2‴); ^{13}CNMR (125 MHz, CD_3OD): δ 208.1 (C-14), 173.4 (C-1‴), 172.7 (C-1″), 172.2 (C-8), 157.1 (C-1), 134.1 (C-11), 131.7 (C-3), 131.3 (C-5), 130.2 (C-10), 127.1 (C-2), 124.8 (C-4), 117.7 (C-6), 101.2 (C-1′), 80.0 (C-9), 76.6 (C-3′), 76.3 (C-5′), 75.7 (C-2′), 72.4 (C-4′), 64.8 (C-7), 65.3 (C-6′), 37.6 (C-13), 28.0 (C-12), 21.8 (C-2″), 21.5 (C-2‴); FABMS *m/z* 509 $[M + H]^+$; HRFABMS *m/z* 531.1481 $[M + Na]^+$ (calcd. for 531.1478).

The BV2 microglial cells were maintained in DMEM (Sigma) supplemented with 10% FBS, 100 IU/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma) at 37 °C in a humidified incubator containing 5% CO_2 gas. Compounds **1**–**12** were dissolved in DMSO (final concentration, < 0.1%). The purity of the tested compounds was verified to be above 95% by using an HPLC-UV system. For those assays, the cells were seeded in 48-well plates at a density of 4×10^5 cells/mL and incubated overnight. BV2 cells were treated with vehicle or compounds **1**–**12** at concentrations from 1 μM to 100 μM for 24 h. Inhibitory activity of each compound on LPS-induced NO production in BV2 cells was assessed by using the Griess assay [22]. L-NIL [L–N6-(1-iminoethyl)lysine, > 97% purity] used as a positive control was purchased from Sigma. Male ICR (Harlan Sprague–Dawley; 4 weeks old) mice, weighing 25–30 g each, were used after a 1-week adaptation period at room temperature under a 12-h light cycle and fed *ad libitum* with free access to water. Ten mice were used per group. All experiments and the method used for euthanasia were according to the guidelines of the Institutional Animal Care and Use Committee at Seoul National University (SNU-120430–1, 16-Nov-2010). Mice were orally treated with the sample. Amnesia was subcutaneously induced in mice with scopolamine (Sigma, 1 mg/kg body weight s.c.). All of the samples for the *in vivo* test were dissolved in 0.5% CMC (carboxylmethyl cellulose; Sigma). Donepezil (purity > 98%) was from Sigma. The passive avoidance test was performed as described in our previous report [23], and the method details were included in the Supporting Information. After the passive avoidance test, the mice were immediately euthanized with urethane (1.5 g/kg) to allow measurement of antioxidant enzyme activity levels. The cerebral cortex and hippocampus of the mice were rapidly dissected and homogenized. The homogenates were centrifuged and the supernatant used for measurement of antioxidant enzyme activity and GSH content. The SOD activity was determined by using the xanthine–xan-

thine oxidase reaction method [24]. The GR activity was measured by using a method based on the reduction of GSSG by GR in the presence of NADPH [25]. The activity of GPx was determined by quantifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide [26]. Total GSH content in the supernatant was determined spectrophotometrically by using an enzymatic cycling method [27]. Protein concentration was determined by using a bicinchoninic acid (BCA) kit (Sigma) with bovine serum albumin as a standard. Data from the passive avoidance tests were expressed as mean ± SEM, while data for the level of the antioxidant activity were expressed as mean ± SD. Passive avoidance latencies and antioxidant activity values were analyzed by one-way ANOVA. The data were considered to be statistically significant when the probability (p) value was 0.05 or less.

Supporting information

Original spectral data of **1** and **2** as well as detailed descriptions of bioassay protocols are available as Supporting Information.

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Conflict of Interest

There is no conflict of interest.

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