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Identification of Neuroactive Constituents of the Ethyl Acetate Fraction from Cyperi Rhizoma Using Bioactivity-Guided Fractionation

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Abstract

Cyperi Rhizoma (CR), the rhizome of *Cyperus rotundus* L., exhibits neuroprotective effects in *in vitro* and *in vivo* models of neuronal diseases. Nevertheless, no study has aimed at finding the neuroactive constituent(s) of CR. In this study, we identified active compounds in a CR extract (CRE) using bioactivity-guided fractionation. We first compared the anti-oxidative and neuroprotective activities of four fractions and the CRE total extract. Only the ethyl acetate (EA) fraction revealed strong activity, and further isolation from the bioactive EA fraction yielded nine constituents: scirpusin A (1), scirpusin B (2), luteolin (3), 6'-acetyl-3,6-diferuloylsucrose (4), 4',6' diacetyl-3,6-diferuloylsucrose (5), *p*-coumaric acid (6), ferulic acid (7), pinellic acid (8), and fulgidic acid (9). The activities of constituents 1-9 were assessed in terms of anti-oxidative, neuroprotective, anti-inflammatory, and anti-amyloid-β activities. Constituents 1, 2, and 3 exhibited strong activities; constituents 1 and 2 were characterized for the first time in this study. These results provide evidence for the value of CRE as a source of multi-functional neuroprotectants, and constituents 1 and 2 may represent new candidates for further development in therapeutic use against neurodegenerative diseases.

Key Words: Cyperi Rhizoma, Scirpusin A, Scirpusin B, Neuroprotection, Bioactivity-guided fractionation

INTRODUCTION

Neurodegenerative diseases (NDs) include a variety of conditions arising from the chronic breakdown and deterioration of neurons in the central nervous system; Alzheimer's disease (AD) and Parkinson's disease (PD) are the two best-known NDs (Houghton and Howes, 2005). Although the etiology of AD and PD is still not fully understood, it is clear that neurodegeneration in these diseases is multifactorial; several mechanisms have been implicated in a cascade of events involving many biochemical and signaling pathways, such as oxidative stress, neuroinflammation, dysregulation of protein aggregation (e.g., amyloid-β (Aβ) in AD and Lewy bodies in PD), metabolic impairment, mitochondrial instability, reduced clearance of toxins, DNA damage, and apoptosis (Mandel *et al*., 2008; Kim and Oh, 2012). Present clinical treatments for AD and PD only improve the symptoms and cause side effects during or after long-term administration, because the therapeutic approaches merely address a single mechanism (Houghton and Howes, 2005; Mandel *et al*., 2008). Thus, there is a continuing need to develop new compounds that simultaneously act on two or more pharmacological targets as disease-modifying therapies for AD and PD.

Natural products have a special role in modern drug development, evidenced by the fact that roughly half of the drugs currently in clinical use were derived from them (Decker, 2011). Especially, natural products have a high potential to be developed into optimum pharmaceuticals and nutraceuticals for NDs because their multiple properties can effectively target multifactorial diseases such as AD and PD (Decker, 2011). For

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example, major advances in the treatment of AD have included the use of acetylcholinesterase inhibitors, such as galantamine from *Galanthus nivalis* L., huperzine A from *Huperzia serrata* (Thunb. ex Murray) Trevis., and physostigmine from *Physostigma venenosum* Balf. (Houghton and Howes, 2005). Likewise, bromocriptine, pergolide, cabergoline, and lisuride from ergot, *Claviceps purpurea* (Fr.) Tul., have dopaminergic receptor-stimulating effects and are now used clinically for PD patients (Houghton and Howes, 2005).

Cyperi Rhizoma (CR) is the rhizome of *Cyperus rotundus* L., a sedge of the Cyperaceae family that grows naturally in tropical and temperate regions, and has been used for the treatment of several diseases, including stomach disorders and menstrual or emotional disturbances in women in Korea, China, Japan, and other Asian countries (Kim and Park, 1997; Jung *et al*., 2013). Several studies have investigated the pharmacological effects of CR, including its anti-diabetic, antibacterial, anti-apoptotic, anti-inflammatory, and anti-oxidative activities (Natarajan *et al*., 2006; Kilani *et al*., 2008). Previously, we demonstrated that a CR extract (CRE) exhibited neuroprotective effects in *in vitro* and *in vivo* PD models (Lee *et al*., 2010; Kim *et al*., 2013). CRE attenuated the neuronal damage induced by 6-hydroxydopamine (6-OHDA) in both PC12 and primary dopaminergic cells by inhibiting reactive oxygen species (ROS) and nitric oxide (NO) generation, mitochondrial membrane reduction, and caspase-3 activity, suggesting that the neuroprotective effects of CRE involve its anti-oxidative and anti-apoptotic activities (Lee *et al*., 2010). Additionally, CRE protected dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine stress in estrogen-deprived mice via inhibition of mitochondrial Bcl-2 reduction and Bax elevation, cytosolic cytochrome c elevation, and caspase-3 activity (Kim *et al*., 2013). These results demonstrate that CRE has neuroprotective effects due to its phytochemical constituents.

Although previous studies on the neuroprotective effects of CRE, specifically its anti-oxidative and anti-apoptotic activities, have been reported, no study has aimed at finding its neuroactive constituents. In this study, we sought to find the active constituents of CRE using bioactivity-guided fractionation. To explore the active constituents of CRE, we first compared the anti-oxidative activities of four fractions and the CRE total extract using radical-scavenging assays and their protective effects against 6OHDA-induced neurotoxicity in PC12 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. We then compared the anti-oxidative, neuroprotective, anti-inflammatory, and anti-Aβ activities of the constituents isolated from the bioactive fraction.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin (P/S) were purchased from Hyclone Laboratories Inc. (Logan, UT, USA). Horse serum was purchased from Gibco Industries Inc. (Auckland, NZ). MTT, 6-OHDA, collagen, Griess reagent, corticosterone, 2,2-azinobis-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-2-picrylhydrazyl (DPPH), thioflavin T (ThT), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). $A\beta_{1-42}$ peptide was purchased from American Peptide (Sunnyvale, CA, USA).

Plant material

The rhizomes of *Cyperus rotundus* L. (Cyperaceae) were obtained from a domestic Korean market (Kyungdong Crude Drugs Market, Seoul, Republic of Korea), in June 2011. The origin of the herbal material was identified by Prof. Dae Sik Jang and a voucher specimen (CYRO1-2011) has been deposited in the Lab. of Natural Product Medicine, College of Pharmacy, Kyung Hee University, Republic of Korea.

Extraction and isolation

The dried and milled plant material (2.8 kg) was extracted with 10 L of 80% EtOH three times by maceration. The extracts were combined and concentrated in vacuo at 40°C to give the 80% EtOH extract (399 g). A portion of the 80% EtOH extract (392 g) was suspended in H_2O (2 L) and successively extracted with *n*-hexane (3×2 L), ethyl acetate (EA) (3×2 L) and BuOH (3×2 L) to give *n*-hexane- (45.8 g), EA- (23.5 g), BuOH- (52.4 g) and water-soluble extracts (270.3 g), respectively. Based on the initial biological testing, we chose the EA-soluble extract for detailed phytochemical investigation. A portion of the EA-soluble extract (15.0 g) was chromatographed over silica gel (6.5×41 cm, 70-230 mesh) as stationary phase with a CH_2Cl_2-MeOH gradient [99:1 (11.5 L), 49:1 (3.5 L), 19:1 (5.0 L), 9:1 (11.0 L), 4:1 (4.0 L), 1:1 (2.0 L), 0:1 (2.0 L)] as mobile phase to afford 17 pooled fractions (E01~E17). Fraction E10 [eluted with CH_2Cl_2-MeOH (19:1 v/v); 1.19 g] was subjected to a Sephadex LH-20 column (3.5×58.5 cm) eluting with CH_2Cl_2-MeOH mixture (1:1, 600 mL) to produce 6 subfractions (E10-1~E10-6). Constituent 7 (8.3 mg) was purified from the subfraction E10-5 (35.0 mg) by using a flash chromatographic system (26 g C18 flash column, RediSep®Rf, Teledyne Isco Inc. (Lincoln, NE, USA) with MeOH-H_2O (30:70 to 33:67 v/v, 7 mL/min). Fraction E11 [eluted with CH_2Cl_2-MeOH (9:1 v/v); 1.04 g] was subjected to a silica gel column (3.8×28 cm, 230-400 mesh) with CH_2Cl_2-MeOH-H_2O mixture (9:1:0.1, 1.0 L) to produce 8 subfractions (E11-1~E11-8). Fraction E11-6 (225.7 mg) was fractionated using a Sephadex LH-20 column (3.5×58.5 cm) with CH_2Cl_2-MeOH mixture (1:1, 500 mL) to afford constituent 5 (7.2 mg). Fraction E12 [eluted with CH_2Cl_2-MeOH (9:1 v/v); 690 mg] was subjected to a silica gel column (3.8×28 cm, 230-400 mesh) with CH_2Cl_2-MeOH-H_2O mixture (9:1:0.1, 1.5 L) to produce 7 subfractions (E12-1~E12-7). Constituents 6 (6.9 mg) and 4 (8.2 mg) were purified by preparative HPLC (YMC-Pack ODS A, MeOH-H_2O, 40:60 to 75:25 v/v, 7.5 mL/min) from the subfraction E12-6 (99.7 mg). Fraction E13 [eluted with CH_2Cl_2-MeOH (9:1 v/v); 1.09 g] was subjected to a silica gel column (3.8×28 cm, 230-400 mesh) with CH_2Cl_2-MeOH-H_2O mixture (8.5:1.5:0.1, 1.5 L) to produce 7 subfractions (E13-1~E13-7). Constituents 3 (5.0 mg) and 1 (9.4 mg) were isolated from the subfractions E13-2 (238.8 mg) and E13-4 (90.5 mg), respectively, by repeated chromatography. Fraction E14 [eluted with CH_2Cl_2-MeOH (9:1 v/v); 444 mg] was subjected to a silica gel column (3.8×28 cm, 230-400 mesh) with CH_2Cl_2-MeOH-H_2O mixture (8:1.8:0.2, 1.0 L) to afford constituents 2 (9.6 mg) and 9 (8.5 mg). Fraction E15 [eluted with CH_2Cl_2-MeOH (4:1 v/v); 590 mg] was subjected to a reversed phase column chromatography (3.6×24 cm, YMC gel) with MeOH-H_2O gradient (1:1, 3:2, 4:1, 1:0, 500 mL each eluent) to purify constituent 8 (10.4 mg).

Measurement of ABTS radical cation scavenging activity

ABTS solution of 7.4 mM was added to 2.6 mM potassium persulfate for 1 day before starting the experiment in the dark. Fractions and total extract of CRE at various concentrations of 2-500 μg/mL and constituents 1-9 at various concentrations of 0.1-100 μM were mixed with 7.4 mM ABTS solution with 2.6 mM potassium persulfate. After incubation at room temperature for 5 min, the absorbance of the mixture was determined at 732 nm using a spectrophotometer (VersaMax microplate reader; Molecular Device, Sunnyvale, CA, USA). Also, the activity was expressed as half maximal inhibiting concentration (IC_{50}) which is defined as the concentration of fractions and total extract of CRE and constituents 1-9 required to scavenge 50% of ABTS radical cations.

Measurement of DPPH radical scavenging activity

Fractions and total extract of CRE at various concentrations of 2-500 μg/mL and constituents 1-9 at various concentrations of 0.1-100 μM were mixed with 0.2 mM DPPH ethanol solution (1:1). After incubation at room temperature in the dark for 30 min; the absorbance of the mixture was determined at 517 nm using a spectrophotometer (VersaMax microplate reader; Molecular Device, Sunnyvale, CA, USA). Also, the activity was expressed as IC_{50} which is defined as the concentration of fractions and total extract of CRE and constituents 1-9 required to scavenge 50% of DPPH radicals.

Cell culture and treatment

PC12 cell line, a rat pheochromocytoma, was obtained from the Korea Cell Line Bank (KCLB, Seoul, Republic of Korea). PC12 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 10% heat-inactivated horse serum and 1% P/S in a 5% CO_2 incubation at 37°C. The culture medium was changed every three days and PC12 cells were subcultured about twice a week. PC12 cells were seeded at a density of 2.5×10^5 cells/mL on 96-well plates. After 24 h of incubation, PC12 cells were treated with fractions and total extract of CRE (10 μg/mL) or constituents 1-9 (10 μM) for 1 h and then incubated without or with 6-OHDA (100 μM) for a further 3 h. BV-2 cell line was obtained from the KCLB (Seoul, Republic of Korea) and has both the phenotypic and functional properties of reactive microglia cells and was used in this study. BV-2 cells were maintained in DMEM supplemented with 5% heat-inactivated FBS and 1% P/S in a 5% CO_2 incubation at 37°C. The culture medium was changed every day and BV-2 cells were subcultured about once a day. BV-2 cells were seeded at a density of 3.0×10^5 cells/mL on 96-well plates. After 24 h of incubation, BV-2 cells were treated with constituents 1-9 (10 μM) for 1 h and then stimulated with LPS (100 ng/mL) for a further 23 h. HT22 cell line, a mouse hippocampal neuronal precursor cell, was obtained from the KCLB (Seoul, Republic of Korea). HT22 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% P/S in a 5% CO_2 incubation at 37°C. The culture medium was changed every two days and HT22 cells were subcultured about three times a week. HT22 cells were seeded at a density of 1.0×10^5 cells/mL on 96-well plates. After 24 h of incubation, HT22 cells were treated with constituents 1-9 (10 μM) for 1 h and then incubated without or with corticosterone (100 μM) for a further 48 h.

Table 1. Radical scavenging activities of fractions and total extract of CRE

	Sample	ABTS IC_{50} (μg/mL)	DPPH IC_{50} (μg/mL)
SBE[a]	Total extract	32.44	30.16
CRE	Hexane fraction	145.72	224.52
	EA fraction	12.00	21.25
	BuOH fraction	26.45	49.44
	Water fraction	> 500	> 500
	Total extract	156.07	262.63

[a]Water extract of Scutellariae Radix (SBE): a positive control.

Measurement of cell viability

Treated cells were incubated with 0.5 mg/mL of MTT at 37°C in a CO_2 incubator for 3 h. MTT medium was carefully aspirated from the wells and the formazan dye was eluted using DMSO. The plate was shaken on a shaker to dissolve the blue MTT-formazan. The absorbance at 570 nm was measured using a spectrophotometer (Versamax microplate reader, Molecular Device; Sunnyvale, CA, USA) and then expressed as a percentage of the control group value.

Measurement of NO production

Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 μL) was harvested and mixed with an equal volume of Griess reagent in the dark at room temperature. After 10 min, the absorbance at 550 nm was measured using a spectrophotometer (Versamax microplate reader, Molecular Devices; Sunnyvale, CA, USA). For each experiment, freshly prepared $NaNO_2$ that had been serially diluted was used as a standard, in parallel with culture supernatants.

Measurement of Aβ aggregation

The solutions of monomeric $A\beta_{1-42}$ (5 μL of 100 μM in DMSO) and 5 μL of samples or PBS were added to 40 μL PBS at pH 7.4. The resulting mixture was incubated for 2 h at room temperature. To the resulting mixture, 150 μL of ThT solution (5 μM in 50 mM glycine-NaOH at pH 8.5) was added and incubated for 30 min. The ThT fluorescence was measured at 520 nm with excitation at 470 nm in FLUOstar Omega multimode microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

Statistical analysis

All statistical parameters were calculated using GraphPad Prism 5.01 software (GraphPad Software Inc., San Diego, USA). Values were expressed as the mean ± standard error of the mean (SEM). The results were analyzed by one-way analysis of variance followed by the Tukey's *post hoc* test. Differences with a *p*-value less than 0.05 were considered statistically significant.

RESULTS

EA fraction of CRE showed the highest radical scavenging activities among the fractions and total extract of CRE

Firstly, we compared the anti-oxidative activities of four

fractions and CRE total extract to find the bioactive fraction of CRE by performing ABTS and DPPH free radical scavenging activity assays. The water extract of Scutellariae Radix (SBE) was used as a positive control due to its remarkable anti-oxidative and neuroprotective activities (Gao *et al*., 1999). In both assays, the EA fraction of CRE exhibited the most potent radical scavenging activities, showing higher activity than SBE (Table 1).

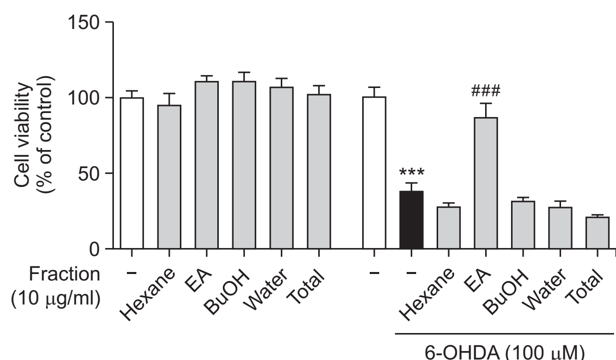

Fig. 1. Protective effects of fractions and total extract of CRE against 6-OHDA in PC12 cells. Cells were treated with fractions and total extract of CRE (10 μg/mL) for 1 h and incubated without or with 6-OHDA (100 μM) for a further 3 h. Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 4 h). Values are indicated as the mean ± SEM. ***$p<0.001$; mean values were significantly different from the control group. ###$p<0.001$; mean values were significantly different from the 6-OHDA only treated group.

EA fraction of CRE exhibited the protective effect against 6-OHDA neurotoxicity in PC12 cells

To compare the activities of fractions and CRE total extract in PC12 cells stressed with 6-OHDA, we performed an MTT assay. 6-OHDA, a hydroxylated analog of the neurotransmitter dopamine, generates ROS and is one of the most common neurotoxins used in degenerative models of catecholaminergic neurons and dopaminergic neurons (Schober, 2004). Treatment of the fractions and CRE total extract (10 μg/mL) for 4 h had no influence on cell proliferation and caused no apparent cell toxicity. Incubation with 6-OHDA (100 μM) reduced cell viability by 37.77 ± 5.87% compared with the control group, whereas only pretreatment with the EA fraction of CRE showed significant protective effects, increasing cell viability to 87.28 ± 9.20% (Fig. 1). These results are consistent with the anti-oxidative activities of the EA fraction of CRE. Taken together, the EA fraction (among the four fractions and CRE total extract) showed strong anti-oxidative and neuroprotective activities.

Constituents 1-9 isolated from EA fraction of CRE were identified

Here, we considered that the EA fraction may contain the most active constituent(s). To identify the active constituent(s) from the EA fraction of CRE, we isolated nine compounds. Repeated chromatography of the EA fraction resulted in the isolation and characterization of two stilbenes (1 and 2), one flavonoid (3), two phenolic glycosides (4 and 5), two phenylpropanoids (6 and 7), and two fatty acids (8 and 9). The structures of these known compounds were identified as scirpusin A (1) (Lam *et al*., 2008), scirpusin B (2) (Kobayashi *et al*., 2006), luteolin (3) (Han *et al*., 2007), 6'acetyl-3,6-diferuloylsucrose (4) (Nakano *et al*., 1986), 4',6'-diacetyl-3,6-diferuloylsucrose

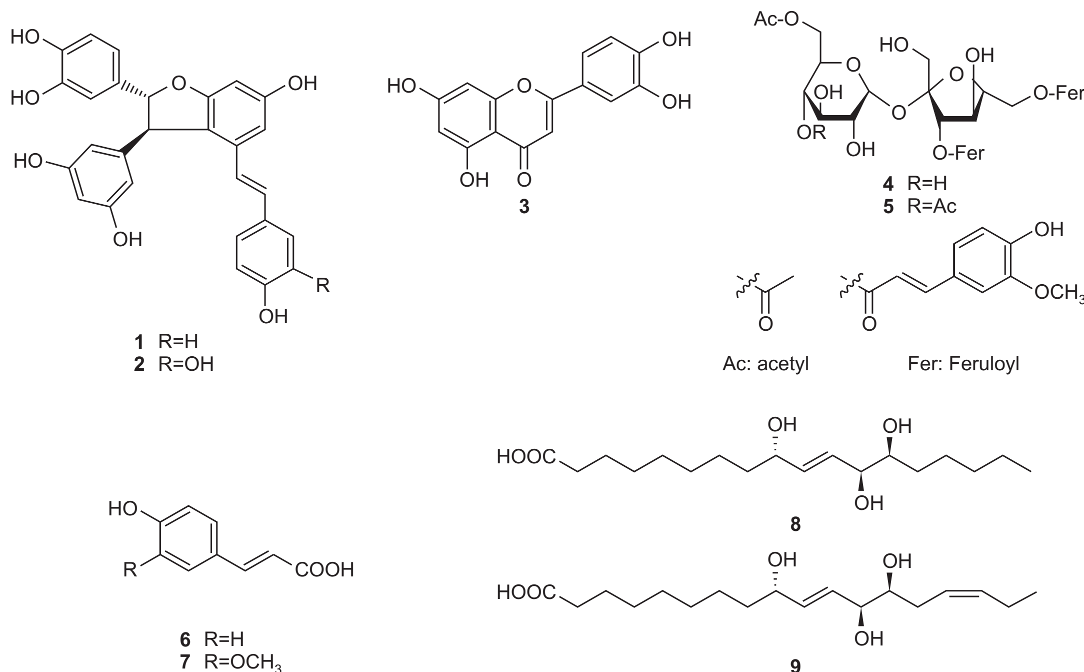

Fig. 2. Chemical structures of constituents 1-9 as followings; (1) scirpusin A, (2) scirpusin B, (3) luteolin, (4) 6'-acetyl-3,6-diferuloylsucrose, (5) 4',6' diacetyl-3,6-diferuloylsucrose, (6) *p*-coumaric acid, (7) ferulic acid, (8) pinellic acid, and (9) fulgidic acid.

Table 2. Radical scavenging activities of constituents 1-9

Sample	ABTS IC_{50} (μM)	DPPH IC_{50} (μM)
Curcumin[a]	4.34	17.26
Constituent 1	10.20	20.33
Constituent 2	6.88	8.40
Constituent 3	18.80	11.05
Constituent 4	9.91	29.08
Constituent 5	9.42	28.99
Constituent 6	20.52	> 500
Constituent 7	19.05	30.10
Constituent 8	> 500	> 500
Constituent 9	> 500	> 500

[a]Curcumin: a positive control.

(5) (Miyase and Ueno, 1993), *p*-coumaric acid (6) (Swislocka *et al*., 2012), ferulic acid (7) (Yoshioka *et al*., 2004), pinellic acid (8) (Hong and Oh, 2012), and fulgidic acid (9) (Kurashina *et al*., 2011) by spectroscopic (^{1}H NMR, ^{13}C NMR, 2D NMR, and MS) measurements and comparison with published values (Fig. 2).

Constituents 2 and 3 showed the higher radical scavenging activities than other constituents

We compared the anti-oxidative activities of constituents 1-9 using ABTS and DPPH free radical scavenging activity assays. As shown in Table 2, constituent 2 and 3 exhibited the most potent radical-scavenging activity compared with curcumin, the positive control (Ak and Gülçin, 2008). In Table 2, constituent 6 showed a very low activity in DPPH assay, compared with ABTS assay. This gap of anti-oxidative activities depending on assays are similarly shown in other reports (Yang *et al*., 2011; Badanai *et al*., 2015). It is reported that compound 6, a hydroxyl cinnamic acid derivative, has only one hydroxyl group and has no reaction with DPPH regardless of incubation time or concentrations, thus, this phenolic compound shows a slow kinetic reaction with DPPH (Brand-Williams *et al*., 1995; Von Gadow *et al*., 1997; Roginsky and Lissi, 2005).

Constituents 1-3 significantly protected neuronal cells against neurotoxins in *in vitro*

To compare the neuroprotective effects of constituents 1-9 against 6-OHDA toxicity in PC12 cells, we performed an MTT assay. Among the isolated constituents, constituents 2 and 3 showed significant neuroprotective effects against 6-OHDA in PC12 cells (Fig. 3A). We also investigated whether constituents 1-9 protects HT22 hippocampal cells against corticosterone exposure. Stress is another factor in NDs and affects various brain areas, including the hippocampus (Sharvit *et al*., 2015). When stressful conditions occur, corticosterone, a steroid hormone synthesized in rodents, is typically elevated and induces decreased cell viability and distinct morphological changes in the HT22 cell line, a mouse hippocampal neuronal precursor cell (Xu *et al*., 2011). Among the nine isolated constituents, constituents 1 and 2 (10 μM) significantly protected hippocampal neurons against chronic corticosterone exposure in HT22 cells (Fig. 3B).

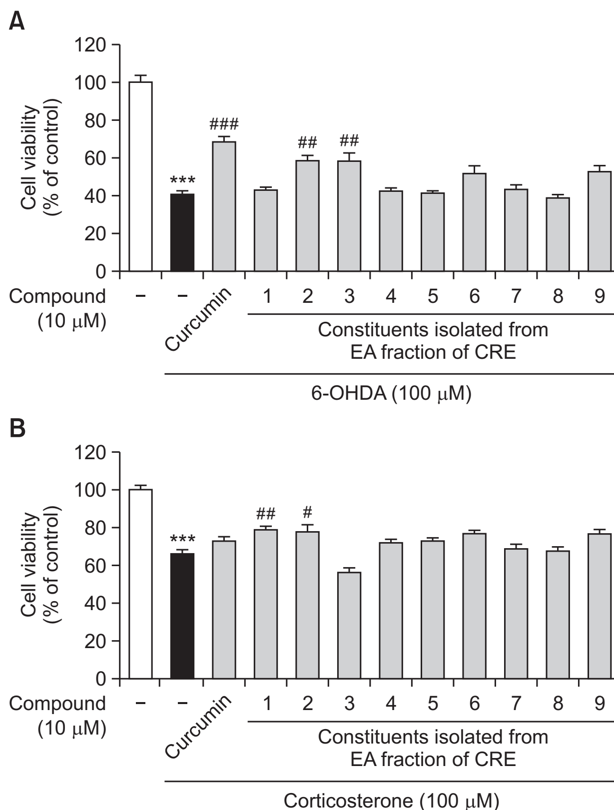

Fig. 3. Protective effects of constituents 1-9 against neurotoxins in *in vitro*. (A) Cells were treated with constituents 1-9 (10 μM) for 1 h and incubated with 6-OHDA (100 μM) for a further 3 h. Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 4 h). (B) Cells were treated with constituents 1-9 (10 μM) for 1 h and incubated with corticosterone (100 μM) for a further 48 h. Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 49 h). Values are indicated as the mean ± SEM. ***$p<0.001$; mean values were significantly different from the control group. #$p<0.05$, ##$p<0.01$ and ###$p<0.001$; mean values were significantly different from the 6-OHDA or corticosterone only treated group.

Constituent 3 showed the strongest NO inhibition in LPS-activated BV-2 cells

Although oxidative stress has been linked to NDs, inflammation in the central nervous system is also closely associated with neuronal damage and neurodegeneration (Wyss-Coray and Mucke, 2002). Many factors, including NO and free radicals such as superoxide produced by activated microglia, are proinflammatory and neurotoxic (Liu and Hong, 2003). Thus, regulation of microglial activation contributes to the suppression of neurodegeneration via neuroinflammatory regulation (Ha *et al*., 2012a). We investigated the anti-inflammatory effects of constituents 1-9 in LPS-stimulated BV-2 microglial cells. LPS, a major component of the outer membrane of Gram-negative bacteria, is a potent activator of microglia (Ha *et al*., 2012b). Treatment with curcumin, a positive control (Jin *et al*., 2007), and constituents 1-9 (10 μM) for 24 h did not influence cell viability according to the MTT assay (Fig. 4A). Constituents 1, 3, 4, 5, 6, and 7 significantly reduced LPS-induced NO production, and constituent 3 had the most potent anti-inflammatory activity (Fig. 4B).

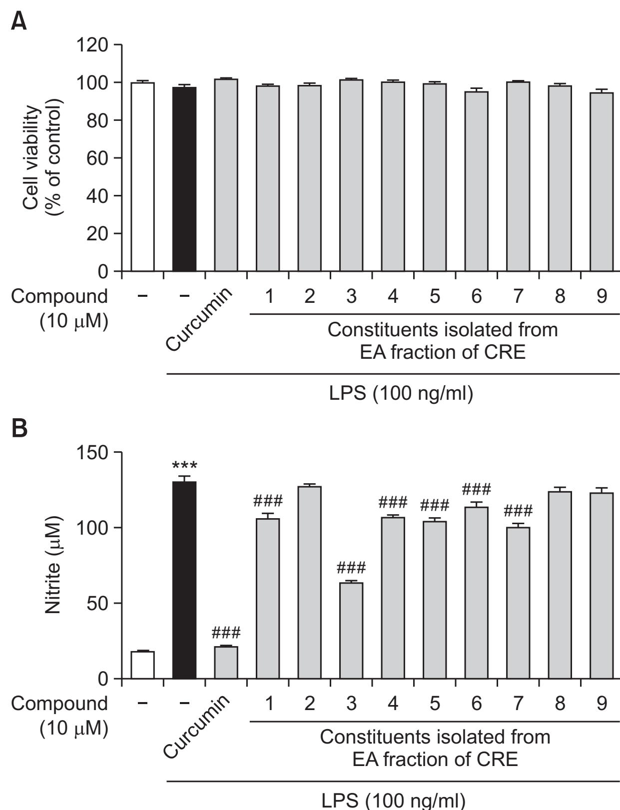

Fig. 4. Inhibitory effects of constituents 1-9 on NO production in LPS-activated BV-2 cells. Cells were treated with 1-9 (10 μM) for 1 h and stimulated with LPS (100 ng/mL) for a further 23 h. The culture medium was then collected for measurement of secreted NO. (A) Cell viabilities are expressed as a percentage of the controls (cells treated with vehicle for 24 h). (B) NO production in BV-2 cells was assayed by measuring the levels of nitrite in the supernatant fluid using the Griess reagent. Values are indicated as the mean ± SEM. ***p<0.001; mean values were significantly different from the control group. ##p<0.01 and ###p<0.001; mean values were significantly different from the LPS only treated group.

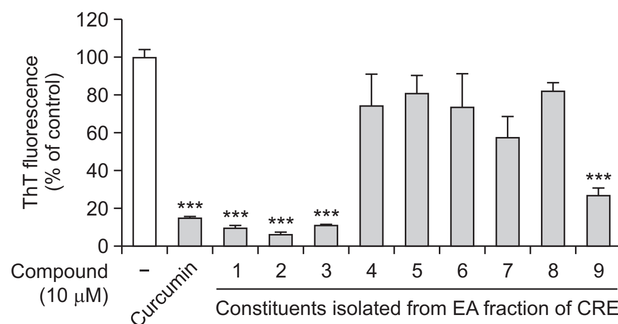

Fig. 5. Inhibitory effects of constituents 1-9 on Aβ aggregation. Values are indicated as the mean ± SEM. ***p<0.001; mean values were significantly different from the control group.

Constituents 1, 2, 3, and 9 revealed the significant anti-Aβ aggregation effect

Aβ acts as a neurotoxin to cells in culture via multiple pathways and its toxicity is correlated with the degree of peptide aggregation (Rivière *et al*., 2010). Inhibition of Aβ fibril formation is an attractive therapeutic and preventive strategy in the development of disease-modifying drugs for AD (Fujiwara *et al*., 2009). We compared the inhibitory effects of constituents 1-9 on Aβ aggregation using fluorescence spectroscopy with ThT, a cationic benzothiazole dye, which is used widely for the identification and quantification of Aβ fibrils *in vitro* (Vassar and Culling, 1959). Treatment with curcumin and constituents 1, 2, 3, and 9 (100 μM) significantly inhibited the aggregation of monomeric $A\beta_{1-42}$ (100 μM), as demonstrated by reduced ThT fluorescence intensity compared with the control group (Fig. 5).

DISCUSSION

In this study, we found that EA fraction is the most bio-active fraction of CRE by comparing the anti-oxidative effects and protective activities against 6OHDA-induced neurotoxicity in PC12 cells. And then we isolated and identified the nine constituents (constituents 1-9) from EA fraction of CRE. We compared the anti-oxidative, neuroprotective, anti-inflammatory, and anti-Aβ activities of the constituents 1-9 and found the neuroactive constituents.

As shown in the present study, constituents 1-3 (among constituents 1-9 isolated from EA fraction of CRE) exhibited strong anti-oxidative, neuroprotective, anti-inflammatory, and anti-Aβ activities. On the basis of their chemical structures, constituents 1-3 are natural polyphenolic compounds. Numerous studies have reported that polyphenols in natural products prevent NDs through different mechanisms, including oxidative stress prevention and modulation of enzymes and receptors (Bastianetto *et al*., 2009; Essa *et al*., 2012; Ferreres *et al*., 2013). Thus, the multiple activities of constituents 1-3 might be due to their chemical structures. More experimental evidences on the multiple activities of them have been reported. Previous studies demonstrated that constituent 1 protects against singlet oxygen-induced DNA strand breakage (Kong *et al*., 2010). Furthermore, constituent 1 has β-secretase and Aβ inhibitory activities (Jeon *et al*., 2007; Rivière *et al*., 2010; Richard *et al*., 2011), but the direct neuroprotective effects of constituent 1 were demonstrated for the first time in the present study. Several studies have reported that constituent 2 exhibits anti-oxidative, anti-diabetic, and anti-photoaging activities, as well as vasorelaxing effects (Kobayashi *et al*., 2006; Sano *et al*., 2011; Maruki-Uchida *et al*., 2013; Tran *et al*., 2014), while no study on the neuroprotective effects of constituent 2 has been reported. However, further investigation is necessary to confirm these effects in animal models and to determine the molecular mechanisms underlying the neuroprotection of constituents 1 and 2. Moreover, further optimization is necessary before use of these constituents as lead compounds for the treatment of NDs. Regarding constituent 3, recent studies have shown that constituent 3 has neuroprotective effects. Constituent 3 protects dopaminergic neurons in *in vitro* and *in vivo* PD models (Yoo *et al*., 2013; Zhu *et al*., 2014). Moreover, constituent 3 ameliorates scopolamine-induced amnesia (Patil *et al*., 2014). The present study demonstrated that constituents 1-3 have neuroprotective effects by reducing oxidative damage, neuroinflammation, and Aβ protein aggregation.

Due to the multifactorial nature of NDs, they are considered to be among the most enigmatic diseases and place the me-

dicinal chemist working in this field in a challenging situation (Decker, 2011). The present one-target paradigm for anti-ND treatment appears to be clinically unsuccessful (Kim and Oh, 2012). Future trends could involve the use of multi-functional drugs that act in different ways by mechanisms such as anti-oxidative and anti-inflammatory activities and inhibition of the formation of fibrillary tangles and Aβ plaques (Houghton and Howes, 2005). The current data show that constituents 1 and 2 are novel multi-functional compounds that exert a variety of activities, including anti-oxidative, neuroprotective, anti-inflammatory, and anti-Aβ activities.

In conclusion, our study identified the neuroactive constituents of CRE using bioactivity-guided fractionation. We compared the anti-oxidative and neuroprotective activities of four fractions and CRE total extract, and found that only the EA fraction of CRE exhibits strong anti-oxidative and neuroprotective activities. We then isolated nine constituents from the EA fraction and compared their anti-oxidative, neuroprotective, and anti-Aβ activities. Constituents 1-3 from the EA fraction showed the most potential as multi-functional neuroprotectants through their anti-oxidative, anti-inflammatory, and anti-Aβ activities. Moreover, constituents 1 and 2 may represent new candidates for further development as therapeutics against NDs.

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