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The Protective Effects of Phenolic Constituents from *Gastrodia elata* on the Cytotoxicity Induced by KCl and Glutamate

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Seven phenolic compounds (**1**-**7**) were isolated from the tubers of *Gastrodia elata*. Their structures were elucidated on the basis of MS and NMR spectral data. *p*-Ethoxymethyl phenyl-*O*-*β*-*D*-glucoside (**1**) was proved to be a new compound, with *N*-(*p*-hydroxybenzyl)-adenosine (**7**) isolated from this plant for the first time. In this study, the protective effects of the six constituents (**1**-**6**) on PC12 cells against the cytotoxicity induced by KCl and glutamate were also investigated. The viability of the PC12 cells was significantly enhanced by pretreatment with the six phenolic constituents.

Key words: *Gastrodia elata*, Phenolic constituents, Neuroprotection, PC12 cells, MTT assay

INTRODUCTION

The dried tubers of *Gastrodia elata* Blume (Family Orchidaceae), officially listed in the Chinese pharmacopoeia, have been used for the medicinal treatment of headaches, migraine, dizziness, tetanus, epilepsy, neuralgia and paralysis, as well as other neuralgic and nervous disorders. A variety of studies have shown the extract of *G. elata* Blume (GEB), as well as its active components and preparations, play a neuroprotective role by affecting the excitotoxicity, nitric monoxide (NO) system, neuroglia, biomembrance, oxidative neurotoxicity and apoptosis (Sun *et al.*, 2004). An earlier study showed that vanillin and gastrodin can exert sedative and anticonvulsive effects (Wu *et al.*, 1989). GEB significantly inhibits the increase of lipid peroxide levels and increases the SOD activity in the rat brain with a ferric chloride-induced seizure (Liu and Mori., 1992). Other studies have shown that extracts of GEB exert an inhibitory effect on kainic acid binding to the glutamate receptor (Andersson *et al.*, 1995), suggesting an anti-epileptic effect. Subsequent research has demonstrated that the extracts of GEB exert anticonvulsive effects against kainate-induced seizures in rats (Hsieh *et al.*, 1999), with the ether fraction attenuating the decrease in γ-aminobutyric acid (GABA) and increase in the glutamate content following a pentylenetetrazole-induced seizure (Huh *et al.*, 1995; Ha *et al.*, 2000). Recently, it has been reported that the extracts of GEB protect against glutamate-induced apoptosis in IMR-32 human neuroblastoma cells (Lee *et al.*, 1999) and dramatically reduce the extent of neuronal cell death in IMR-32 human neuroblastoma cells treated with amyloids-beta peptide (Kim *et al.*, 2003). Extracts of GEB protect serum-deprived apoptosis through activation of the serine/thereosine kinase-dependent pathway and suppression of the JNK activity (Huang *et al.*, 2004). GEB, as well as 4-hydroxybenzyl alcohol, also prevent brain damage through the increased expression of genes encoding antioxidant proteins following transient focal cerebral ischemia (Yu *et al.*, 2005b). More recently, an extract of GEB has been reported to have significant anxiolytic-like effects via the serotonergic and GABAergic nervous systems (Jung *et al.*, 2006).

Previous investigations on GEB revealed the presence of several phenolic compounds (Hayashi *et al.*, 2002; Huang *et al.*, 2005); with further investigations having led to the isolation of a new phenolic glucoside (**1**), along with several known compounds (**2**-**7**). In this paper, the isolation and structural elucidation of these compounds, as well as their protective effects on PC12 cells against cytotoxicity induced by KCl and glutamate, are reported.

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MATERIALS AND METHODS

General

Melting points were determined using a Yanaco MP-S3 melting point apparatus, and are reported uncorrected. ESI-MS and EI-MS were obtained using VG-70SE and QP5050A mass spectrometers, respectively. One-dimensional (1D) and 2D NMR spectra were recorded in DMSO on a Bruker ARX 300 spectrometer (^{1}H, 300MHz; ^{13}C, 75MHz), with the chemical shifts referenced to δ using TMS as an internal standard. Column chromatography was performed on silica gel (200-300 mesh), YWG-C_{18} resin (15-30 μm), Sephadex LH-20 and AB-8 macropore adsorptive resin (40-60 mesh).

Plant material

The tubers of *Gastrodia elata* Blume (Orchidaceae) were collected in Kunming city, Yunnan province, during November, 2002, and identified by Prof. Fa-kui Chen, Shenyang Pharmaceutical University. A voucher specimen has been deposited in the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China.

Extraction and isolation

Tubers of GEB (10 kg) were extracted with 80% ethanol (80 L × 3) for 2 hours under reflux. The 80% EtOH extracts were combined and evaporated under reduced pressure to give 1150 g of residue, which was subsequently suspended in H_2O (5 L) and partitioned with $CHCl_3$ (5 L × 3). The H_2O layer (1080 g) was successively subjected to chromatography on an AB-8 resin (8 kg × 2) column, using H_2O, and 30, 60 and 95% EtOH as eluents. The 30% EtOH fraction (115 g) was subjected to silica gel (1 kg) column chromatography, eluted with $CHCl_3$-MeOH, to give fractions I-VI and **3** (100:15, 260 mg). Subfractions I (100: 2, 270 mg) and II (100:5, 310 mg) were purified by silica gel column ($CHCl_3$-MeOH) to give **2** (100:1, 10 mg) and **7** (20:1, 12 mg), respectively. Further purification of subfraction III (10:1, 7 g) was performed by employing reversed-phase column chromatography, eluted with MeOH-H_2O, using YWG-C_{18} column (50 g) and silica gel column chromatographies, eluted with $CHCl_3$-MeOH (100:8), to give **1** (3 mg), **5** (3 mg) and **6** (4 mg). The 60% ethanol extract (66 g) was subjected to chromatography on silica gel (300 g), eluted with $CHCl_3$-MeOH (100:1), and purified on a Sephadex LH-20 (MeOH) column to yield **4** (5 mg).

p-Ethoxymethyl-phenyl-*O*-β-*D*-glucoside (1)

White powder (acetone); m.p. 110-112 °C; ESI-MS *m/z* (intens.): 337 $[M+Na]^+$ (2.6×10^7), 353 $[M+K]^+$(1.7×10^7) and 349 $[M+Cl]^-$ (3.7×10^6); ^{1}H-NMR (DMSO-d_6) δ_H: 7.24 (2H, d, J = 8.1 Hz, H-3, 5), 7.00 (2H, d, J = 8.1 Hz, H-2, 6), 4.37 (2H, s, H-7), 3.45 (2H, q, J = 6.9 Hz, H-8), 1.13 (3H, t, J = 6.9 Hz, H-9) and 4.85 (1H, d, J = 7.2 Hz, H-1'); ^{13}C-NMR (DMSO-d_6) δ_C: 156.8 (C-1), 132.0 (C-4), 128.9 (C-3, C-5), 116.1 (C-2, C-6), 71.2 (C-7), 64.7 (C-8), 15.2 (C-9) and 100.8 (C-1'), 77.0 (C-3'), 76.7 (C-5'), 73.3 (C-2'), 69.8 (C-4'), 60.8 (C-6').

N-(*p*-Hydroxybenzyl)-adenosine (7)

White needle (MeOH-$CHCl_3$); m.p. 201-204 °C; EI-MS *m/z* (rel. int., %): 373 $[M]^+$ (7), 241 (45), 178 (34), 164 (75), 135 (100), 122 (22), 108 (35), 78 (15); ^{1}H-NMR (DMSO-d_6) δ_H: 8.35 (1H, s, H-8), 8.27 (1H, br.s, 6-NH), 8.20 (1H, s, H-2), 7.16 (2H, d, J = 8.1 Hz, H-2", 6"), 6.68 (2H, d, J = 8.1 Hz, H-3", 5"), 4.64 (2H, s, H-7"), 5.89 (1H, d, J = 6.3 Hz, H-1'), 4.62 (1H, m, H-3'), 4.15 (1H, m, H-2'), 3.96 (1H, m, H-4'), 3.69 (1H, m, H_a-5'), 3.58 (1H, m, H_b-5'); ^{13}C-NMR (DMSO-d_6) δ_C: 156.1 (C-6), 152.3 (C-2), 148.5 (C-4), 139.8 (C-8), 119.8 (C-5), 87.9 (C-1'), 70.6 (C-2'), 73.5 (C-3'), 85.9 (C-4'), 61.7 (C-5'), 154.5 (C-1"), 130.2 (C-4"), 128.6 (C-2", C-6"), 114.9 (C-3", C-5"), 42.5 (C-7").

Reagents and cell culture

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Trypan blue were purchased from Sigma chemical (USA). PC12 cells were purchased from Cell Bank of Chinese Academic of Science. The cells were cultured in RPMI-1640 medium, supplemented with 5% fetal bovine serum, 10% heat-inactivated horse serum (Gibco, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2.

Drug solutions

Compounds (**1-3** and **5-6**) were dissolved in Phosphate buffered saline (PBS) to make stock solutions, and compound **4** dissolved in DMSO, then diluted in PBS at different concentrations, and used immediately. Control cultures were performed in the presence of DMSO in the same solution. In all assays, the final concentration of DMSO in the culture medium was less than 0.1%.

MTT assay

Cells were seeds in 96-well plates, at a density of 5000 cells per well. The cultures were grown at 37 °C for 12 h, with the media then changed to those containing varying concentrations of the compounds for up to 24 h. After treatment of the PC12 cells with KCl (4 mM) and glutamate (400 μM) for 40 min and 24 h, respectively, MTT solution (5 mg/mL in PBS) was added to the 96-well plates (0.5 mg/mL) and the cells allowed to incubate for a further 4 h at 37 °C. The medium with MTT was removed,

and 100 μl DMSO added to each well to dissolve the formazan crystals. The extent of MTT reduction was measured at 540/620 nm on a plate reader (Tecan, Austria).

Statistical analysis

Data are expressed as the mean±SD from three independence experiments. The significance of inter-group differences was evaluated by a two-way analyses of variance (ANOVA) followed by Student's *t*-tests. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Seven phenolic compounds (Fig. 1) were isolated from the 80% EtOH extracts of *G. elata*. Of these, five compounds were identified as 4-hydroxybenzyl alcohol (**2**), gastrodin (**3**), 4-hydroxybenzaldehyde (**4**), 4-methylphenyl-*β*-*D*-glucopyranoside (**5**) and 3, 5-dimethoxybenzoic acid-4-*O*-*β*-*D*-glucopyranoside (**6**) by matching their NMR spectroscopic data with those previously reported (Zhou *et al.*, 1980; Huang *et al.*, 2005). Compounds **1** and **7** were determined from their MS and NMR spectra data.

Compound **1** was obtained as a white powder from acetone. Its molecular formula was determined as $C_{15}H_{22}O_7$ by ESI-MS at *m/z* 337.0 $[M+Na]^+$ 348.9 $[M+Cl]^-$ and NMR data. The ^{1}H-NMR spectrum of **1** showed an AA'BB' spin-spin coupling system at δ_H 7.24 (2H, d, J = 8.1 Hz) and 7.00 (2H, d, J = 8.1 Hz), which was ascribable to a 1, 4-disubstituted aromatic ring. One singlet due to oxygenated methylene protons was found at δ_H 4.37 (2H, s). A pair of coupled proton signals at δ_H 1.13 (3H, t, J = 6.9 Hz) and 3.45 (2H, q, J = 6.9 Hz) were ascribed to an ethoxy group. The presence of a 1, 4-disubstituted aromatic ring was confirmed by the six aromatic signals at δ_C 116.1 (C-2, C-6), 128.9 (C-3, C-5), 132.0 (C-4) and 156.8 (C-1) in the ^{13}C-NMR spectrum. The carbon signals at δ_C 71.2, 64.7 and 15.2 were assigned to two oxygenated methylenes (C-7, C-8) and a methyl group (C-9), respectively. Furthermore, the ^{13}C-NMR spectrum of **1** displayed the characteristic signals of a glucose nucleus: δ_C 100.8 (C-1'), 77.0 (C-3'), 76.7 (C-5'), 73.3 (C-2'), 69.8 (C-4') and 60.8 (C-6'). The doublet of the glucose anomeric proton appeared at δ 4.85, with a biaxial coupling constant $J_{1',2'}$ = 7.2 Hz; thus, confirming the β-configuration of the D-glucosyl unit. Cross-peaks between the isolated methylene protons (δ_H 4.37) and 116.1 and 64.7 (C-8) occurred in the HMBC spectrum, suggesting the presence of an ethoxymethylphenyl group. In the HMBC spectrum, the anomeric proton showed three-bond correlations with the aromatic carbon 156.8 (C-1), which clearly indicated the glycosylation position. On the basis of the above spectral data, compound **1** was determined as *p*-ethoxymethyl phenyl-*O*-*β*-*D*-glucoside. The assignments

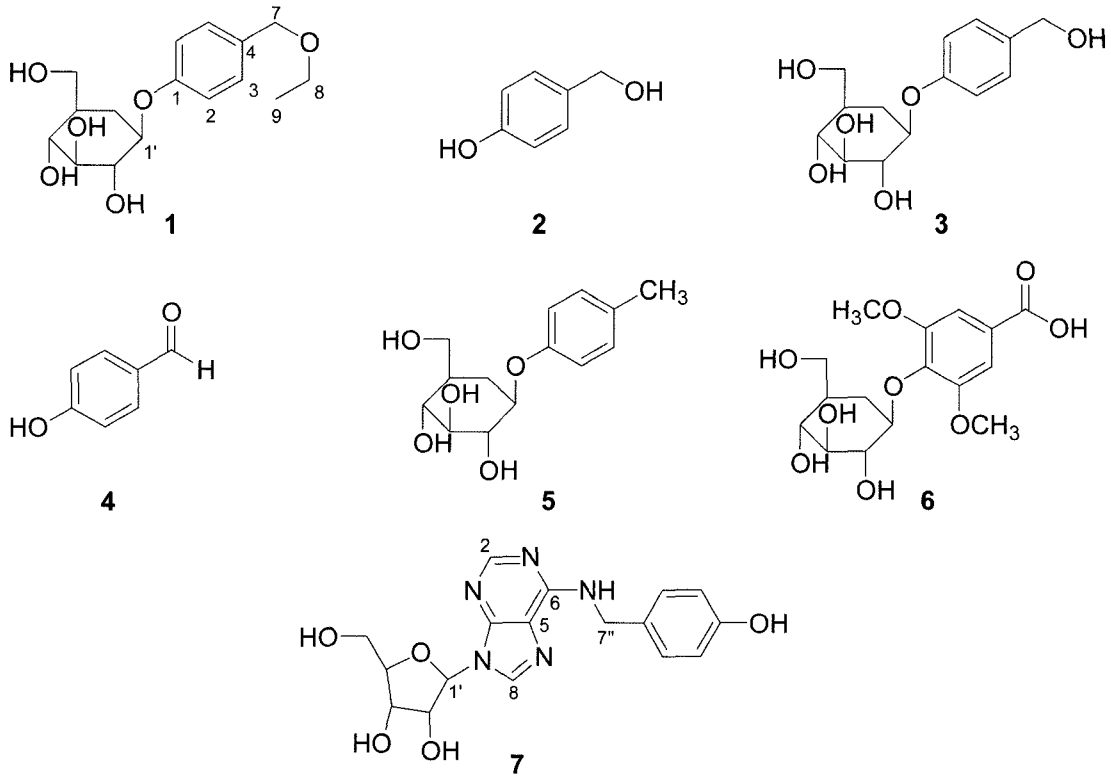

Fig. 1. Structures of compounds (**1-7**) isolated from *Gastrodia elata*

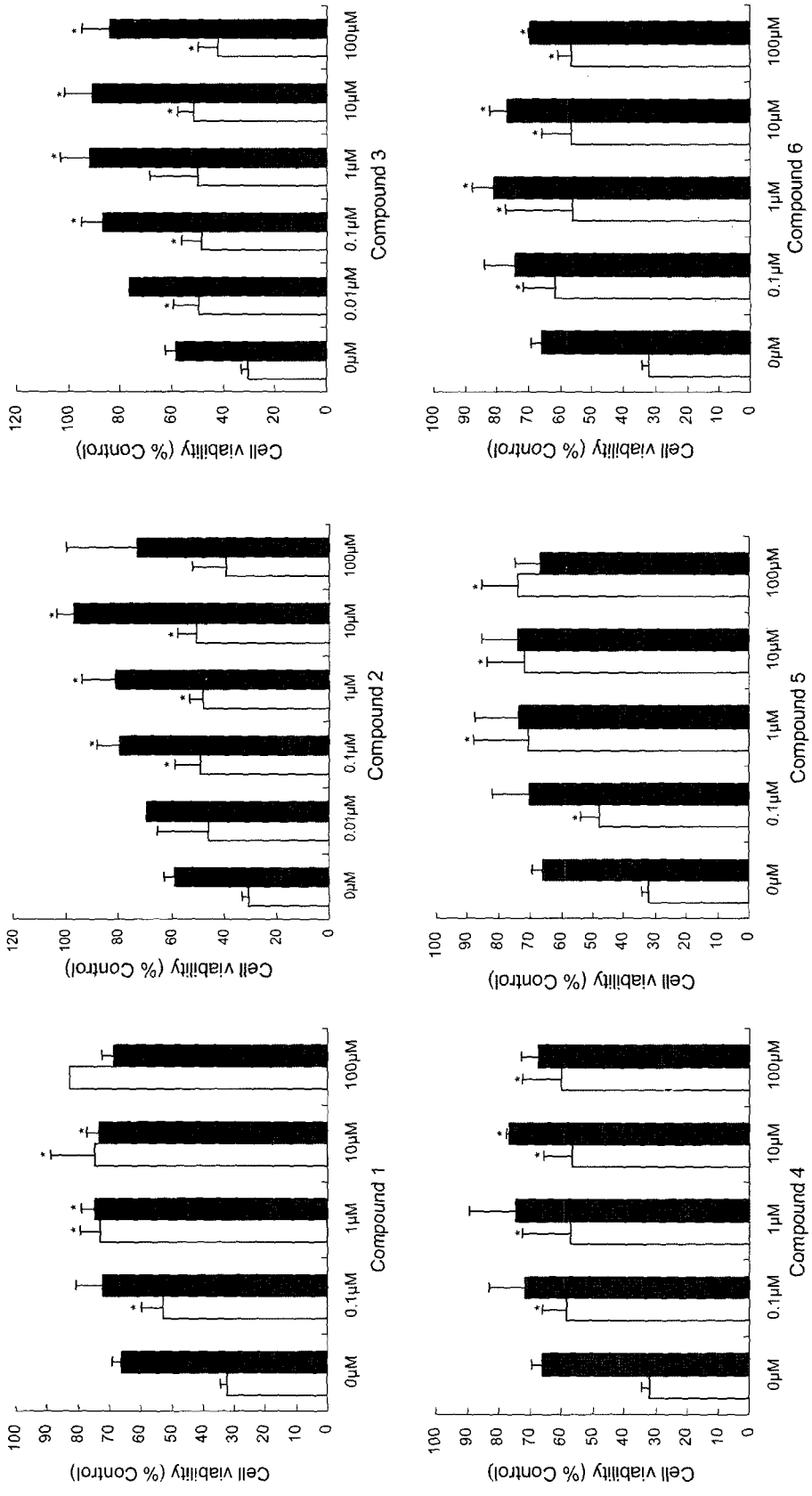

Fig. 2. Protection afforded by the phenolic constituents extracted from *Gastrodia elata* against KCl and glutamate-induced neurotoxicity in PC12 cells. Values are expressed as the mean ± SD. Cells in 96-well plates were treated with various concentrations of compounds (1-6) for 12 h; the cells were exposed to 400 μM glutamate for 24 h (or 4 mM KCl for 40 min), then measured using the MTT method. Control: without KCl, glutamate and compounds (1~6). (*) $p < 0.05$ in comparison with cells exposed to either KCl or glutamate alone. □ KCl,ne. ■ glutamate.

of the chemical shifts of **1** were deduced unequivocally by HMBC, HMQC and ^{1}H-^{1}H COSY experiments.

Compound **7** was obtained as white needles from MeOH-$CHCl_3$. Its molecular formula was deduced to be $C_{17}H_{19}N_5O_5$ on the basis of EI-MS and NMR spectra data. The ^{1}H-NMR spectrum of **7** presented two doublets at δ_H 7.16 (2H, d, J = 8.1 Hz) and 6.68 (2H, d, J = 8.1Hz), which were ascribed to a 1, 4-disubstituted aromatic ring, and a singlet at δ_H 4.64 (2H, s) due to methylene protons. Comparing the ^{1}H and ^{13}C NMR spectral data of **7** with those of adenosine (Yu and Yang., 1999), it was found that the two compounds were very similar, with the exception of data corresponding to a *p*-hydroxy benzyl group. In the HMBC spectrum of **7**, proton signals at δ_H 4.64 (H-7") showed long-range correlations with the carbons at δ_C 156.1 (C-6) and 119.8 (C-5). Therefore, the structure of **7** was deduced to be *N*-(*p*-hydroxybenzyl) adenosine. This compound is a kind of natural cytokinin, which is a derivative of adenosine substituted at the *N*-6 position with a benzyl moiety (Vaňková *et al.*, 1998).

Neuroprotective effect

Glutamate is thought to be the major excitory neurotransmitter in the central nervous system and the cause of a specific pattern of neurodegeneration in the brain of experimental animals in the primary culture of brain neurons and in some cultured neuronal cell lines, including PC12 cells. In PC12 cells, a high concentration glutamate inhibits cysteine uptake and depletes intracellular glutathione, which lead to the accumulation of reactive oxygen species (ROS), and ultimately to cell death (Yu *et al.*, 2005a). In the present study, KCl and glutamate-induced cytotoxicity in neuronal-like PC12 cells was used as a model to screen the six phenolic compounds (**1-6**) isolated from *G. elata* as potentially therapeutic neuro-protectants. The viability of PC12 cells was significantly enhanced by pretreatment with all six phenolic constituents compared with the cells treated with KCl and glutamate alone (Fig. 2). The compounds exerted differential levels of protection against KCl and glutamate-induced cell death. Among compounds **1-6**, **1** and **5** inhibited the KCl-induced cytotoxicity, as measured by the MTT reduction, by 80 and 78% at a concentration of 100 μM, respectively. Although the other four compounds (**2**, **3**, **4** and **6**) demonstrated moderate protective activities, the cell viabilities of these groups showed significant differences from the group treated with KCl alone. As with KCl, their protective roles on the PC12 cells against the cytotoxicity induced by glutamate noticeably increased with increasing concentrations, from 0.1~10 μM, which were also dose-dependent within the tested range. Of the six compounds, **2** and **3,** at concentrations ranging from 0.01 to 10 μM, and **1**, **4**, **5** and **6,** at concentrations ranging from 0.1 to 10 μM, exhibited the highest protection. When the concentrations of **1, 2, 4** and **5** were greater than 10 μM and those of **3** and **6** were greater than 1 μM, the protections induced were weaker. The results indicate the two models had similar phenomena, in that they could protect PC12 cells against the cytotoxicity induced by KCl and glutamate.

An increasing number of reports have provided evidence implicating oxidative stress as a major pathogenic mechanism in neurodegeneration, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS). Oxidant over production leads to oxidative molecular damage of tissues. Therefore, protecting neurons from oxidative injures may provide useful therapeutic potentials for the protection or treatment of neurodegenerative disorders caused by oxidative stress (Yu *et al.*, 2005a). GEB components have been shown to be directly responsible for scavenging free radicals, decreasing lipid peroxidation and reducing free radical generation (Liu and Mori, 1992 and 1993; Ha *et al.*, 2001; Kim *et al.*, 2003; Yu *et al.*, 2005b). Thus, one of the mechanisms by which compounds exert the neuro-protective effect observed in this study may be mediated by this mechanism.

Taken together, in the present study, seven phenolics (**1-7**), including one novel compound (**1**), were isolated and identified from the 80% ethanol extract of *G. elata*; the protective actions of compounds (**1-6**) were also assessed via the damage caused to PC12 cells due to high concentrations of KCl and glutamate. These results also suggest that GEB could have novel therapeutic potential in treating neurotoxicity or could give rise to the opportunity for exploration, at least in part, of its phenolic components as neuro-protectants.

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