Natural Product Sciences
13(2) : 101-104 (2007)

Cytoprotective Activity of *Belamcanda chinensis* Rhizome Against Glutamate-Induced Oxidative Injury in HT22 Cells

Gil-Saeng Jeong[1], Ren-Bo An[2,3], Seung-Hwan Oh[4], Dae Gill Kang[5], Ho Sub Lee[5], and Youn-Chul Kim[2]*

[1]X-ray Microscopy Research Center, Wonkwang University, Iksan, 570-749, Korea
[2]College of Pharmacy, Wonkwang University, Iksan, 570-749, Korea
[3]College of Pharmacy, Yanbian University, Yanji, Jilin 133000, China
[4]Central R&D Center, Kwang Dong Pharmaceutical Co., Pyongtaek, 459-020, Korea
[5]Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, 570-749, Korea

Abstract – Four isoflavones including tectorigenin (**1**), irisflorentin (**2**), irigenin (**3**), and tectoridin (**4**) have been isolated from the 70% EtOH extract of *Belamcanda chinensis* rhizome. The evaluation for protective effect of compounds **1** - **4** against glutamate-induced cytotoxicity in hippocampal HT22 cell line was conducted. Compound **1** showed significant protective effect with an EC_{50} value of 67.25 ± 1.2 μM, whereas compounds **2** - **4** were inactive. These results suggest that compound **1** may possess the neuroprotective activity against oxidative cellular injuries.
Keywords – Tectorigenin, Isoflavone, *belamcanda chinensis*, glutamate-induced cytotoxicity, HT22 cells

Introduction

Oxidative stress is considered to play an important role in a variety of neurodegenerative disorders of central nervous system, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemia (Behl *et al.*, 1994; Colye and Puttfarcken, 1993; Makkesbery, 1997; Simonian and Coly, 1996). HT22 cell line is a subclone of the HT4 hippocampal cell line, and has been used as one of the useful models for studying the mechanism of oxidative toxicity (Davis and Maher, 1994). Glutamate, the major neurotransmitter in the mammalian brain, is known to induce oxidative stress. Thus, the aim of this work was to find substances for the protection of the cytotoxic effect of glutamate in hippocampal HT22 cells from medicinal plants, and would be valuable for potential therapeutic use.

Belamcanda chinensis (L.) DC. belongs to Iridaceae family. The dried rhizome of this plant has been used as an expectorant and agent for smoothing the sore throat in oriental medicine (Zhu, 1998). As a part of our continuing research to find substances with protective effect on HT22 cells from medicinal plants (Kang *et al.*, 2005), this paper describes the isolation of four isoflavones from the rhizome of *B. chinensis*, and evaluation of their protective effects against glutamate-induced cytotoxicity in HT22 cells.

*Author for correspondence
Fax: +82-63-852-8837; E-mail: yckim@wonkwang.ac.kr

Experimental

Chemicals and instruments – NMR spectra were recorded in DMSO-d_6 using a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C), and chemical shifts are quoted versus tetramethylsilane, ESI-MS spectra were measured on a Quattro LC-MS (Micromass). Column chromatography was performed on Silica gel 60 (70-230 mesh, Merck), Sephadex LH-20 (Pharmacia, Sweden), and YMC-GEL ODS-A (S-75 mm, YMC). In TLC silica gel 60 F_{254} plate (Merck) were used. Spots were detected under UV light or after spraying with 10% H_2SO_4 reagent, followed by heating. Glutamate, trolox, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA).

Plant material and isolation – The dried rhizome of *B. chinensis* was purchased from the University Oriental Drugstore, Iksan, Korea in June 2006. A voucher specimen (No. WP06-189) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). The dried rhizome of *B. chinensis* (1 kg) was extracted twice with 70% aqueous EtOH (2 L) under the ultrasonic condition for 3 h. The 70% EtOH extract (259 g) was suspended in H_2O (1 L) and partitioned successively with *n*-hexane (800 mL × 2) and $CHCl_3$ (800 mL × 2) to yield *n*-hexane-soluble (7.71 g) and $CHCl_3$-soluble extract (41.94 g). The

$CHCl_3$-soluble extract was subjected to column chromatography (CC) on silica gel, which was using *n*-hexane-EtOAc (4 : 1 → 2 : 1) to give four fractions (Fr. A-D). Fr. A (1.75 g) was subjected to silica gel CC with $CHCl_3$-MeOH (25 : 1) to get three subfractions (Fr. A1-A3). Fr. A2 (540 mg) was further chromatographed on a Sephadex LH-20 with $CHCl_3$-MeOH (25 : 1) to afford compound **1** (387 mg, 0.0387 w/w%). Fr. B (1.8 g) was chromatographed on YMC gel (60% MeOH in H_2O) to give compound **2** (70.4 mg, 0.007 w/w%) together with one subfraction (Fr. B1). Fr. B1 (50 mg) was further chromatographed on a Sephadex LH-20 with CH_2Cl_2-MeOH (20 : 1) to yield compound **3** (19.2 mg, 0.0019 w/w%). Fr. D (1.0 g) was purified by silica gel CC with $CHCl_3$-MeOH (20 : 1) to afford compound **4** (242 mg, 0.024 w/w%).

Tectorigenin (1) – Pale yellow solid, (–)-ESI-MS *m/z* 299 $[M - H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 8.32 (1H, s, H-2), 7.37 (2H, d, J = 8.7 Hz, H-2, 6), 6.82 (2H, d, J = 8.7 Hz, H-3, 5), 6.49 (1H, s, H-8), 3.74 (3H, s, $OC\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 181.1 (C-4), 158.0 (C-9), 157.9 (C-4), 154.6 (C-2), 153.8 (C-7), 153.3 (C-5), 131.9 (C-6), 130.7 (C-2, 6), 122.3 (C-3), 121.7 (C-1), 115.6 (C-3, 5), 105.4 (C-10), 94.4 (C-8), 60.5 ($O\underline{C}H_3$).

Irisflorentin (2) – Pale yellow solid, (–)-ESI-MS *m/z* 385 $[M - H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 8.31 (1H, s, H-2), 7.03 (1H, s, H-8), 6.83 (2H, s, H-2, 6), 6.18 (2H, s, -O-$C\underline{H}_2$-O-), 3.90 (3H, s, $OC\underline{H}_3$), 3.79 (6H, s, 2 × $OC\underline{H}_3$), 3.68 (3H, s, $OC\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 174.3 (C-4), 154.4 (C-7), 153.2 (C-2), 153.0 (C-3, 5), 152.6 (C-9), 141.0 (C-5), 137.8 (C-4), 136.5 (C-6), 128.0 (C-1), 124.7 (C-3), 113.7 (C-10), 107.3 (C-2, 6), 103.2 (-O-$\underline{C}H_2$-O-), 94.1 (C-8), 61.3 ($O\underline{C}H_3$), 60.6 ($O\underline{C}H_3$), 56.5 (2 × $O\underline{C}H_3$).

Irigenin (3) – Pale yellow solid, (–)-ESI-MS *m/z* 359 $[M - H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 8.37 (1H, s, H-2), 6.71 (1H, d, J = 1.8 Hz, H-2), 6.66 (1H, d, J = 1.8 Hz, H-6), 6.49 (1H, s, H-8), 3.78 (3H, s, $OC\underline{H}_3$), 3.74 (3H, s, $OC\underline{H}_3$), 3.69 (3H, s, $OC\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 180.7 (C-4), 158.5 (C-9), 155.2 (C-2), 153.8 (C-7), 153.4 (C-5), 153.2 (C-5), 150.8 (C-3), 136.9 (C-4), 132.1 (C-6), 126.6 (C-1), 122.2 (C-3), 110.9 (C-2), 105.2 (C-10), 105.0 (C-6), 94.5 (C-8), 60.4 (2 × $O\underline{C}H_3$), 56.3 ($O\underline{C}H_3$).

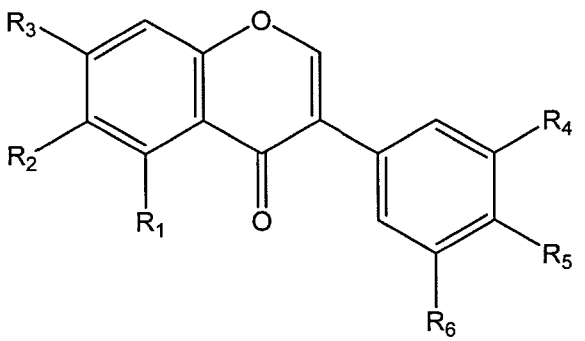
Tectoridin (4) – Pale yellow solid, (–)-ESI-MS *m/z* 461 $[M - H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 8.45 (1H, s, H-2), 7.40 (2H, d, J = 8.2 Hz, H-2, 6), 6.88 (1H, s, H-8), 6.82 (2H, d, J = 8.2 Hz, H-3, 5), 5.09 (1H, d, J = 7.4 Hz, Glc-1), 3.76 (3H, s, $OC\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 181.3 (C-4), 158.0 (C-4), 157.9 (C-9), 155.2 (C-2), 153.4 (C-7), 153.0 (C-5), 133.0 (C-6), 130.7 (C-2, 6), 122.6 (C-3), 121.6 (C-1), 115.6 (C-3, 5), 107.0 (C-10), 100.7 (Glc-1), 94.5 (C-8), 77.8 (Glc-5), 77.2 (Glc-3), 73.7 (Glc-2), 70.2 (Glc-4), 61.2 (Glc-6), 60.8 ($O\underline{C}H_3$).

Cell culture – The mouse hippocampal HT22 cells, which is a subclone of the HT4 hippocampal cell line, were received from Dr. Inhee-Mook (Seoul National University, Seoul, Korea) and were maintained at 1 × 10^6 cells/ml culture in DMEM supplemented with 10% heat inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were co-incubated for 24 hours in presence of compounds **1 - 4** at the concentrations of 20, 40, 80, 160 mM and 5 mM glutamate. Cell viability was measured spectrophotometrically using MTT assay. The samples were dissolved initially in DMSO (stock solution) and then diluted with the medium solution. The final DMSO concentration in each experimental and control well was kept constant at 0.1%, and this final concentration showed no relevant effects of DMSO on cellular growth and survival in our assay.

MTT assay – MTT cytotoxicity assay was performed according to the method previously described (Mosmann, 1983). MTT solution was added at a concentration 50 mg/mL into each well. After 4 hours of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 ml DMSO. Optical density at 570 nm was determined with a microplate reader. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability. Trolox was used as a positive control. EC_{50} values for protective effects (defined as percentage viability versus the respective control) were calculated by linear regression using mean values, and are expressed as means ± S.D. of three independent experiments.

Results and Discussion

In the present study, we investigated the 70% aqueous EtOH extract of the rhizome of *B. chinensis* with the aim of identifying natural compounds for the protection of the cytotoxic effects of glutamate in hippocampal HT22 cells. Phytochemical fractionation of $CHCl_3$-soluble fraction led to the isolation of four compounds (**1 - 4**). The structures of isolated compounds were identified as tectorigenin (**1**) (Lee *et al.*, 1989), irisflorentin (**2**) (Eu *et al.*, 1991), irigenin (**3**) (Lee *et al.*, 1989), and tectoridin (**4**) (Lee *et al.*, 1989) by comparing data with those previously reported (Fig. 1). Compounds **1 - 4** were tested for their cytoprotective activity in the *in vitro* assay system. Tectorigenin (**1**) showed the protective effect in a concentration-dependent



1 : $R_1 = R_3 = R_5 = OH$, $R_2 = OCH_3$, $R_4 = R_6 = H$
2 : $R_1 = R_4 = R_5 = R_6 = OCH_3$, R_2-O-CH_2-O-R_3
3 : $R_1 = R_3 = R_4 = OH$, $R_2 = R_5 = R_6 = OCH_3$
4 : $R_1 = R_5 = OH$, $R_2 = OCH_3$, R_3 = O-Glc, $R_4 = R_6 = H$

Fig. 1. Chemical structures of compounds **1** - **4**.

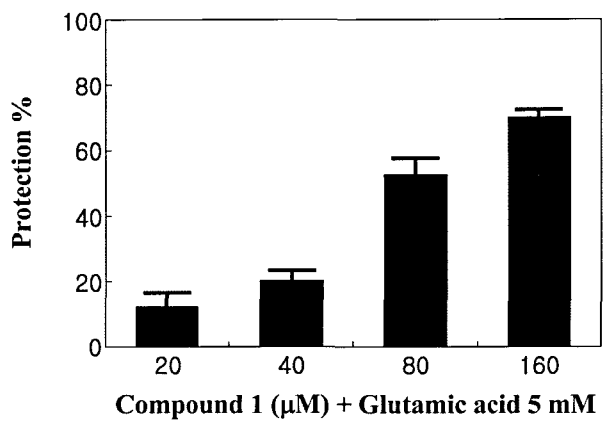


Fig. 2. Cytoprotective effect of compound **1** against glutamate-induced cytotoxicity in HT22 cells. Cells were co-incubated for 24 hours in presence of indicated concentrations of compound **1** and 5 mM glutamate. Cell viability was measured spectrophtometrically using MTT assay. Each column represents the mean± S.D. from three independent experiments.

manner against glutamate-induced cytotoxicity in mouse hippocampal HT22 cells with EC_{50} value of 67.25 ± 1.2 μM (Fig. 2). Com-pounds **2** - **4** did not showed cytoprotec-tive effects up to the concentration of 160 μM. Tectorigenin (**1**) is one of the major constituents of *B. chinensis*, known to possess various biological activities including hepato-protective (Lee *et al.*, 2005), anti-anaphylaxis (Park *et al.*, 2004), hypoglycemic and hypolipidemic (Lee *et al.*, 2000), and PGE2 production inhibitory effect (Shin *et al.*, 1999), however, the neuro-protective effect of **1** has not been reported previously to the best of our knowledge.

Oxidative stress plays a pivotal role in neurodegenera-tive diseases (Colye and Puttfarcken, 1993), and induces cell death through the oxidation of various cellular components. Especially, the central nervous system (CNS) is susceptible to oxidative stress because of its high oxygen turnover. Glutamate is the major excitatory neurotransmitter in the mammalian brain. It is well-known glutamate-induced oxidative stress is a key factor in the neuropathology of both acute and chronic neurodegenerative disorders (Colye and Puttfarcken, 1993). While the mouse hippocampal HT22 cells lack functional glutamate receptors, glutamate induces oxidative stress by inhibiting the uptake of cystine into the cells, which results in a depletion of intracellular concentrations of antioxidant molecule glutathione (Maher and Davis, 1996). HT22 cell line has been used as one of the useful models for studying the mechanism of oxidative brain toxicity (Davis and Maher, 1994). Thus, antioxidants may protect the aging brain against oxidative stress damage associated with CNS disorders (Grundman *et al.*, 2002). Based on above evidences, we have demon-strated that an isoflavone tectorigenin (**1**) may possess the neuroprotective activity against oxidative cellular injuries, and it would be also need further evaluation as potential neuroprotective agents.

Acknowledgements

This study was supported by grants of the Oriental Medicine R&D Project (03-PJ9-PG6-SO02-0001), Ministry of Health & Welfare, Republic of Korea.

References

Behl, C., Davis, J.B., Lesley, R., and Schubert, D., Hydrogen peroxide mediates amyloid b protein toxicity. *Cell* **77**, 817-827 (1994).

Colye, J.T. and Puttfarcken, P., Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262**, 689-695 (1993).

Davis, J.B. and Maher, P., Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line. *Brain Res.* **652**, 169-173 (1994).

Eu, G.H., Woo, W.S., Chung, H.S., and Woo, E.H., Isoflavonoids of *Belamcanda chinensis* (II). *Kor. J. Pharmacogn.* **22**, 13-17 (1991).

Grundman, M., Grundman, M., and Delaney, P., Antioxidant strategies for Alzheimer's disease. *Proc. Nutr. Soc.* **61**, 191-202 (2002).

Kang, T.H., Baek, H.Y., and Kim, Y.C., Protective effect of Jakyak-Gancho-Tang extract and its constituents against *t*-BHP-induced oxidative damage in HT22 cells. *Am. J. Chin. Med.* **33**, 181-189 (2005).

Lee, H.U., Bae, E.A., and Kim, D.H., Hepatoprotective effect of tectoridin and tectorigenin on *tert*-butyl hydroperoxide-induced liver injury. *J. Pharmacol. Sci.* **97**, 541-544 (2005).

Lee, K.T., Sohn, I.C., Kim, D.H., Choi, J.W., Kwon, S.H., and Park, H.J., Hypoglycemic and hypolipidemic effects of tectorigenin and kaikasaponin III in the streptozotocin-induced diabetic rat and their antioxidant activity *in vitro*. *Arch. Pharm. Res.* **23**, 461-466 (2000).

Lee, S.O., Woo, W.S., Woo, E.H., and Kim, K.S., Isoflavonoids of *Belamcanda chinensis*. *Kor. J. Pharmacogn.* **20**, 219-222 (1989).

Maher, P. and Davis, J.B., The role of monoamine metabolism in oxidative glutamate toxicity. *J. Neurosci.* **16**, 6394-6401 (1996).

Makkesbery, W.R., Oxidative stress hypothesis in Alzhiemer's disease.

Free Radical Bio. Med. **23**, 134-147 (1997).
Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).
Park, E.K., Shin, Y.W., Lee, H.U., Lee, C.S., and Kim, D.H., Passive cutaneous anaphylaxis-inhibitory action of tectorigenin, a metabolite of tectoridin by intestinal microflora. *Biol. Pharm. Bull.* **27**, 1099-1102 (2004).
Shin, K.H., Kim, Y.P., Lim, S.S., Lee, S., Ryu, N., Yamada, M., and Ohuchi, K., Inhibition of prostaglandin E2 production by the isoflavones tectorigenin and tectoridin isolated from the rhizomes of *Belamcanda chinensis*. *Planta Med.* **65**, 776-777 (1999).
Simonian, N.A. and Coly, J.T., Oxidative stress in neurodegenerative diseases. *Ann. Rev. Pharmacol.* **36**, 83-106 (1996).
Zhu, Y.-P., *Chinese Materia Medica*, Harwood Academic Publishers, Amsterdam, 1998.

(Accepted February 5, 2007)