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Cytoprotective Constituents of *Alpinia katsumadai* Seeds Against Glutamate-Induced Oxidative Injury in HT22 Cells

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Abstract – Glutamate-induced oxidative injury contributes to neuronal degeneration in many central nervous system (CNS) diseases, such as epilepsy and ischemia. Bioassay-guided fractionation of the MeOH extract of the seeds of *Alpinia katsumadai* Hayata (Zingiberaceae) furnished three phenolic compounds, alpinetin (**1**), pinocembrin (**2**), and (+)-catechin (**3**). Compounds **2** and **3** showed the potent neuroprotective effects on glutamate-induced neurotoxicity and reactive oxygen species (ROS) generation in the mouse hippocampal HT22 cells. In addition, Compounds **2** and **3** showed significant DPPH free radical scavenging effect. These results suggest that compounds **2** and **3** could be the effective candidates for the treatment of ROS-related neurological diseases.
Keywords – Glutamate-induced toxicity, *Alpinia katsumadai*, HT22, DPPH, Cytoprotective

Introduction

The seeds of *Alpinia katsumadai* (Zingiberaceae) were used as an antiemetic and a stomachic in oriental medicine (Zhu, 1998). Previous phytochemical investigations of *A. katsumadai* have reported a variety of diarylheptanoids (Kuroyanagi *et al.*, 1983), flavonoids (Yushiro *et al.*, 1968), monoterpenes, sesquiterpenoids (Saiki *et al.*, 1978; Lawrence *et al.*, 1972), and stilbenes (Yang *et al.*, 1999).

Oxidative stress, defined as the accumulation of reactive oxygen species (ROS) caused by enhancement of ROS production or by suppression of ROS destruction, plays a pivotal role in neurodegeneration associated with ischemia, trauma, and other neurodegenerative diseases (Coyle and Puttfarcken, 1993; Satoh *et al.*, 1999; 2006; Satoh and Lipton, 2007). It is also well-known that the accumulation of ROS in neurons results in lipid peroxidation, protein oxidation, DNA damage, and finally cell death. Thus, antioxidant is one of the potential targets of drug development for neuroprotection (Satoh *et al.*, 2006; Satoh and Lipton, 2007). As a model of oxidative stress-induced cell death, we studied the glutamate-induced cytotoxicity in HT22 cells, a neuronal cell line that originated from the mouse hippocampus. HT22 cells have been used as a useful model for studying the mechanism of oxidative glutamate toxicity (Davis and Maher, 1994). Excitotoxicity is not involved in the cell death since glutamate receptor antagonists did not have any effects (Davis and Maher, 1994). Rather, glutamate inhibits cysteine uptake, which subsequently leads to depletion of glutathione levels (Davis and Maher, 1994), increased production of ROS and elevated Ca^{2+} levels (Sagara *et al.*, 2002). As a part of our continuing research to find substances with protective effect on HT22 cells from medicinal plants (An *et al.*, 2006), this paper deals the isolation of three compounds from the seeds of *A. katsumadai,* and their protective effects against glutamate-induced cytotoxicity in HT22 cells.

Experimental

Chemicals and instruments – NMR spectra were recorded using a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^1H, 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) and Sep-Pak® (C18 - 5 g, 20 mL, Waters). In TLC silica gel F_{254} plate (Merck) were used. Spots

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were detected under UV light or after spraying with 10% H_2SO_4 reagent, flowed by heating. Trolox, L-glutamate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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 – *Alpinia katsumadai* seeds were purchased from the University Oriental herbal drugstore, Iksan, Korea, in November 2006, and a voucher specimen (No. WP 06-420) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Dried and pulverized *A. katsumadai* seeds (1 kg) were extracted twice with MeOH (5 L) under the ultrasonic condition for 3 h. The MeOH extract (43.0 g) was suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The $CHCl_3$-soluble extract (15.2 g) was subjected to silica gel column chromatography eluting with $CHCl_3$-MeOH (35 : 1) to give compound **1** (8.5 mg) and three fractions (Fr. A-C). Fr. B (6.7 g) was subjected to silica gel column chromatography eluting with $CHCl_3$-MeOH (20 : 1) to afford compound **2** (189 mg). The EtOAc-soluble extract (3.43 g) was subjected to silica gel column chromatography eluting with $CHCl_3$-MeOH-H_2O (7 : 3 : 0.3) to yield four fractions (Fr. E-H). Fr. F (500 mg) was chromatographed on silica gel column with CH_2Cl_2-MeOH (4 : 1) to get three subfractions (Fr. F1-F3). Fr. F1 (440 mg) was purified by reversed-phase Sep-Pak® eluting with 10% MeOH in H_2O to give compound **3** (192 mg).

Alpinetin (1) – Light yellow crystal; (–)-ESI-MS *m/z* 269 $[M-H]^-$, ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 7.30 - 7.57 (5H, m, H-2', 3', 4', 5', 6'), 6.52 (1H, d, J = 2 Hz, H-8), 6.47 (1H, d, J = 2 Hz, H-6), 5.53 (1H, dd, J = 12.8, 2.3 Hz, H-2), 3.78 (3H, s, 5-OC$\underline{H}_3$) 3.15 (1H, dd, J = 15.1, 12.8 Hz, H-3a), 2.90 (1H, dd, J = 15.1, 2.3 Hz, H-3b); ^{13}C-NMR (pyridine-d_5, 125 MHz) δ: 187.7 (C-4), 166.1 (C-7), 165.2 (C-5), 163.3 (C-9), 139.9 (C-1'), 128.8 (C-3', 5'), 128.6 (C-4'), 126.7 (C-2', 6'), 105.6 (C-10), 96.7 (C-6), 94.5 (C-8), 79.3 (C-2), 55.7 (5-O$\underline{C}H_3$), 46.2 (C-3).

Pinocembrin (2) – Yellow crystal; (-)-ESI-MS *m/z* 255 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ: 7.33 - 7.48 (5H, m, H-2', 3', 4', 5', 6'), 5.93 (1H, d, J = 2.1 Hz, H-8), 5.90 (1H, d, J = 2.1 Hz, H-6), 5.42 (1H, dd, J = 12.6, 2.5 Hz, H-2), 3.07 (1H, dd, J = 17.2, 12.6 Hz, H-3a), 2.75 (1H, dd, J = 17.2, 2.5 Hz, H-3b); ^{13}C-NMR (CD_3OD, 125 MHz) δ: 196.0 (C-4), 167.1 (C-7), 164.2 (C-5), 163.3 (C-9), 139.1 (C-1'), 128.4 (C-3', 4', 5'), 126.0 (C-2', 6'), 102.1 (C-10), 95.9 (C-6), 94.9 (C-8), 79.1 (C-2), 42.9 (C-3).

(+)-Catechin (3) – White powder; (–)-ESI-MS *m/z* 289 $[M-H]^-$, ^{1}H-NMR (acetone-d_6, 500 MHz) δ: 6.88 (1H, d, J = 1.8 Hz, H-2') , 6.78 (1H, d, J = 7.8 Hz, H-5') , 6.74 (1H, d, J = 8.2, 1.8 Hz, H-6'), 6.01 (1H, d, J = 2.2 Hz, H-6), 5.86 (1H, d, J = 2.1 Hz, H-8), 4.55 (1H, d, J = 7.4 Hz, H-2), 3.98 (1H, m, H-3), 2.90 (1H, dd, J = 15.8, 5 Hz, H-4b), 2.52 (1H, dd, J = 15.8, 8.3 Hz, H-4a); ^{13}C-NMR (acetone-d_6, 125 MHz) δ: 156.9 (C-5), 156.4 (C-7), 156.1 (C-9), 144.9 (C-3'), 144.8 (C-4'), 131.4 (C-1'), 119.3 (C-6'), 114.9 (C-5'), 114.4 (C-2'), 99.8 (C-10), 95.3 (C-6), 94.6 (C-8), 81.9 (C-2), 67.5 (C-3), 28.4 (C-4).

Cell culture – The mouse hippocampal HT22 cells, which is 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 μg/ml), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The experiment was performed with three groups: the control, glutamate-treated and sample pre-treated groups. Cells of the control group received neither glutamate nor the sample treatment. Those of the glutamate-treated group were incubated with glutamate at a concentration of 5 mM for 12 hours, and those of the sample pre-treated group received sample treatment for 1 hours prior to exposure to glutamate. 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 μg/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 μL 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. The results were compared using one-way ANOVA and Tukey's multiple comparison test. Statistically significant differences between groups were defined as having P values of < 0.01. Calculations were performed using GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, USA).

DPPH radical scavenging assay – The scavenging action of DPPH radical was measured as follows (An *et al*., 2005). The reaction mixture contained 1 mL of 0.1 mM DPPH-ethanol solution, 1 mL of ethanol, 0.95 mL of 0.05 M Tris-HCl buffer (pH 7.4) and 50 mL of either test samples or deionized water (control). Reduction of the DPPH free radical was measured by reading the absorbance at 517 nm exactly 30 sec after adding the samples. L-Ascorbic acid was used as a positive control. The absorbance of the sample alone was subtracted as the blank from that of the reaction mixture. DPPH radical scavenging activity of the sample was expressed as the IC_{50} value, which required concentration to inhibit DPPH radical formation by 50%, determined from the log dose-inhibition curve.

Results and Discussion

In the present study aimed at the identification of plant-derived secondary metabolites with cytoprotective activity on glutamate-induced cytotoxicity in HT22 cells, the MeOH extract of the seeds of *A. Katsumadai* was investigated. The $CHCl_3$- and EtOAC-soluble fractions of MeOH extract showed the promising cytoprotective activity (data not shown), and subsequent activity-guided fraction led us to the isolation of three compounds (**1 - 3**). The structures of **1 - 3** were identified as alpinetin (Itokawa *et al*., 1981), pinocembrin (Liu *et al*., 1992), and (+)-catechin (Nahrstedt *et al*., 1987), respectively, on the basis of comparisons of MS, ^{1}H-NMR, and ^{13}C-NMR data with those reported in the literature (Fig. **1**).

Increasing reports have provided evidences implicating oxidative stress as a major pathogenic mechanism in neurodegeneration such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Emerit *et al*., 2004). Oxidant overproduction leads to oxidative molecular damage of the tissue (Zhu *et al*., 2004). Therefore, protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment of neurodegenerative disorders caused by oxidative stress (Behl and Moosmann, 2002; Bastianetto and Quirion, 2002).

Glutamate is the main excitatory neurotransmitter in the CNS, reaching concentrations of 1 - 10 mM in the synaptic cleft and intraneuronal compartments (Dzubay and Jahr, 1999). Disturbance of glutamate levels is the primary cause of neuronal death in stroke, mechanical trauma and seizure, and it is considered to play a role in some chronic neurodegenerative disorders such as Parkinson's or Alzheimer's diseases (Lipton, 2004). In

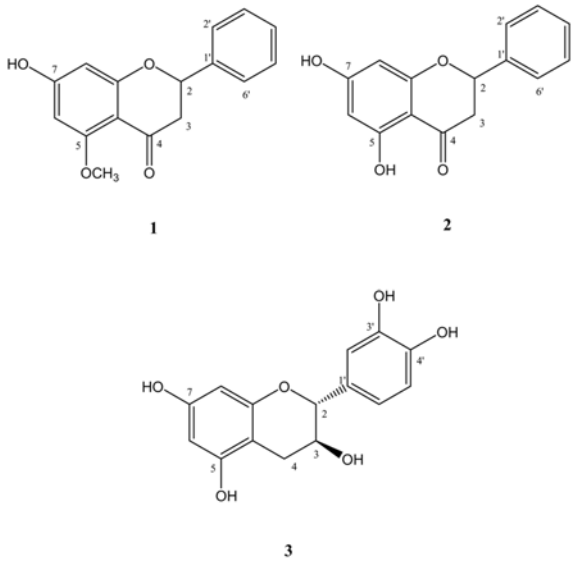

Fig. 1. Chemical structures of compounds **1-3**.

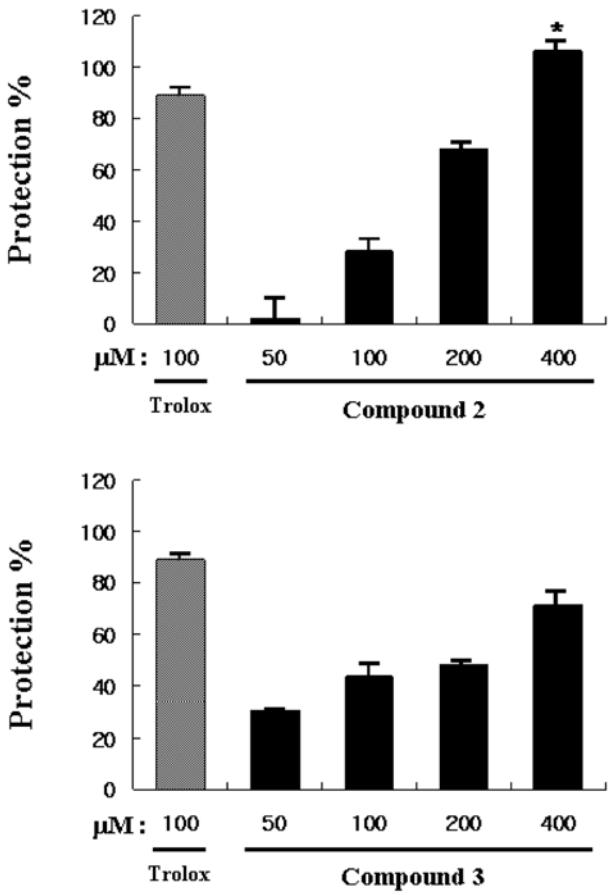

Fig. 2. The cytoprotective effects of compounds **2** and **3** on glutamate-induced cytotoxicity in HT22 cells. Cytotoxicity was assessed after 12-h incubation period with 100 mM of glutamate in DMEM medium. Each value represents the mean ± S.D. of three experiments. Significantly different from the control; *p<0.05. Trolox was used as positive control.

HT22 cells, a mouse hipocampal cell line, lacking ionotropic glutamate receptors, the high concentration glutamate inhibits cystine uptake and depletes intracellular glutathione, which leads to the accumulation of reactive oxygen species (ROS) and ultimately causes cell death (Froissard and Duval, 1994).

To evaluate the *in vitro* neuroprotective effects of isolated compounds from *A. katsumadai,* protective effects on glutamate-induced cytotoxicity in HT22 cells were tested. Among isolated compounds, compounds **2** and **3** exhibited protective effects against glutamate-induced cytotoxicity in HT22 cells, with effective protection ratios of 68.3 ± 0.7 and 48.2 ± 0.9%, respectively, at a concentration of 100 μM (Fig. **2**). Trolox, well known for its antioxidative efficiency, was used as a reference substance, and showed protect ratio of 88.2 ± 0.2% at a concentration of 100 μM. In addition, to estimate the antioxidative effects of isolated compounds, free radical scavenging activity of compounds **1 - 3** was evaluated by the interaction with stable free radical DPPH (data not shown). Among these compounds, compounds **2** and **3** showed potent scavenging effects on DPPH radical exhibiting IC_{50} value of 10.43 and 87.23 μM, respectively. L-Ascorbic acid, well-known for its anti-radical efficiency, was used as a positive control and exhibited the IC_{50} value of 65.98 μM.

In conclusion, three phenolic compounds (**1 - 3**) from *A. katsumadai* were isolated. The protective effect of compounds **2** and **3** against glutamate-induced cytotoxicity in mouse hippocampal HT22 cells appears to be related to its free radical scavenging effects and these results suggest that compounds **2** and **3** can be valuable source of potential neuroprotective agents.

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