Neuroprotective effects of 4,5-dimethoxypyrocatechol isolated from *Cynanchum paniculatum* on HT22 cells

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ABSTRACT

Background: *Cynanchum paniculatum*, belongs to the family Asclepiadaceae and is used to treat various diseases, such as invigorate blood, alleviate edema and to relieve pain and toxicity for a long time. **Materials and Methods:** 4,5-Dimethoxypyrocatechol was isolated from the 80% methanol extract of *C. paniculatum* and its neuroprotective effect was evaluated by MTT assay. **Results:** 4,5-Dimethoxypyrocatechol had neuroprotective effect on the glutamate-induced cellular oxidative death in HT22 cells. **Conclusion:** Furthermore, we found that reactive oxygen species (ROS) accumulation and calcium concentration by oxidative stress were reduced by 4,5-dimethoxypyrocatechol in HT22 cells.

Key words: *Cynanchum paniculatum,* 4,5-dimethoxypyrocatechol, HT22 cells, neuroprotective effect

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INTRODUCTION

Oxidative stress was implicated in various neurodegenerative diseases including Alzheimer's disease (AD).[1] Oxidative stress causes neuronal cell death and necrosis resulting in neuronal diseases.[2] Glutamate is the major excitatory neurotransmitter in brain and can contribute to neuronal cell death.[3,4] High levels of extracellular glutamate generate excitotoxicity, and this causes calcium (Ca^{2+}) to enter cells, leading to neuronal damage via increased reactive oxygen species (ROS) levels.[5,6] Glutamate induced oxidative stress results in the depletion of glutathione and accumulation of ROS levels by inhibiting the uptake of cystine through the glutamate/cystine antiporter system $x(c)^-$.[7,8]

The immortalized mouse hippocampal HT22 cells, a sub-line of HT4 cells, have been used as an *in vitro* hippocampal cholinergic neuronal model for studying the mechanism of oxidative glutamate toxicity.[9]

The whole plant or root of *Cynanchum paniculatum* Kitagawa (Asclepiadaceae family) that is found in Korea, China, and Japan has been used to treat many diseases, such as rheumatoid arthritis, lumbago, abdominal pain, vomiting, and acute gastro-enteritis, and it used as an anodyne. There are several reports in the literature indicating that *C. paniculatum* has the anti-inflammatory and antinociceptive effects.[10] *C. paniculatum* is known to contain compounds such as paeonol, C_{21} steroids, glycosides, and alkaloids. Paeonol is a major active compound in *C. paniculatum*.[11,12] We isolated 10 compounds, paeonol, 4-acetylphenol, 2,5-dihydroxy-4-methoxyacetophenone, 2,3-dihydroxy-4-methoxyacetophenone, acetoveratrone, 2,5-dimethoxyhydroquinone, vanillic acid, resacetophenone, m-acetylphenol, and 3,5-dimethoxy-hydroquinone from *C. paniculatum* and investigated their neuroprotective effects.[13]

The aim of this study was to isolate additional compounds from *C. paniculatum* and to evaluate their neuroprotective effect against glutamate-induced oxidative stress in mouse HT22 cells.

MATERIALS AND METHODS

Plant materials

The roots of *C. paniculatum* were purchased from Kyungdong traditional herbal market in Seoul and were identified by Dr. Young Bae Seo, a professor of the College of Oriental Medicine, Daejeon University (Daejeon, Korea). A voucher specimen (No. CJ021M) was deposited in the Department of Biomaterials Engineering in Kangwon National University.

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Extraction and isolation

Roots of *C. paniculatum* (5.2 kg) were extracted using 80% methanol by ultrasonication-assisted extraction at room temperature. The extract was partitioned with n-hexane, $CHCl_3$, EtOAc, and n-BuOH. The $CHCl_3$ fraction (36.86 g) was subjected to silica gel column chromatography (CC) to obtain eight (A–H) fractions. Compound A was isolated from C by Sephadex-LH 20 CC and preparative HPLC on a C_{18} YMC hydrosphere (250 mm × 20 mm I.D. S-5 μm).

HT22 cell culture

Mouse hippocampal HT22 cells were obtained from Seoul National University, Korea. HT22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of cell viability

Cell viability was evaluated by MTT assay. HT22 cells (6.7×10^4 cells/300 μL) were seeded into 48-well plates. After 24 h of incubation, the cells were treated with various concentrations of samples and 50 μM trolox, a well-known antioxidant, and were further incubated for 1 h. Then, 2 mM glutamate was added to the DMEM medium for 24 h. MTT solution (1 mg/mL) was applied to the cells and incubated for 3 h. The formazan formed was dissolved in 300 μL of dimethyl sulfoxide (DMSO). Optical density was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Neuroprotective activity of samples was investigated by relative protection ratio (%).

Determination of ROS level

The level of intracellular ROS was detected using 2'7'-dichlorofluorescein diacetate (H2-DCF-DA) in HT22 cells. Cells (6.74×10^4/well) were treated with 2 mM glutamate in the presence or absence of sample. After 8 h of glutamate treatment, the cells were stained with 10 μM DCF-DA in Hanks' balanced salt solution for 30 min in a dark room. The cells were then extracted with 1% Triton X-100 in PBS for 10 min at 37°C. Fluorescence was recorded with an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

Measurement of Ca^{2+} concentration

Cytosolic Ca^{2+} concentration in HT22 cells was measured by fluorescence assay using the Fura-2AM. HT22 cells (6.74×10^4/well) were plated into 48-well plates. After treatment of 10, 50, and 100 μM of sample and 2 μM Fura-AM, glutamate was added to each well. After 2 h, the cells were washed with PBS and then suspend in 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 400 nm and an emission wavelength of 535 nm.

DPPH radical scavenging assay

The DPPH radical scavenging assay was performed for the determination of free radical-scavenging activity by the decreases in absorbance of DPPH solution. Various concentrations of sample (150 μL) were added to 150 μL of 0.4 mM DPPH-methanol solution into a 96-well microplate. After 30 min at darkroom, reduction of the DPPH free radical was measured by recording the absorbance at 517 nm. L-Ascorbic acid was used as a positive control. The assay was repeated three times. Percentage inhibition was calculated using the formula, (1 − sample absorbance/only DPPH absorbance) ×100.

RESULTS

Isolation of a compound from *C. paniculatum*

We isolated compound A from $CHCl_3$ fraction of *C. paniculatum* and elucidated it using a ^1H and ^{13}C nuclear magnetic resonance (NMR). The structure of isolated compound A was identified as 4,5-dimethoxypyrocatechol by comparison with a previously reported spectroscopic data and its structure [Figure 1].

Cell viability

To investigate whether 4,5-dimethoxypyrocatechol protects against glutamate-induced neuronal cell death in HT22 cell, MTT assay was performed. The relative protection (%) of 4,5-dimethoxypyrocatechol was 2.19% and 29.59% at 50 μM and 100 μM, respectively [Figure 2].

ROS measurement

To evaluate the effect of 4,5-dimethoxypyrocatechol on the intracellular ROS induced by oxidative stress, ROS levels were determined by 2'7'-dichlorofluorescein diacetate (H2-DCF-DA) in HT22 cells.[14] The level of ROS of glutamate-stimulated cells was recorded as 125.14%. The level of ROS on 4,5-dimethoxypyrocatechol-treated cells was recorded as 123.74%, 104.23%, and 103.36% at 10, 50, and 100 μM, respectively. 4,5-Dimethoxypyrocatechol suppressed glutamate-induced intracellular ROS production in cells [Figure 3].

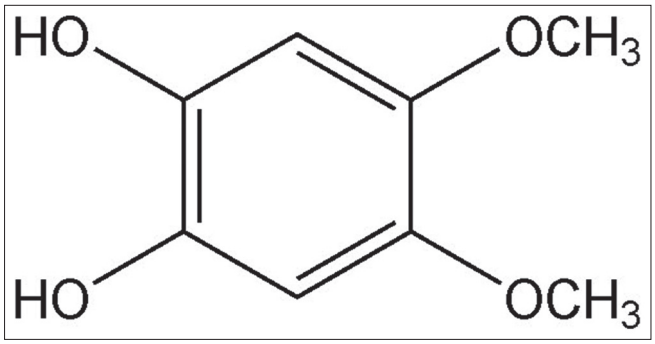

Figure 1: The chemical structures of 4,5-dimethoxypyrocatechol

Determination of intracellular Ca^{2+} concentration

For the measurement of intracellular Ca^{2+} concentration, 2 μM Fura-AM was used. Exposure of HT22 cells to glutamate-induced oxidative stress resulted in 167.99% increase in Ca^{2+} level. However, increase in the Ca^{2+} level was reduced by 4,5-dimethoxypyrocatechol (100 μM), and the value of Ca^{2+} value was recorded as 106.28% [Figure 4].

Antioxidant activity

To evaluate the antioxidant ability of 4,5-dimethoxy pyrocatechol, DPPH radical scavenging activity was assayed. As shown in Figure 5, 4,5-dimethoxypyrocatechol showed prominent DPPH inhibitory activity with IC_{50} values of 931.75 μM.

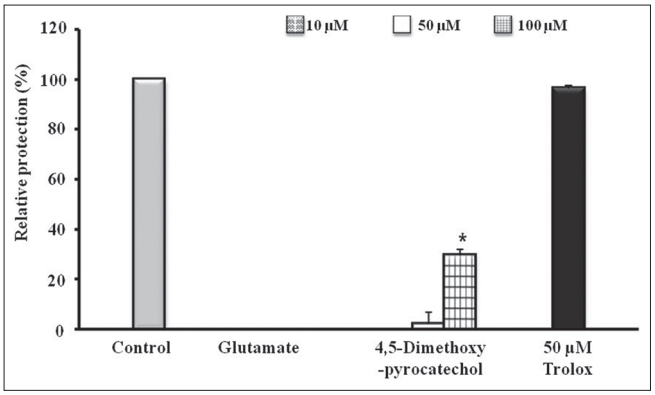

Figure 2: The neuroprotective effects of 4,5-dimethoxypyrocatechol on glutamate-induced cytotoxicity in HT22 cells. HT22 cells were treated with 10, 50, and 100 μM of 4,5-dimethoxypyrocatechol, and then incubated for 24 h with glutamate (2 mM). Positive control, trolox (50 μM) exhibited relative protective activity (96.39 ± 1.25%). Each bar represents the mean ± SD of three independent experiments. **P*< 0.05, ***P* < 0.01, ****P* < 0.001 vs. SCRT (ANOVA)

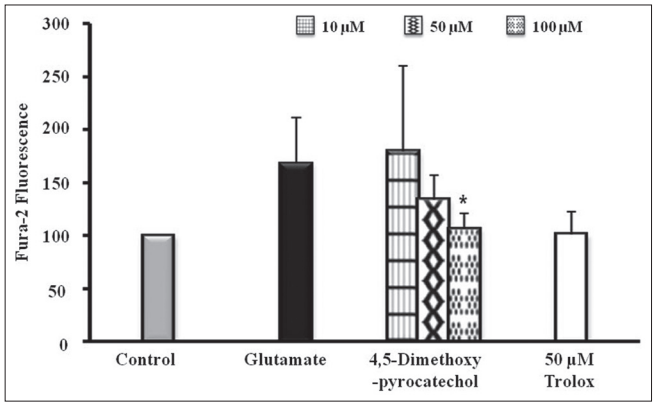

Figure 4: Effect of homosyringaldehyde on intracellular Ca^{2+} accumulation in glutamate-treated HT22 cells. 4,5-Dimethoxypyrocatechol was treated with 2 μM Fura-2 AM 1 h before exposure to glutamate. The alteration of Ca^{2+} concentration measured 2 h after glutamate treated. The data present means ± SD. **P*< 0.05, ***P* < 0.01 and ****P* < 0.001 vs. glutamate-injured cells (ANOVA)

DISCUSSION

Glutamate-induced oxidative cell death is mediated by c-jun N-terminal kinase (JNK), one of the mitogen-activated protein kinase and various apoptosis-inducing factors.[15] Glutamate toxicity can block the glutamate/cystine antiporter system $x(c)^-$, followed by the depletion of glutathione. Ca^{2+} accumulation and generation of ROS is a common pathway of many forms of cell death.[16,17] HT22 cell is a good *in vitro* model system for screening for agents that may prevent glutamate-induced oxidative cell death. HT22 cell also expresses essential cholinergic markers, such as choline acetyltransferase, muscarinic acetylcholine receptors, and high-affinity choline transporter, but it does not have functional iontropic receptors, such as the *N*-methyl-D-asparatate (NMDA) receptor.[18,19]

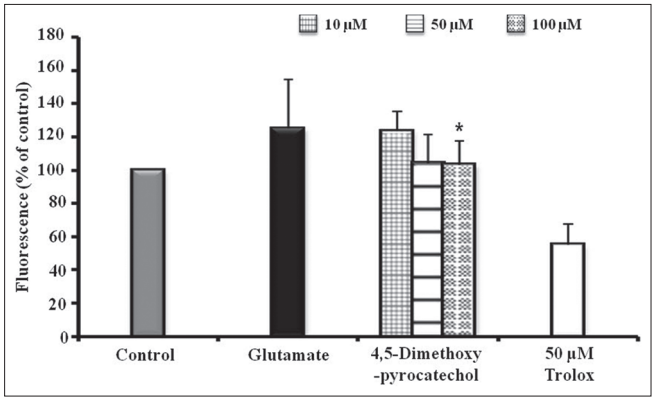

Figure 3: Inhibition of reactive oxygen species generation. HT22 cells were treated with 10, 50, and 100 μM of 4,5-dimethoxypyrocatechol and then exposed to 2 mM glutamate for 8 h increased ROS production. Trolox (10 μM) was used as a positive control. Each bar represents the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. glutamate-injured cells (ANOVA)

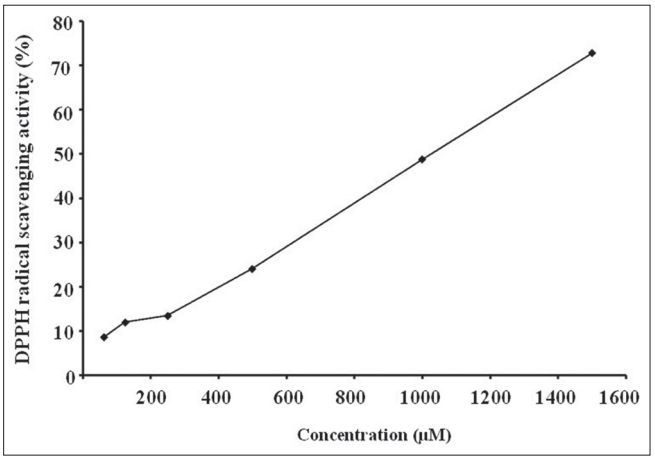

Figure 5: Anti-oxidant effects of 4,5-dimethoxypyrocatechol; DPPH radical scavenging activity.**P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control (ANOVA)

Our previous study showed that *C. paniculatum* was found to exhibit neuroprotective effect and isolated neuroprotective phenolic compounds.[13] In addition, the present study isolated compound from *C. paniculatum* and identified it as 4,5-dimethoxypyrocatechol. Also, 4,5-dimethoxypyrocatechol showed neuroprotective effect against glutamate-induced cell death in HT22 cell. 4,5-Dimethoxypyrocatechol dose-dependently reduced cell death and exhibited significant neuroprotective effect at 100 μM. To better understand the underlying mechanism of neuroprotective effect of 4,5-dimethoxypyrocatechol from oxidative glutamate toxicity, we examined inhibitory effect of ROS production and intracellular Ca^{2+} elevation. The result of these experiments showed that 4,5-dimethoxypyrocatechol reduced glutamate-induced ROS reduction and attenuated acceleration of Ca^{2+} fluxes in HT22 cells.

In addition, to evaluate the antioxidative effect of 4,5-dimethoxypyrocatechol, DPPH radical scavenging activity was investigated. DPPH radical is a stable free radical, which has been widely used to evaluate the radical-scavenging ability of antioxidants. 4,5-Dimethoxypyrocatechol showed scavenging activity for DPPH radical.

4,5-Dimethoxypyrocatechol has a catechol structure and is a phenolic compound. Many studies have demonstrated that phenolic compounds have more effective antioxidant activity and neuroprotective effect against the cytotoxicity of glutamate and hydrogen peroxide. This neuroprotective effect of phenolic compounds might be due to its antioxidant activity.[20,21]

In conclusion, 4,5-dimethoxypyrocatechol from *C. paniculatum* showed involvement of neuroprotection to prevent glutamate-induced oxidative cell death through its activity to suppress ROS, inhibition of Ca^{2+} concentration, and antioxidantive effect. Therefore, 4,5-dimethoxypyrocatechol may be a valuable source of potential neuroprotective agents.

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