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NEUROPROTECTIVE EFFECTS OF OLEIFERIN F ON MITOCHONDRIAL-MEDIATED APOPTOSIS IN GLUTAMATE-INDUCED HT22 CELLS

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Abstract: Neuronal disease is caused by neuronal cell damage, and a high concentration of glutamate causes neuronal cell death. The purpose of this experiment was to confirm the protective effect of Oleiferin F (OF) on glutamate-induced apoptosis in HT22 cells. Oleiferin F inhibited the death of glutamate-induced HT22 cells in a dose-dependent manner as confirmed by annexin V/propidium iodide (PI) double staining. Oleiferin F reduced disruption of the mitochondrial membrane potential ($\Delta\Psi m$), p53 expression, the Bax/Bcl-2 expression ratio and mitochondrial apoptosis-inducing factor (AIF) protein release. Oleiferin F also reduced levels of the cleaved form of caspase pathway proteins (PARP, caspase-9, caspase-3), which lead to apoptosis. These results reveal the mechanism underlying the neuroprotective effect of Oleiferin F in HT22 cells. Oleiferin F may be useful in preventing or treating neurological diseases.

Keywords: glutamate; Oleiferin F; apoptosis; neuroprotective effect.

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1. INTRODUCTION

Neurodegenerative diseases such as Alzheimer's and Parkinson's are caused by damage to neuronal cells [1]. One cause of neuronal cell damage is excessive glutamate, which is absorbed by nerve tissue and destroys neuronal cell membranes. In the mammalian central nervous system (CNS), glutamate is an important excitatory neurotransmitter and plays an important role in neural development including learning and memory [2]. However, high concentrations of glutamate result in cytotoxicity, apoptosis and ultimately neurological diseases [3]. When excess glutamate is absorbed into the nerve tissue, glutamate receptors are activated and induce excessive calcium (Ca^{2+}) uptake into the cells, leading to altered expression of pro/anti-apoptotic regulators such as Bax and Bcl-2 [4-5]. p53 is associated with DNA damage and expresses Bax protein, which is pro-apoptotic [6]. Bax causes the collapse of mitochondrial membrane potential ($\Delta\Psi m$), resulting in release of apoptosis inducing factor (AIF) and cytochrome c from the mitochondria [7-9]. Cytochrome c activates the caspase cascade leading to DNA cleavage and apoptosis [10-12]. HT22 cells, derived from mouse hippocampal neuronal cells, lack functional ionotropic glutamate receptors. Therefore, HT22 cells are useful models for glutamate toxicity studies [13].

Oleiferin F (OF) is a lignin that can be extracted from *Schisandra chinensis* or *Myristica fragrans*. In addition, it has a tetrahydrofuran structure (Fig. 1) and has been known to have neuroprotective effects [14-15].

Although OF is known to have memory enhancement and neuroprotective effects, the detailed mechanism underlying its protective effect against glutamate-induced apoptosis is not yet known. Therefore, the aim of this study was to examine the mechanism of neuroprotective effect of OF in glutamate-induced HT22 cells.

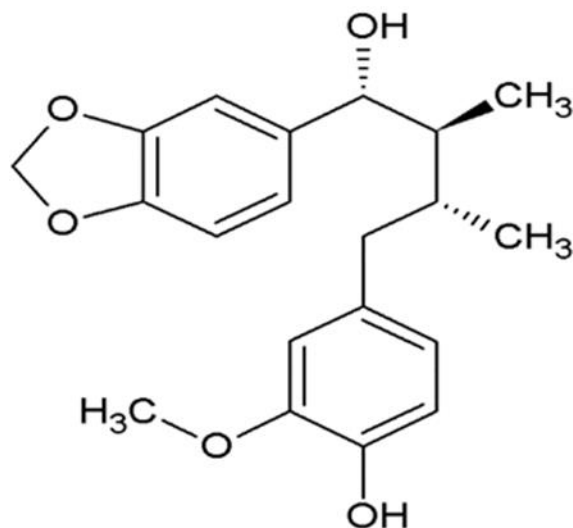


Fig. 1. The chemical structure of Oleiferin F

2. MATERIALS AND METHODS

2.1. Cell viability assay

HT22 cells were seeded at a density of 4×10^3 cells / well in 96-well plates and cultured for 24 h in a humidified 5% CO_2 incubator. The cells were then treated with 10 mM glutamate and the indicated amount of OF for 24 h. Then, 5 mg/ml MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) in DMEM was applied for 2 h at 37°C in a 5% CO_2 incubator. The medium was removed and the cells were treated with 100 μl of dimethyl sulfoxide (DMSO) for 5 min. Activity was measured at 540 nm using a microplate reader (BioTek Instruments, Korea). Quercetin is known to have a neuroprotective effect and was used as a positive control [16].

2.2. Flow cytometry analysis

2.2.1. FITC-annexin V/PI double staining

To investigate the type of cell death in glutamate-induced HT22 cells, cells were seeded at a density of 5×10^5 cells/dish. After 24 h, cells were treated with 10 mM glutamate and OF and incubated for 24 h. Cells were harvested with scraper and washed with PBS. Then, fluorochrome-conjugated annexin V was added to the cell suspension and stained for 15min. Cells were washed

with binding buffer and stained in the dark for 20 min with propidium iodide (PI) solution. They were then washed with binding buffer and measured by flow cytometry (FACS, ACEA Biosciences Inc.). The data were analyzed using NovoExpress software (ACEA Biosciences, Inc.).

2.2.2. JC-1 staining

The JC-1 [5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl-benzimidazoly-carbocyanine iodide; BioTium. Glowing Products for Science™] staining reagent was used to measure mitochondrial membrane potential (△Ψm). After 2 μM of JC-1 was placed in each well and stained at 37°C for 30 min, the fluorescent intensities of JC-1 retained by 30,000 cells per sample were measured on a flow cytometer with 530 nm (green) and 590 nm (red) channels and analyzed using NovoExpress software (ACEA Biosciences, Inc.).

2.3. Fluorescence microscope analysis

The HT22 cells were seeded in six-well plates with a density of 2×10^5 cells/well and incubated for 24 h. The cells were treated with 10 mM glutamate and OF for 24 h. Then, 2 μM of JC-1 was suspended in the medium, treated in each well, and stained at 37°C for 30 min. Stained cells were washed and analyzed by fluorescence microscopy (Thermo Fisher Scientific Inc.).

2.4. Western blot analysis

The HT22 cells treated with 10 mM glutamate and an indicated amount of OF were washed with PBS and lysed using RIPA buffer supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Then, cell lysates were centrifuged at 14,000 rpm for 15 min at 4°C, protein extracts were collected and protein concentrations were determined using the DC Bio-Rad Protein Array Kit. Protein samples were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene fluoride membrane (PALL Life Science, Port Washington, NY, USA). Membranes were pre-incubated with blocking solution [5% (w/v) skim milk in tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TBS-T)] for 1 h and then incubated with anti- β-Actin (Cell Signaling Technology Inc.), anti-p53 (BD PharmingenTM), anti-Bcl-2 (Cell Signaling Technology Inc.), anti-Bax (Santa Cruz Biotechnology Inc.), anti-poly (ADP-ribose) polymerase

(PARP; Cell Signaling Technology Inc.), anti-caspase-9 (Santa Cruz Biotechnology Inc.), anti-caspase-3 (Cell Signaling Technology Inc.) or anti-apoptosis inducing factor (AIF; Santa Cruz Biotechnology Inc. Dallas, TX, USA) at 4°C overnight. All primary antibodies were diluted 1:2000 in blocking solution. The membranes were washed three times for 10 min with TBS-T, and then probed with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:4,000 diluted in blocking solution; Sigma-Aldrich) or anti-mouse IgG (1:4,000 diluted in blocking solution; Sigma-Aldrich) antibody at room temperature for 2 h. The membranes were washed three times for 10 min with TBS-T. Proteins were detected with Super Signal™ West Pico or Femto Chemiluminescence System (Thermo Fisher Scientific Inc.) and immune response bands were normalized to the control group using Image J software.

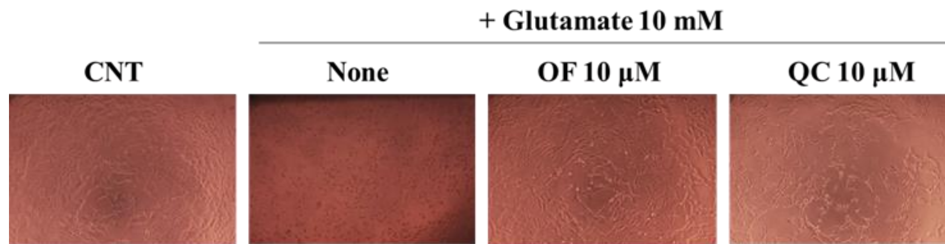
2.5. Statistical analysis

All results are presented as mean ± standard deviation (SD) or standard error (SE). The Student's t-test was used to compare the untreated control group and treated groups (***$p < 0.001$, **$p < 0.01$).

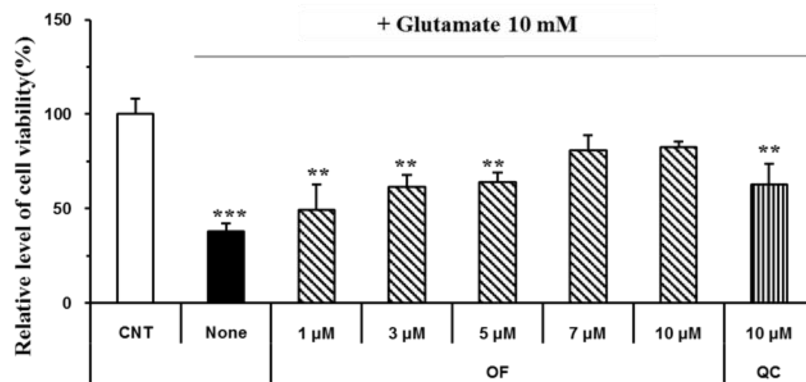
3. RESULT

3.1. The protective effects of OF against glutamate-mediated cell death

MTT analysis was performed to determine whether OF has neuroprotective effects against glutamate-induced neuronal cell death. Viability of cells exposed to 10 mM glutamate decreased to 38.0 ± 4.0% compared with the control (100 ± 8.1%), but OF prevented glutamate-induced HT22 cell death in a dose-dependent manner (Fig. 2b). In addition, HT22 cells treated with 10 μM OF showed a morphology more similar to that of control cells than glutamate-treated cells (Fig. 2a).



(a)



(b)

Fig. 2. Inhibitory effects of glutamate-induced HT22 cell death. (a) Observation of cell morphology. (b) The cell viability assay. ***$p < 0.001$ and **$p < 0.01$ compared to control cells. QC was used as a positive control.

Based on this data, annexin V / PI double staining was performed to determine the type of cell death. FITC-Annexin V / PI double staining was analysed by flow cytometry, and the extent of apoptosis during glutamate-induced cell death and the protective effect of OF treatment were determined (Fig. 3a).

Fewer glutamate-induced cells (42.6%) survived compared to the control (91.1%), but OF treatment increased survival in a dose-dependent manner (Fig. 3b). In addition, the apoptosis ratio was increased in glutamate-induced cells (55.5%) compared to the control (8.8%), but that was dose-dependently decreased by OF treatment (Fig. 3C).

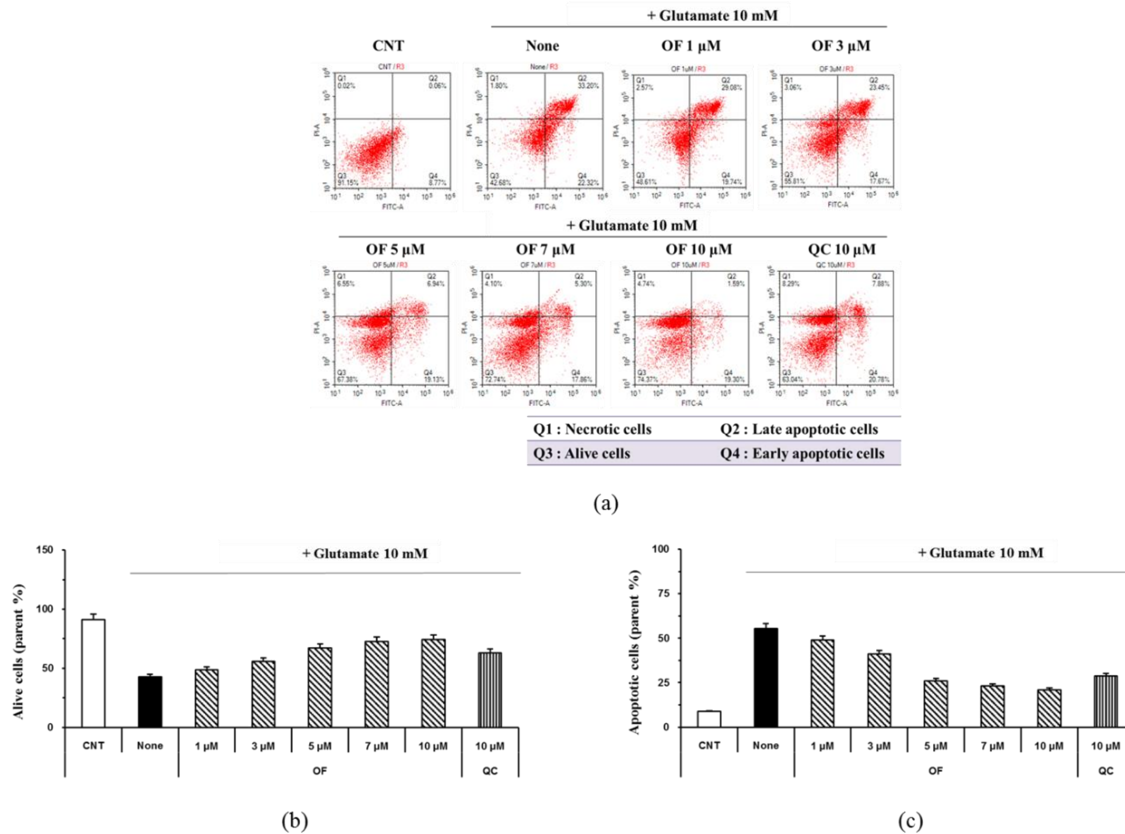


Fig. 3. Inhibitory effects of OF on glutamate-induced HT22 apoptosis. (a) FITC-annexin V/PI to evaluate the number of apoptotic and necrotic cells, respectively. (b, c) Images were quantitatively analyzed using NovoExpress software. (b) Bars denote the percentage of living cells. (c) Bars denote the percentage of apoptotic cells.

These studies show that OF has a protective effect against death of HT22 cells caused by high concentrations of glutamate (10 mM).

3.2. The inhibitory effects of OF on glutamate-induced disruption of mitochondrial membrane potential (△Ψm)

Mitochondrial membrane potential (△Ψm) was analysed using fluorescence microscopy, flow cytometry to further understand the role of OF in glutamate-induced apoptosis (Figure 4).

Fluorescence microscopy results showed that 10 mM glutamate treatment resulted in a significant decrease in JC-1 red/green fluorescence ratios (0.54 ± 0.02%), indicating destruction of the mitochondrial membrane potential (△Ψm) by glutamate toxicity (Fig. 4a). The red/green ratio indicated that the mitochondrial membrane potential (ΔΨm) was dose-dependently restored by OF treatment (Fig. 4b). Fluorescence intensities of JC-1 monomer (green fluorescence) and aggregates

(red fluorescence) were determined using flow cytometry to more accurately investigate the mitochondrial membrane potential (ΔΨm) (Fig. 4c). The proportion of red/green was reduced (0.55 ± 0.03%) in 10 mM glutamate-treated cells compared to the control (1%) but increased after OF treatment in a dose-dependent manner (Fig. 4d). These results suggest that OF inhibits disruption of mitochondrial membrane potential (ΔΨm) in glutamate-induced HT22 cells, protecting the mitochondria and facilitating energy synthesis.

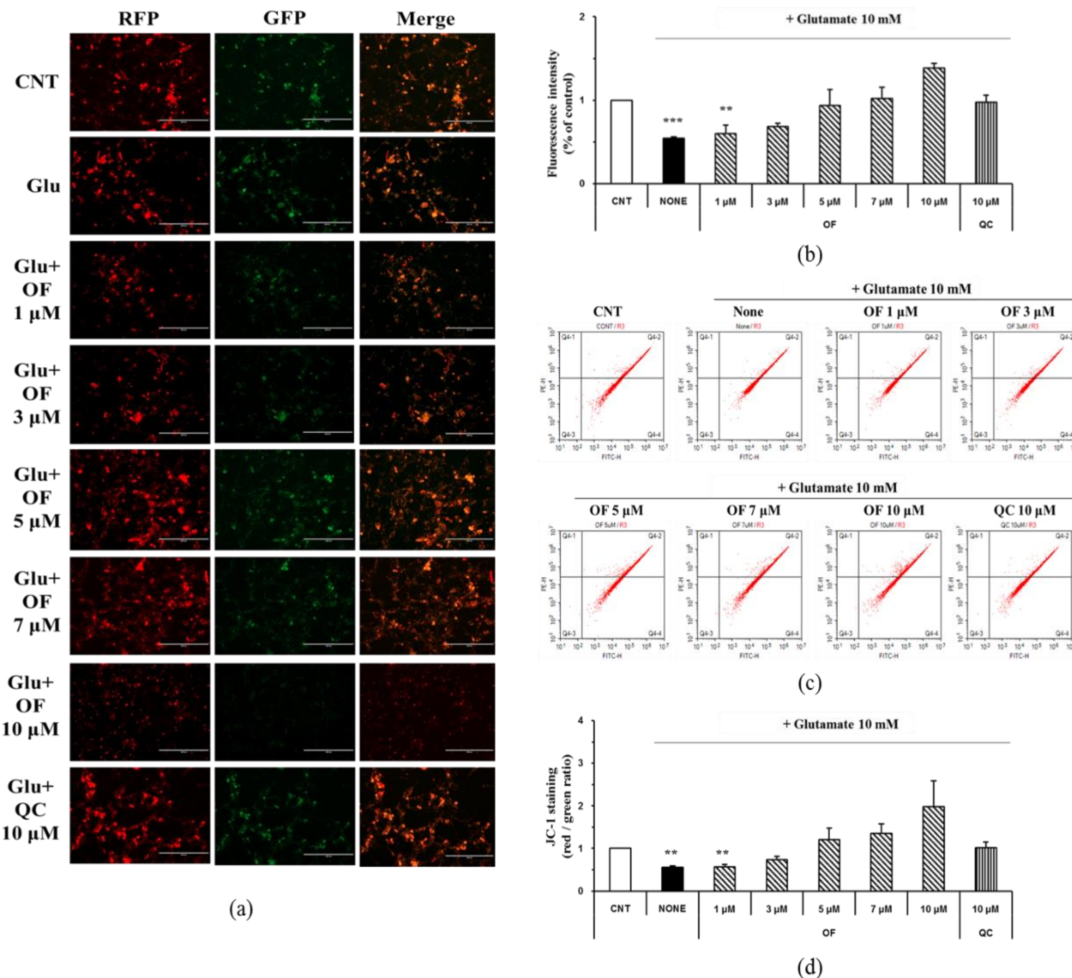


Fig. 4. The effects of OF on disruption of glutamate-induced mitochondrial membrane potential (ΔΨm). (a) JC-1 staining and images were obtained by fluorescence microscopy. (b) The fluorescence intensity was measured using the Image J program. (c) JC-1 staining was performed and analyzed with a flow cytometer. (d) The red/green percentage was analyzed using NovoExpress software. ***$p < 0.001$ and **$p < 0.01$ compared to control cells.

3.3. The effects of OF on changes in Bax, Bcl-2 and p53 expression induced by glutamate

Bcl-2 is an anti-apoptotic protein that inhibits apoptosis. When mitochondrial membrane disruption occurs, pro-apoptotic Bax is activated, and AIF and cytochrome c are released to activate the caspase pathway. Therefore, it is important to determine the levels of expression of proteins such as Bax and Bcl-2, which are mitochondria-mediated proteins involved in apoptosis, and the Bax/Bcl-2 ratios. It is also important to examine p53 protein expression involved in Bax protein expression. When HT22 cells were treated with 10 mM glutamate, Bcl-2 protein expression levels decreased and Bax and p53 protein expression levels increased (Fig. 5a). In contrast, treatment with OF dose-dependently increased Bcl-2 protein expression levels and decreased Bax and p53 expression levels (Fig. 5b-d). The expression ratio of Bax/Bcl-2 was also decreased (Fig. 5e). These data suggest that OF protects against glutamate-induced mitochondrial damage by regulating expression of proteins associated with mitochondria (Bcl-2 up-regulation, Bax and p53 down-regulation).

3.4. The effects of OF on AIF and caspase cascade protein expression

When mitochondria are damaged by the Bax protein, mitochondrial AIF and cytochrome c are released into the cytosol. Since AIF induces apoptosis, suppression of its release may be a good strategy to prevent apoptosis. Thus, AIF and caspase cascade expression were confirmed via western blot analysis (Fig. 6, 7). When cells were treated with 10 mM glutamate, AIF was released into the cytoplasm from the mitochondria, but this release was dose-dependently inhibited by OF treatment (Fig. 6b, c). The expression of cleaved forms of PARP, caspase-9, caspase-3 increased when HT22 cells were treated with 10 mM glutamate. On the other hand, treatment with OF decreased expression of these proteins in a dose-dependent manner (Fig 7b-d). These data suggest that OF inhibits the release of AIF in mitochondria, ultimately inhibiting the induction of apoptosis by decreasing expression of cleaved caspase pathway proteins.

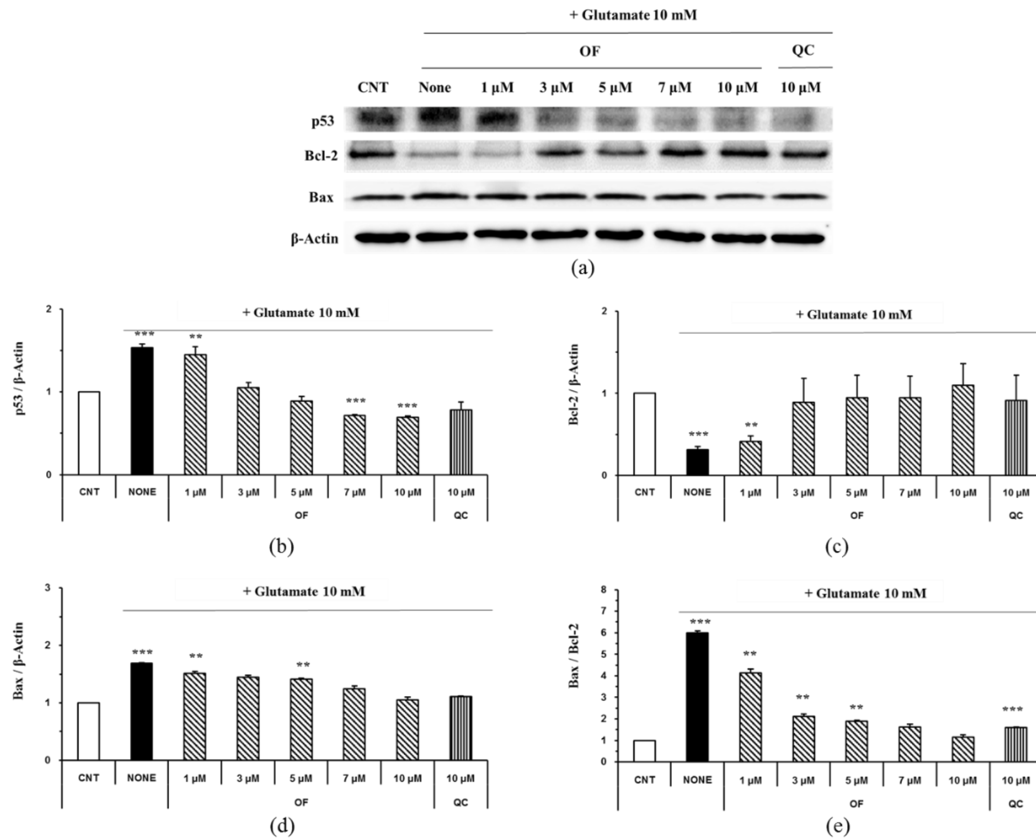


Fig. 5. The effects of OF on Bcl-2, Bax and p53 expression in glutamate-induced HT22 cells. (a) HT22 cells were exposed to 10 mM of glutamate in the presence of OF for 24 h and western blot analysis was performed. β-actin was used as a loading control. (b, d) Bars indicate increases in p53 and Bax compared with the control cells. (c) Bars indicate decreases in Bcl-2 compared with the control cells. (e) Bars indicate increases in the Bax/Bcl-2 ratio compared with the control cells. ***$p < 0.001$ and **$p < 0.01$ compared with the control cells.

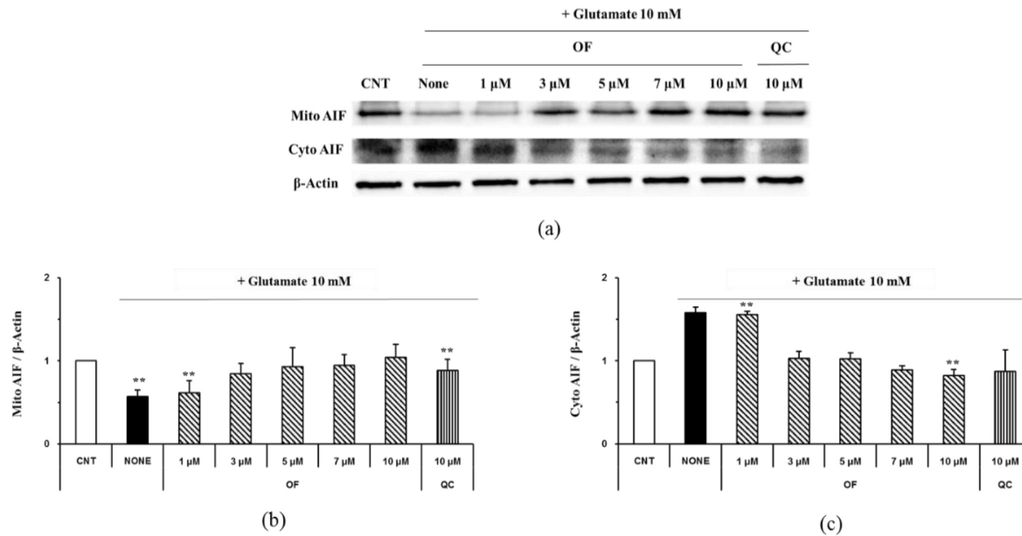


Fig. 6. The inhibitory effect of OF on the release of AIF into mitochondria in glutamate-induced HT22 cells. (a) HT22 cells were exposed to 10 mM glutamate in the presence of OF for 24 h, mitochondria and cytosol were separated, and western blot analysis was performed. (b, c) Glutamate-induced HT22 cells induced release of AIF in mitochondria compared to the control, and OF suppressed release in a dose-dependent manner. **p < 0.01 compared with the control cells.

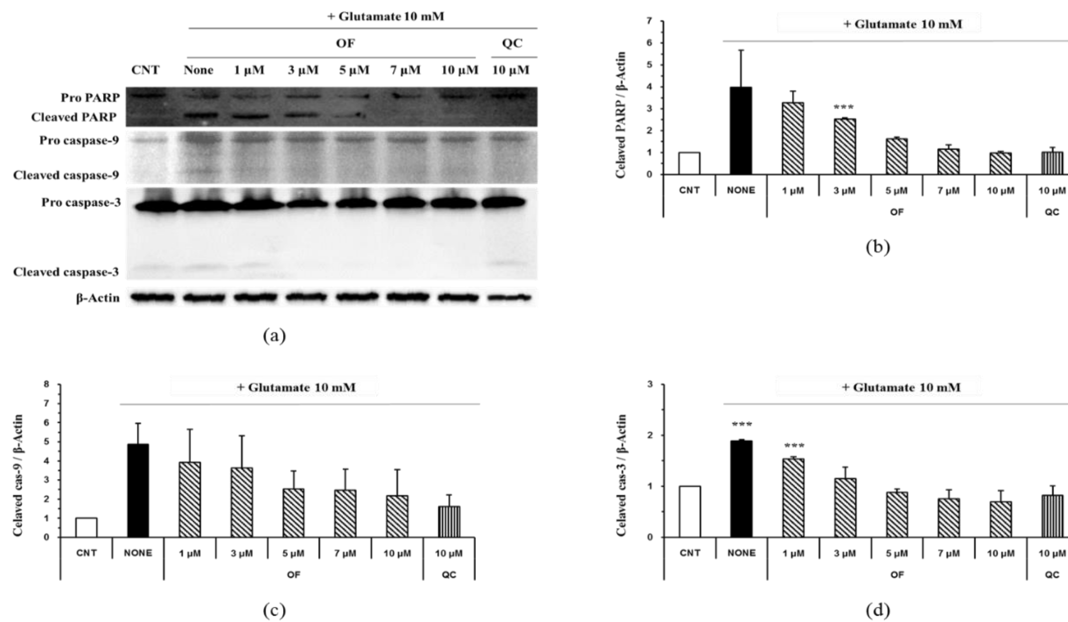


Fig. 7. The effects of OF on PARP, caspase-9, caspase-3 expression in glutamate-induced HT22 cells. (a) HT22 cells were exposed to 10 mM of glutamate in the presence OF for 24 h and western blot analysis was performed. (b-d) Bars indicate the increases in cleaved caspase-3, cleaved caspase-9, and cleaved PARP compared with the control cells. ***p < 0.001 compared with the control cells.

4. DISCUSSION

Glutamate-induced oxidative stress and Ca^{2+} influx cause apoptosis and necrosis of HT22 cells [17]. Glutamate toxicity was mainly induced by glutamate receptor-mediated excitatory toxicity and ROS-mediated oxidation in neurons [18]. Treatment of OF in glutamate-induced HT22 cells resulted in a dose-dependent protective effect to reduce neuronal cell death.

Increased intracellular ROS levels due to excessive Ca^{2+} influx interfere with mitochondrial dysfunction, resulting to cell death [19]. The disruption of mitochondrial membrane potential ($\Delta\Psi m$) thus induced is regulated by various proteins such as Bcl-2 and Bax [20]. The development of neurodegenerative diseases is deeply associated with mitochondrial dysfunction. Therefore, looking at the proteins involved can be a good target.

Cell death of glutamate-induced HT22 cells was confirmed by FITC-annexin V / PI double staining, and OF compounds inhibited cell death in a dose-dependent manner. OF also activated mitochondrial membrane potential. The role of the Bcl-2 protein family, such as anti-apoptosis or pro-apoptosis, plays an important role in mitochondria [21]. Changes in the ratio of Bax/Bcl-2 can affect the regulation of mitochondrial membrane permeability and release of certain pro-apoptotic is proteins such as AIF. In addition, the p53 protein activates pro-apoptosis (Bax) protein. OF reduced the expression ratio of Bax/Bcl-2 in glutamate-induced HT22 cells and inhibited p53 protein activation. In addition, OF can inhibit apoptosis by inhibiting the release of AIF in the mitochondria.

Caspase-cascade is an important pathway mediating apoptosis, which is essential for the initiation and execution of apoptosis. OF inhibited caspase pathway apoptosis by reducing the expression levels of caspase-3 and caspase-9 in truncated forms in HT22 cells treated with glutamate. PARP is a transcription factor activated by caspase signaling and is involved in numerous cellular processes such as DNA repair and apoptosis [22]. OF inhibits DNA damage by inhibiting the expression level of the cleaved form PARP caused by glutamate.

OF has beneficial effects such as antioxidant and anticancer effects, but the effect of OF on cell survival and cell death due to glutamate toxicity was not yet known. Therefore, we investigated

the expression of various proteins that mediate mitochondria in glutamate-induced HT22 cells. OF can help you find drugs that can prevent and treat neurodegenerative diseases such as Alzheimer's disease.

5. CONCLUSION

High concentrations of glutamate cause neuronal cell death, leading to multiple neurological diseases. Therefore, finding a natural compound that inhibits apoptosis may be helpful for neuroprotection. OF, a lignin, has been extracted from *Schisandra chinensis* and *Myristica fragrans*. OF extracted from *Schisandra chinensis* has neuroprotective effects. However, the mechanisms underlying OF's ability to inhibit neuronal cell death mechanisms are not understood. We explored the neuroprotective effects of OF on glutamate-induced HT22 neuronal cells. OF inhibited apoptosis by regulating the expression of mitochondrial-mediated proteins, protecting against mitochondrial membrane disruption and inhibiting apoptosis in glutamate-induced HT22 cells. Furthermore, OF inhibited the expression of cleaved caspase pathway proteins and inhibited apoptosis and DNA cleavage. These findings suggest that OF treatment protects neuronal cells against apoptosis.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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