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Protective Effect of Extracts from *Euryale ferox* against Glutamate-induced Cytotoxicity in Neuronal Cells

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Abstract – Oxidative stress plays an important role in neuronal cell death, which is associated with neurodegenerative conditions such as Alzheimer's and Parkinson's disease. This study evaluated the neuroprotective effect of *Euryale ferox* (EF) extracts against glutamate-induced cytotoxicity in hybridoma N18-RE-105 cells. Specifically, neuroprotective effects of methanol and ethanol extracts were evaluated by the MTT reduction assay. The ethanol extracts of EF displayed dose dependent protection against neuronal cell death induced by 20 mM of glutamate. Furthermore, the ethanol extracts of EF was sequentially fractionated with hexane, diethyl ether, ethyl acetate, and water layer according to degree of polarity. The hexane fractions exhibited neuroprotective effect against glutamate-stressed N18-RE-105 cells. Overall, results suggest that EF extracts can potentially be used as chemotherapeutic agents against neuronal diseases.
Keywords – Oxidative stress, Neuroprotective, *Euryale ferox*, Glutamate, N18-RE-105 cells

Introduction

Oxidative stress has long been associated with the development of neurodegenerative conditions such as Alzheimer's, Parkinson's, and Huntington's disease, whereby nerve cells are unable to deal with an imbalance of reactive oxygen species (ROS) generated as by products of normal and irregular metabolic processes (Ito *et al*., 2006; Choi *et al*., 2007). This imbalance is caused by deregulation of electron transport, activation of certain enzymes, and/or the loss of antioxidants which results in the accumulation of ROS such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals (Hou *et al*., 2003; Kazushi *et al*., 2007). Accumulation of ROS results in damage to major cellular macromolecules including lipids, proteins, and nucleic acids (Jeon *et al*., 2006).

Glutamate is an excitatory amino acid, and one of the major neurotransmitters in the central nervous system (CNS) of mammals (Greenwood and Connolly, 2007). Studies have shown that glutamate and related excitatory amino acid analogs cause specific neurodegenerative disorders in the brain of experimental animals, in primary cultures of brain neurons, and in cultured neuronal cell lines (Claudia and Catarina, 1997). Glutamate-induced neurotoxicity has been suggested to precipitate oxidative stress in the brain via two mechanisms (Monaghan *et al*., 1989). The first one is thought to be mediated through three types of excitatory amino acid receptors, while for the second mechanism it has been proposed that elevated levels of extracellular glutamate inhibit cystine uptake, which causes a decrease in intracellular antioxidant glutathione (GSH) and accumulation of ROS (Tan *et al*., 1998).

In-depth understanding of oxidative stress and its underlying molecular mechanisms are critical to the development of therapeutic agents. Currently, there are no cures for neurodegenerative disorders, with most available therapies focusing on symptomatic treatment only. Accordingly, new approaches are being explored. Unfortunately, current therapeutic modalities for advanced diseases are limited. Some patients have turned to alternative options such as herbal treatments, which are extensively used by some cultures throughout the world (Yoon *et al*., 2007; Lee *et al*., 2008). The popularity of herbal medicines has gained momentum as their pharmacological applications and low toxicity are being discovered through extensive testing (Kodach *et al*., 2006).

In this study, we screened 435 varieties of herbal medicines and determined that *Euryale ferox* (EF) extracts exhibited neuroprotective effects against glutamate-induced cytotoxicity in N18-RE-105 cells. EF is edible

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and fried seeds or puffs are extremely nutritious (Das *et al*., 2006). EF is widely used in traditional oriental medicine to treat a variety of diseases, including chronic diarrhea, excessive leucorrhea, kidney problems, and hypofunction of the spleen (Row *et al*., 2007). Recently, it has also been shown to reduce myocardial ischemic reperfusion injury, and possess immunostimulant activity (Puri *et al*., 2000). However, the neuroprotective effects of EF are yet to be assessed in detail.

The purpose of this research was to conduct a baseline study where the neuroprotective effects of EF extracts are examined against glutamate-stressed N18-RE-105 cells.

Experimental

Materials and Preparation of Various Extracts from EF – *Euryale ferox* (EF) was obtained from Kumkang Pharm Co., Ltd., Masan, South Korea. Ten grams of EF were extracted with 100 mL of methanol and ethanol for 3 days at room temperature and filtered through a Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol and ethanol solvent were then removed by evaporation, and the dried extracts were obtained. The extracts were dissolved in DMSO and stored at −20 °C. Furthermore, the ethanolic extract was partitioned consecutively in a separating funnel using solvents of increasing polarity: hexane, diethyl ether, ethyl aetate, and water. The solvent extracts were the dissolved in DMSO and stored at −20 °C. L-Glutamic acid (monosodium salt hydrate) was purchased from Sigma-Aldrich (St Louis, MO) and dissolved in sterilized distilled water at stock concentration of 1 M. All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Cell Culture and Treatments – Hybridoma N18-RE-105 cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). The cells were maintained in DMEM medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (HS), 1 × HAT supplement, penicillin (100 U/mL), streptomycin (100 mg/mL), and 3.7 g/mL $NaHCO_3$ in a humidified incubator set at 37 °C and 5% CO_2. The extracts of EF were added to the cell culture medium such that the DMSO made up less than 0.5% of the total volume of the culture.

Glutathione (GSH) Measurement – To measure GSH content, cells were suspended in 5% *meta*-phosphoric acid at a concentration of 1×10^5 cells/mL. This suspension was ultrasonic processor and centrifuged at 3000 × g, 4 °C for 10 min, its supernatant was used for GSH measurement according to the manufacturer's instruction. The GSH content of the supernatant was determined with a GSH assay kit (Oxis International Inc., Foster city, OR) (Lonigro *et al*., 2000; Wen *et al*., 2002). The GSH content was expressed as μM of protein or percent of the control.

Morphological Analysis – N18-RE-105 cells were seeded at 1×10^5 cells/mL in 6-well plate and incubated in DMEM medium in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were pretreated with various concentrations of EF extracts. After incubation for 30 min, cells were treated with 20 mM glutamate for 24 h. The cellular morphology was observed using an inverted phase-contrast microscope (Nikon, Tokyo, Japan) at 100 × magnification.

MTT Reduction Assay for Cell Viability – Cell viability was measured with blue formazan that was metabolized from colorless tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide] (MTT) (Sigma) by mitochondrial dehydrogenase, which are active only in live cells. N18-RE-105 cells were preincubated in 96-well plates at a density of 5×10^4 cells/mL for 24 h. Cells with various concentrations of EF extracts were treated with glutamate of 20 mM for 24 h. After incubation, MTT reagent (5 mg/mL) was added to each of the wells, and the plate was incubated for an additional about 1 h at 37 °C. The intracellular formazan product was dissolved in 100 μL of DMSO. For MTT reduction assay, the optical density (OD) of each well was then measured at 540 nm wavelength using an ELISA reader (Model 680, BioRad, Hercules, CA). OD values from untreated control cells were designated 100% as a standard.

Lactate Dehydrogenase (LDH) Release Assay – Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). An LDH release assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). N18-RE-105 cells were seeded in 96-well plates (5×10^4 cells/mL), cultured overnight in the presence of glutamate and treated with various concentrations of EF extracts for 24 h, as described above. The LDH release assay reaction was initiated in a 96 well plate by mixing 50 μL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μL. A colorimetric assay was performed, in which the amount of formazan salt was proportional to the level of LDH activity in the sample. The intensity of the resultant red color measured at 540 nm was therefore proportional to LDH activity.

Statistical Analysis – All data presented were the means of three determinations. Data were analyzed using the SPSS package for Windows (Version 14.0; Chicago, IL) and evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test. The differences were considered significant at $p < 0.05$.

Results and Discussion

Glutathione (GSH) Levels in the Presence of Glutamate – GSH is a major antioxidant, high level of which is essential for protecting cells from oxidative stress, while its depletion has been shown to intensify lipid peroxidation and predispose cells to oxidant damage (Penugonda *et al*., 2005). Astrocytes, which are star-shaped glial cells in the brain and spinal cord, exhibit high concentrations of GSH (Buchkman *et al*., 1993). As mentioned previously, glutamate is the most abundant excitatory neurotransmitter in the CNS and linked to development of neurodegenerative disorders. This study demonstrates that GSH plays an important role in modulating glutamate-induced oxidative damage in neuronal cells. It has been proposed that high concentration of glutamate inhibits cystine uptake, resulting in the loss of intracellular GSH (Wang *et al*., 2006). The intracellular levels of GSH were determined in N18-RE-105 cells incubated with various concentration of glutamate (5, 10, and 20 mM) for 24 h. Results showed that glutamate was drastically reduced the GSH depletion. When cells were exposed to 5, 10 and 20 mM of glutamate, GSH levels were calculated to be 5.9, 4.7 and 4.4 μM GSH/mg protein, respectively, showing a marked decline in comparison with control cells (data not shown). These data indicated that depletion of intracellular GSH may be due to glutamate-induced oxidative stress.

EF Extracts Protect the N18-RE-105 cells from Glutamate-induced Cytotoxicity – In this study, we attempted to investigate the neuronal protection effects of EF on cell viability in glutamate-stressed N18-RE-105 cells. The cells were incubated with EF extracts and 20 mM of glutamate, while determination of cell viability was determined via MTT reduction assay. As shown in Fig. 1, cells treated for 24 h with 20 mM of glutamate had reductions in cell viability of 40% compared to the control. However, after 24 h of exposure to various EF extracts, cell viability increased from 40 to 60% when compared to the viability of glutamate-stressed N18-RE-105 cells. Most significantly, increased cell viability was observed in ethanolic extracts from EF (Fig. 1).

To characterize the effects of ethanolic extracts from EF (EFE) on cell viability in glutamate-stressed N18-RE-105 cells, cells were incubated with EFE and 20 mM of glutamate, while morphological alterations were verified via a phase-contrast microscope. As shown in Fig. 2, the control group exhibited round cell bodies with clear edges and fine dendritic networks. However, after exposure to 20 mM of glutamate for 24 h, many cell showed cytoplasmic shrinkage, either detaching from each other or floating in the medium. In contrast, cultures exposed to glutamate in the presence of EFE appeared remarkably preserved, indicating that EF offered protective effect against oxidative stress (Fig. 2). To further investigate the protective effects of EFE, we performed the LDH release assay. Here, the cells were exposed to various concentrations of EFE and glutamate for 24 h. As expected, EFE reduced cell damage in a dose-dependent manner,

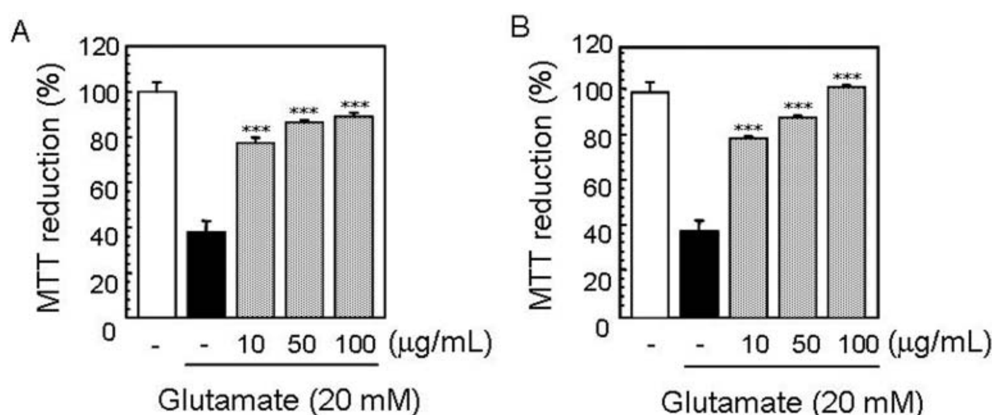

Fig. 1. *Euryale ferox* (EF) extracts protected N18-RE-105 cells from glutamate-induced cytotoxicity. The cells pretreated for 30 min with various concentrations (10, 50, and 100 μg/mL) of extracts from EF (**A**: methanol extracts, **B**: ethanol extracts). The cells were then treated with 20 mM of glutamate for 24 h. After MTT reduction assay, the MTT reduction rate (means ± S.D. of triplicate determination) were calculated by setting each of control survival rate. ***significantly different from glutamate-treated control group (p<0.001).

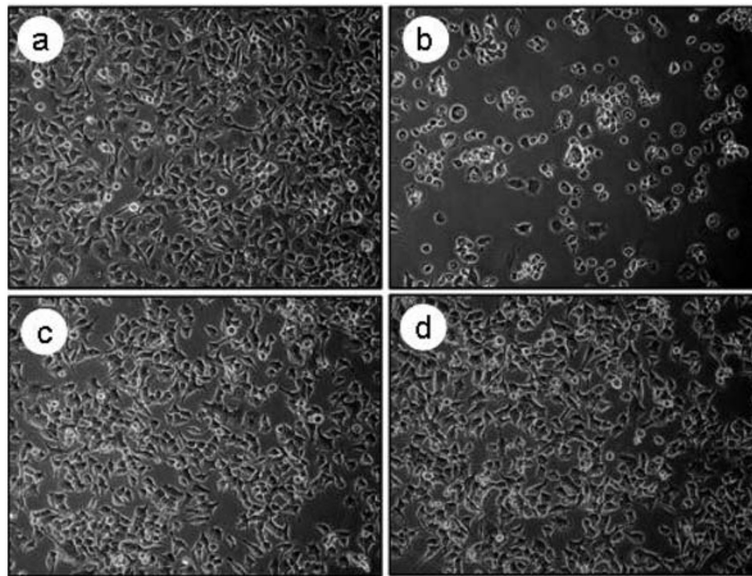

Fig. 2. Analysis of morphological changes by ethanolic extracts of EF (EFE) in N18-RE-105 cells. The cells were exposed to various concentrations of EFE and morphological changes were monitored for 24 h (**a**: control, **b**: 20 mM glutamate, **c**: 20 mM glutamate/50 μg/mL EFE, **d**: 20 mM glutamate/100 μg/mL EFE). Photographs were taken with a phase-contrast microscope at 100 × magnification.

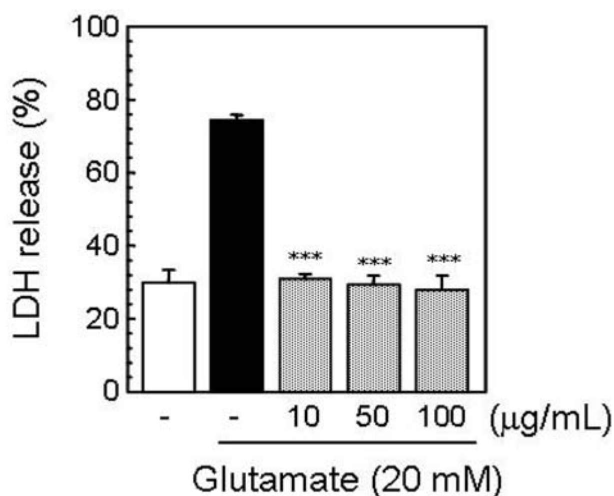

Fig. 3. Protective effect of EFE on LDH leakage in N18-RE-105 cells by glutamate. Cell viability was measured using the LDH release assay in cells exposed to EFE under glutamate-stressed conditions. N18-RE-105 cells pretreated for 30 min with various concentrations of extracts from EFE. The cells were then treated with 20 mM of glutamate for 24 h. Data were normalized to the activity of LDH release from vehicle-treated cell (100%). ***significantly different from glutamate-treated control group ($p < 0.001$).

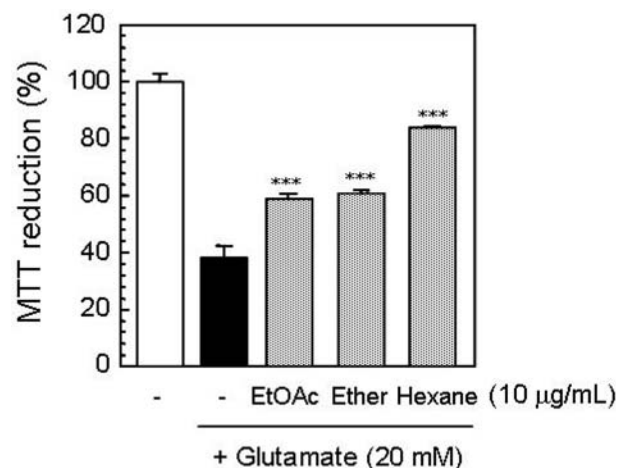

Fig. 4. Protective effect of each fraction in glutamate-stressed N18-RE-105 cells. The cells pretreated for 30 min with 50 and 100 μg/mL of fractions from EFE. The cells were then treated with 20 mM of glutamate for 24 h. After MTT reduction assay, the MTT reduction rate (means ± S.D. of triplicate determination) were calculated by setting each of control survival rate. ***significantly different from glutamate-treated control group ($p < 0.001$).

this being evident by a 40% decrease in LDH release from the glutamate-stressed N18-RE-105 cells (Fig. 3).

Accordingly, our data show that glutamate-induced cytotoxicity was significantly prevented by treatment with ethanol extract, as indicated by morphological observation and LDH release assay. These results indicate that EF provided significant protection to N18-RE-105 cells from glutamate-induced cytotoxicity.

The Protective Effect of Hexane Fractions from EFE on Glutamate-induced Cytotoxicity – EFE, which showed the strongest neuroprotective effect, was further fractionated sequentially with hexane, diethyl ether, and ethyl acetate according to degree of polarity. We next

examined the effects of EFE fractions on glutamate-stressed N18-RE-105 cells, which were determined by measuring the MTT reduction assay. As showed Fig. 4, cells treated for 24 h with 20 mM of glutamate had reductions in cell viability of 40% compared to control. However, after 24 h of exposure to 10 µg/mL of fraction, cell viability increased from 20 to 40% when compared to viability in glutamate-stressed cells. In particular, the highest cell viability (80%) was achieved in 10 µg/mL of hexane fraction (Fig. 4). Such difference in compositions and contents of phytochemicals can cause different neuroprotective effects among fractions. Ultimately, hexane fractions of EFE exhibited the highest potent protective effect on viability of N18-RE-105 cells. Overall, results indicate that non-polar property materials of EF were greatly prevented in the face of glutamate-induced cytotoxicity.

During the present investigation, the seeds are also of great traditional medicinal value. In conclusion, extracts from EF could ameliorate glutamate-induced oxidative stress, which provided higher viability to N18-RE-105 cells. Additionally, we have provided evidence which appear to support the potential therapeutic benefit of EF in the treatment of neuropathological conditions.

Acknowledgments

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