Antioxidant activity of extracts from *Euryale ferox* seed

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Abbreviations: DPPH, 1, 1-diphenyl-2-picrylhydrazyl; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase

Abstract

Euryale ferox has been widely used in traditional oriental medicine to treat a variety of illness. However, very little is known about the cellular actions by which this plant mediates its therapeutic effects. Various aspects of antioxidant activity were evaluated in total extracts and fractions derived from *Euryale ferox*. Total extracts (IC₅₀ 5.6 µg/ml) showed relatively high level radical scavenging activity toward 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and also enhanced viability of Chinese hamster lung fibroblast (V79-4) cells under exposure to oxidative agents. Upon further fractionation, the highest levels of DPPH radical scavenging and lipid peroxidation inhibitory activities were found in the ethyl acetate and butanol fractions. The ethyl acetate fractions, the butanol fractions, and total extracts of *Euryale ferox* also dose-dependently enhanced the activities of superoxide dismutase, catalase and glutathione peroxidase in V79-4 cells. Of these three antioxidant enzymes, glutathione peroxidase activity was most strongly induced. Taken together, our findings show that *Euryale ferox* contains a significant antioxidant activity and that specific components in the ethyl acetate and butanol fractions may play an important role in mediating these antioxidant properties.

Keywords: Antioxidant activity; Antioxidant enzymes; *Euryale ferox*; Lipid peroxidation; Radical scavenging

Introduction

Oxidative modification of DNA, proteins, lipids and small cellular molecules by reactive oxygen species (ROS)

plays a role in a wide range of common diseases and age-related degenerative conditions (for review see Finkel and Holbrook, 2000). Disease states which are known to be affected by ROS include cardiovascular disease (Witztum, 1993), neurodegenerative diseases such as Alzheimer's disease (Frlch and Riederer, 1995), mutations and cancer (Borek, 1991).

To protect themselves against toxic free radicals and other ROS, cells have developed a variety of antioxidant defenses. These include enzymes such as superoxide dismutase (SOD), which dismutates superoxide; catalase (CAT), which converts hydrogen peroxide into water and oxygen, and glutathione peroxidase (GPX), which destroys toxic peroxides. Other molecules that can counteract ROS include glutathione, flavonoids, ubiquinol-10, glucose, and albumin. External sources of antioxidative protection include vitamins C, E, A, and pro-vitamin A, as well as the minerals selenium and zinc (Halliwell and Gutteridge, 1998).

Phytochemicals from natural products are also important in that they provide further protection against oxidative damage from free radicals. Numerous natural antioxidants have been studied in fruits and vegetables (Cao *et al.*, 1996; Wang *et al.*, 1996; Brown and Rice-Evans, 1998), oilseeds (Deiana *et al.*, 1999), herbs (Kitts *et al.*, 2000), tea (Roedig-Penman and Gordon, 1997), berry crops (Wang and Jiao, 2000), propolis (Banskota *et al.*, 2000), *Ginkgo biloba* (Gohil *et al.*, 2000) and *Panax ginseng* (Keum *et al.*, 2000). The plant used in this study, *Euryale ferox* (Nymphaeaceae), was selected based on its traditional use in Chinese medicine. *Euryale ferox* has been considered to be a tonic and has also been recommended for the treatment of pyoderma, hernia and leukorrhea. Glycosylsterols and cerebrosides were identified in *Euryale ferox* by nuclear magnetic resonance and mass spectroscopic methods (Zhao *et al.*, 1989, 1994). In general, very little research has been done on biological activity of this plant. Puri *et al.* (2000) investigated the effect of certain plant products on cell-mediated immunity (CMI) and humoral immunity when administered to mothers after childbirth. These investigators found that the total extracts of *Euryale ferox* stimulated humoral immunity to a greater extent than CMI.

In the present study, the antioxidative actions of *Euryale ferox* were evaluated by their abilities to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, to protect cell viability and to inhibit the formation of lipid peroxides. The effect of *Euryale ferox* on the activity of antioxidant enzymes such as SOD, CAT and GPX was also investigated.

Materials and Methods

Preparation of plant extracts

Euryale ferox (100 g) was extracted at 80°C in 70% methanol for 3 h. The extract was then filtered and the filtrate was concentrated with a vacuum rotary evaporator (Eyela, Japan) under low pressure. The residue was freeze-dried (Ilsin, Korea) and stored at -70°C. The total extracts were extracted with n-hexane, dichloromethane, ethyl acetate and n-butanol, in a stepwise manner, using separatory funnels. Fractions were collected and the solvents were removed by rotary evaporation. The powder was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give final concentrations of total extracts and fractions ranging from 0.8 to 100 µg/ml.

Cell culture

Chinese hamster lung fibroblast, V79-4 (ATCC CCL-93) cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂ / 95% O₂. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 5% fetal bovine serum (FBS, BioWhittaker, USA), 100 µg/ml of streptomycin, 100 unit/ml of penicillin (Gibco BRL) and 2 mM L-glutamine (Gibco BRL).

DPPH radical scavenging activity

In order to measure the antioxidant activity of *Euryale ferox*, the DPPH free radical scavenging assay was carried out according to the procedure described by Blois *et al.* (1958). Total extracts and fractions of *Euryale ferox* at various concentrations (0.8, 4, 20, and 100 µg/ml) were added to a 1.5 X 10⁻⁴ M solution of DPPH (Sigma, St. Louis, MO, USA) in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm, and the radical scavenging activity was obtained from the following equation:

$$\text{Radical scavenging activity (\%)} = \{(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}}\} \times 100$$

The antioxidant activity of *Euryale ferox* was expressed as IC₅₀. The IC₅₀ value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50%.

Nuclear staining with propidium iodide

Cells used in this study were constantly observed under an inverted phase-contrast microscope (Olympus). V79-4 cells were placed in a 60 mm culture plate at a concentration of 1.2 × 10⁵ cells/ml. Sixteen hr after plating, cells were treated with 100 µg/ml of *Euryale ferox* extracts and after 1 h of further incubation, 1 mM H₂O₂ was added to the culture. After 24 h, cells were harvested by centrifugation and washed with PBS twice.

Cells were then resuspended in PBS at a concentration of 4 × 10⁵ cells/ml and placed onto a microscope slide using a cytospin (Hanil, Korea), which was left at room temperature for dryness. Cells were fixed with cold ethanol in the dark. The fixed cells were washed with PBS and stained with 2.5 µg/ml of propidium iodide and DNase-free RNase (100 µg/ml). The morphology of the stained cells was examined under a fluorescence microscope (E800, Nikon, Japan).

Cell viability

Cell viability was estimated by the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen *et al.*, 1989). V79-4 cells were seeded in a 96 well plate at a concentration of 1.2 × 10⁵ cells/ml. Sixteen h after plating, cells were treated with various concentrations of total extracts, ethyl acetate fractions, or butanol fractions (4, 20, and 100 µg/ml) and 1 h later 1 mM H₂O₂ was added to the culture. Cells were incubated for an additional 24 h at 37°C. During the last 4 h, cells were incubated with 20 µl of MTT stock solution (5 mg/ml) in 200 µl medium at 37°C. Samples were then extracted with acidic isopropanol and the absorbance was measured with an ELISA reader (Bio-Rad, USA) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as mean percentage of viable cells as compared to the respective control cultures.

Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa *et al.* (1979). The cells were exposed to total extracts, ethyl acetate fractions or butanol fractions of *Euryale ferox* at various concentrations (4, 20 and 100 µg/ml) for 60 min, followed by 1 mM H₂O₂ for 60 min. Cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. Samples containing 100 µl of cell lysates were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4.0 ml with distilled water and heated to 95°C for 120 min. After cooling to room temperature, 5.0 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 1500 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. Inhibitory activity towards lipid peroxidation was expressed as IC₅₀.

Assays for antioxidant enzymes

The cells were treated with 4, 20 and 100 µg/ml of total extracts, ethyl acetate fractions or butanol fractions of

Euryale ferox for 60 min. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay, as described below. The method of Bradford (1976) was used to determine protein concentrations. Results are expressed as relative percentage of enzyme activity per mg protein compared with corresponding control cultures.

Superoxide dismutase (SOD) activity

SOD activity was assayed by the nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). NBT is reduced to blue formazan by O_2^- , which has a strong absorbance at 560 nm. The presence of SOD inhibits this reaction. The cells were homogenized in 0.05 M sodium carbonate buffer, pH 10.2. The assay mixture consisted of 0.05 M sodium carbonate buffer, pH 10.2 containing 3 mM xanthine, 0.75 mM NBT, 3 mM EDTA, 1.5 mg/ml BSA and 50 μ l of homogenate. The reaction was initiated by the addition of 50 μ l of xanthine oxidase (0.1 mg/ml) and incubated for 30 min at room temperature. The reaction was stopped by adding 6 mM copper (II) chloride and centrifuged at 1500 rpm for 10 min. The absorbance of blue formazan at 560 nm was then determined in the supernatants.

Catalase (CAT) activity

The reaction mixture contained 12 μ l of 3% (v/v) H_2O_2 and 100 μ l of cell lysates in 50 mM phosphate buffer (pH 7.0) at a final volume of 1.0 ml. Samples were incubated for 2 min at 37°C and the absorbance of the samples were monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of H_2O_2 (Carrillo *et al.*, 1991).

Glutathione peroxidase (GPX) activity

GPX was assayed by the method of Paglia and Valentine (1967). The reaction mixture contained 0.1 M phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM glutathione (GSH), 1 mM NaN_3 , 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell lysates. After incubation for 10 min at 37°C, H_2O_2 was added to each sample at a final concentration of 1 mM. GPX activity was measured as the rate of NADPH oxidation at 340 nm.

Results

DPPH radical scavenging activity of total extracts and fractions of *Euryale ferox*

The antioxidant activities of *Euryale ferox* extracts were evaluated by DPPH free radical scavenging activity, by the protective effect on cell viability, and by inhibition of lipid peroxidation. The DPPH radical scavenging activities of total extracts and fractions are shown in

Figure 1. DPPH radical scavenging activity of total extracts and fractions of *Euryale ferox*. Each experiment was performed at least 3 times and data are expressed as average percent change from control $\pm$ S.D. ● -Total extracts, ○ -Hexane fractions, ▲ -Dichloromethane fractions, △ -Ethyl acetate fractions, ■ -Butanol fractions and -Water fractions.

Figure 2. Protective effect of total extracts, ethyl acetate and butanol fractions of *Euryale ferox* against H_2O_2 -induced oxidative damage in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average percent change from control $\pm$ S.D. ● -Total extracts, △ -Ethyl acetate fractions and ■ -Butanol fractions.

Figure 1. Total extracts of *Euryale ferox* showed relatively high DPPH radical scavenging activity, with an average IC_{50} value of 5.6 μ g/ml. Relative to other fractionated samples, the ethyl acetate and butanol fractions had the highest DPPH radical scavenging activity, with IC_{50} values of 1.5 and 2.2 μ g/ml, respectively.

Protective effect of *Euryale ferox* extracts on cell survival

We measured the protective effect of total extracts, ethyl acetate fractions and butanol fractions on cell survival in H_2O_2 treated cells. Cells were treated with extract samples for 1 h prior to the addition of H_2O_2 . Control cells were treated with H_2O_2 in the presence of vehicle

Figure 3. Protective effect of total extracts of *Euryale ferox* against H_2O_2 -induced apoptosis in V79-4 cells. Cellular morphological changes were observed using a fluorescence microscope at the magnitude of 200X. Photomicrographs of V79-4 cells treated with (A) vehicle only (B) 1 mM H_2O_2 (C) 100 μ g/ml *Euryale ferox* extracts for 1 h prior to the addition of H_2O_2 .

only. The final concentration of DMSO was below 0.1% in this study. There was no cyto-protective effect of DMSO at this concentration. The relative cell survival was determined 24 h later by the MTT assay. As shown in Figure 2, treatment with *Euryale ferox* extracts induced a dose-dependent increase in cell survival. Interestingly, treatment with the ethyl acetate fractions dramatically increased the cell survival of H_2O_2 -treated cells. At a dose of 100 μ g/ml, the ethyl acetate fractions actually stimulated cell survival to a level higher than that observed in control cells.

Reduction of H_2O_2 -induced nuclear fragmentation by *Euryale ferox* extract treatment

In order to analyze the protective effect of *Euryale ferox* extracts on H_2O_2 -induced apoptosis, we fluorescently stained nuclei of V79-4 cells treated with H_2O_2 alone or with both *Euryale ferox* extracts and H_2O_2 . As shown in Figure 3, control cells revealed intact nuclei, but cells treated with 1 mM of H_2O_2 showed significant nuclear fragmentation. However, when we treated cells with *Euryale ferox* extracts 1 h prior to H_2O_2 treatment, dramatic reduction in nuclear fragmentation was observed.

Effect of *Euryale ferox* extracts on lipid peroxidation

We also tested the ability of total extracts, ethyl acetate fractions, and butanol fractions to inhibit lipid peroxidation in H_2O_2 -treated V79-4 cells. Pretreating cells with total extracts modestly reduced lipid peroxidation with an IC_{50} value of 20.5 μ g/ml (Figure 4). The ethyl acetate and butanol fractions were found to have high levels of inhibitory activity, with IC_{50} values of 5.1 and 6.3 μ g/ml, respectively.

Effect of total extracts and fractions of *Euryale ferox* on SOD, CAT and GPX

In order to investigate whether these antioxidant properties of *Euryale ferox* extracts are mediated by an increase in antioxidant enzymes, we measured SOD,

Figure 4. Lipid peroxidation inhibitory activity of total extracts, ethyl acetate and butanol fractions of *Euryale ferox* against H_2O_2 -induced oxidative damage in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average percent change from control $\pm$ S.D. ●-Total extracts, $\triangle$ -Ethyl acetate fractions and ■-Butanol fractions.

CAT and GPX activities in V79-4 cells treated with total extracts or fractionated samples (Figure 5). Treatment with total extracts at doses of 4, 20 and 100 μ g/ml increased SOD activity by 9, 20 and 24%, respectively (Figure 5A). Cells treated with ethyl acetate fractions of *Euryale ferox* exhibited even higher levels of SOD activity (34% increase at 100 μ g/ml). Cells treated with 100 μ g/ml of the butanol fractions exhibited a 26% increase in SOD activity. All samples tested dose-dependently increased SOD activity. Of the samples tested, the ethyl acetate fraction most strongly activated SOD within the concentration range used. The activity of SOD in control cell untreated with extracts was 24.9 ± 1.5 U/mg protein. CAT activity was concomitantly increased when cells were treated with 100 μ g/ml of total extracts (23% increase), the ethyl acetate fractions (32% increase), and the butanol fractions (26% increase) (Figure 5B). Each of the samples dose-dependently increased CAT activity over a range of 4 to 100 μ g/ml. The ethyl acetate fractions most strongly

Figure 5. Effect of *Euryale ferox* on SOD (A), CAT (B) and GPX (C) activity in V79-4 cells. Each experiment was performed at least 3 times and data are expressed as average enzyme unit per mg protein from control $\pm$ S.D. T, total extracts; E, ethyl acetate fractions; B, butanol fractions. Black, gray and white bars indicate 4, 20 and 100 mg/ml of samples, respectively.

increased in CAT activity. The CAT activity of control cells (not treated with extracts) was 14.9 ± 1.9 U/mg protein. GPX activity was also dose-dependently increased by each of the treatment conditions (Figure 5C). In fact, *Euryale ferox* extracts activated GPX more strongly than SOD and CAT activities. At a dose of 100 μ g/ml, total extracts, ethyl acetate fractions, and butanol fractions activated GPX by 50, 65 and 58%, respectively. The activity of GPX in control cells untreated with extracts was 11.7 ± 1.3 U/mg protein.

Discussion

Reactive oxygen species (ROS) are unwanted metabolic by-products of normal aerobic metabolism under high levels of O_2 pressure. High levels of ROS create oxidative stress, which leads to a variety of biochemical and physiological lesions. Such cellular damage often impairs metabolic function and leads to cell death (Finkel and Holbrook, 2000). Cells are protected from ROS-induced damage by a variety of endogenous ROS scavenging enzymes, chemical compounds and natural products. Recently there has been increasing interest in the therapeutic potential of natural medicinal plants as antioxidants in reducing such free radical-induced tissue injury, suggesting that many plants have antioxidant activities that could be therapeutically useful. Kim *et al.* (1996) found ginsenoside Rb₁, a major saponin from *Panax ginseng*, was able to prevent lipid peroxidation. Hepatic lipid peroxide contents were examined in rats treated with extract of *Kalopanax pictus* stem bark (Choi *et al.*, 2001). For this reason, we have characterized the antioxidant properties of *Euryale ferox*, a natural medicinal plant which has been previously reported to have immunostimulatory effects (Puri *et al.*, 2000).

Total extracts of *Euryale ferox* significantly increased DPPH free radical scavenging and inhibited lipid

peroxidation in a dose-dependent manner. Total extracts also enhanced viability in V79-4 cells exposed to H_2O_2 . The protective effect on H_2O_2 -induced apoptosis was observed. Cells exposed to H_2O_2 exhibit distinct morphological features of programmed cell death, such as nuclear fragmentation. Cells which were pretreated with *Euryale ferox* extracts did not show nuclear fragmentation. Their microscopic morphology was very similar to that of the control cells. These data suggested that *Euryale ferox* protected cells from H_2O_2 -induced apoptosis. Detailed mechanistic study of apoptosis inhibitory effect of *Euryale ferox* is now in progress.

Euryale ferox extracts were subjected to a series of organic separation steps and the various fractions were assayed for DPPH radical scavenging activity. These experiments suggested that specific constituents in the ethyl acetate and butanol fractions contribute to the antioxidant activity of *Euryale ferox*. This hypothesis is further supported by the finding that the ethyl acetate and butanol fractions also strongly inhibit lipid peroxidation. Lipids are considered to be extremely susceptible to the oxidation by ROS. In particular, polyunsaturated fatty acids located in cellular membranes and in blood are highly prone to attack, leading to the generation of lipid peroxides (Halliwell and Gutteridge, 1998). Lipid peroxidation is an autocatalytic process which is a common cause of cell death. Therefore, inhibition of lipid peroxidation has been used as an important index of antioxidant activity. In the case of cells treated with the ethyl acetate fractions, cell viability was even higher than in control cells. These data suggest that components within the ethyl acetate fractions may have inherent properties that enhance cell viability, in addition to an anti-oxidative protective effect.

We also observed that treating cells with total extracts, ethyl acetate fractions, and butanol fractions of *Euryale ferox* increased the activity of all antioxidant enzymes examined, including SOD, CAT and GPX. These enzymes are modulated in various diseases

caused by free radical attack (Halliwell and Gutteridge, 1998). Thus, maintaining the balance between the rate of generation of radicals and scavenging of radicals is an essential part of biological homeostasis. It is of particular interest to note that SOD catalyzes the breakdown of O_2^- to O_2 and H_2O_2 , prevents formation of OH^- and has thereby been implicated as an essential defense against the potential toxicity of oxygen. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates H_2O_2 , which needs to be further scavenged by CAT and GPX. Extracts from *Euryale ferox* activated GPX to a greater extent than the other enzymes examined in this study. This appears that can be effectively scavenged H_2O_2 formed by SOD actions. Taken together, these results also suggest that the antioxidant activity of *Euryale ferox* may be due to degradation of H_2O_2 and other peroxides.

In conclusion, extracts from *Euryale ferox* have high levels of DPPH radical scavenging activity, inhibit lipid peroxidation, promote cell viability, protect H_2O_2 -induced apoptosis and enhance the effects of various antioxidant enzymes. These findings strongly suggest that *Euryale ferox* has antioxidant activity. Among various fractionated samples of *Euryale ferox*, the ethyl acetate fractions and butanol fractions exhibited relatively high levels of antioxidant activity. Constituents within these fractions may play an important role as natural antioxidants. Further studies are aimed at isolating and identifying these active constituents and elucidating the mechanisms by which *Euryale ferox* regulates cellular metabolism.

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