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Induction of Antioxidant Enzymes in Phloroglucinol Treated Cells

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ABSTRACT : We investigated the cytoprotective effect of phloroglucinol, which was isolated from *Ecklonia cava* (brown seaweed), against oxidative stress induced cell damage in Chinese hamster lung fibroblast (V79-4) cells. Phloroglucinol was found to scavenge intracellular reactive oxygen species (ROS) generated by γ-ray radiation. In addition, phloroglucinol inhibited cell damage induced by radiation through scavenging ROS. Phloroglucinol increased the superoxide dismutase and glutathione peroxidase activity. Taken together, the results suggest that phloroglucinol protects V79-4 cells against oxidative damage by enhancing the cellular antioxidant enzymes activity.

Key words : **Phloroglucinol, oxidative stress; antioxidant enzymes**

Introduction

Ecklonia cava, which is a brown alga belong to the family Laminariaceae, is abundant marine plant growing in water depth 5-20 m in the coast of Jeju Island in Korea. Recently, *Ecklonia* species have been reported to exhibit radical scavenging activity (Kang *et al.*, 2003a; 2004), anti-plasmin inhibiting activity (Fukuyama *et al.*, 1989, 1990), antimutagenic activity (Lee *et al.*, 1996, 1998; Han *et al.*, 2000), bactericidal activity (Nagayama *et al.*, 2002), HIV-1 reverse transcriptase and protease inhibiting activity (Ahn *et al.*, 2004), and tyrosine inhibitory activity (Park *et al.*, 2000). Phlorotannin components, which are oligomeric polyphenolic of phloroglucinol unit, were identified to be responsible for the biological activities in *Ecklonia* species. Phlorotannin components in *Ecklonia* species include phloroglucinol (1,3,5-trihydroxybenzene), eckol (a closed-chain trimer of phloroglucinol), triphlorethol-A (an open-chain trimer of phloroglucinol), phlorofucofuroeckol (a pentamer), 6,6'-bieckol (a hexamer), and dieckol (a hexamer). During the investigation of cytoprotective components against oxidative stress damaged cells in *E. cava*, phloroglucinol compound possessed cytoprotective effect.

Reactive oxygen species (ROS) are associated with tissue damage and are the contributing factors for inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes (Cooke *et al.*, 1997; Darley-Usmar and Halliwell 1996; Farinati *et al.*, 1998; Laurindo *et al.*, 1991; Nakazono *et al.*, 1991; Palinski *et al.*, 1995; Parthasarathy *et al.*, 1992). For cytoprotection against ROS, cells have developed a variety of antioxidant defense mechanisms. In the present study, we have investigated the protective effect of phloroglucinol on cell damage induced by oxidative stress and the possible mechanism of cytoprotection in terms of antioxidant enzymes.

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Materials and Methods

Preparation of Phloroglucinol

The dried *Ecklonia cava* (4 kg), collected from Jeju Island in Korea, was immersed in 80% methanol at room temperature for 2 days. The aqueous methanol was removed *in vaccuo* to give a brown extract (1 kg), which was partitioned between ethyl acetate and water. The ethyl acetate fraction (230 g) was mixed with celite. The mixed celite was dried and packed into a glass column, and eluted in the order of hexane, methylene chloride, diethyl ether and methanol. The obtained diethyl ether fraction (14 g) was subjected to Sephadex LH-20 chromatography using $CHCl_3$-MeOH

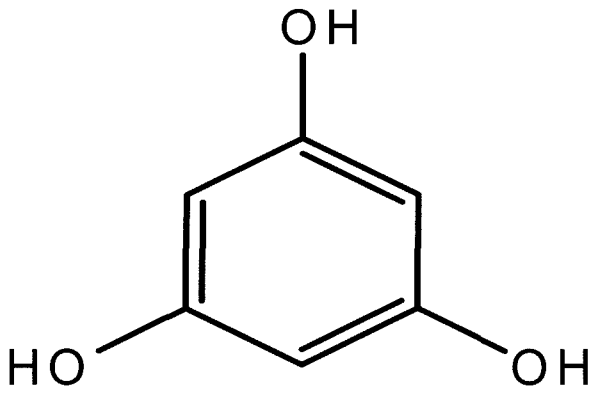

Fig. 1. Chemical structure of phloroglucinol.

gradient solvent (2/1 → 0/1). The phloroglucinol (553 mg) was obtained from these fractions and was identified according to the previously reported method (Fig. 1) (Fukuyama *et al.*, 1989). The purity of phloroglucinol assessed by HPLC was > 90%. Phloroglucinol was freshly dissolved in DMSO; the final concentration of which did not exceed 0. 1%.

Cell Culture

It is reported that lung is an organ sensitive to oxidative stress (Pryor *et al.*, 1998; Murray *et al.*, 2004). To study the effect of phloroglucinol on oxidative stress, we used Chinese hamster lung fibroblasts (V79-4 cells). The V79-4 cells from the American type culture collection, were maintained at 37°C in an incubator with a humidified atmosphere of 5 % CO_2 and cultured in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Intracellular Reactive Oxygen Species Measurement

The DCF-DA method was used to detect the intracellular ROS level (Rosenkranz, 1992). DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichlorofluorescein. The cells were seeded in a 96 well plate at 1×10^5 cells/ml. Sixteen hours after plating, cells were treated with 10 μg/ml of phloroglucinol for 1 h. Plates were irradiated at 10 Gy and the plate was incubated at 37°C for 1 day. After addition of 25 μM of DCF-DA solution, the fluorescence of 2',7'-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission using a PerkinElmer LS-5B spectrofluorometer.

Cell Viability

To determine the effect of phloroglucinol on the viability of V79-4 cells on γ-ray radiation, cells were seeded in a 96 well plate at 1×10^5 cells/ml. Sixteen hours after plating, cells were treated with 10 μg/ml of phloroglucinol for 1 h. Plates were irradiated at 10 Gy and the plate was incubated at 37°C for 4 days and the cell viability was measured using MTT test (Carmichael *et al.*, 1987).

Superoxide Dismutase (SOD) Activity

The V79-4 cells were seeded at 1×10^5 cells/ml, and sixteen hours after plating, the cells were treated with various concentrations of phloroglucinol for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 s. Triton X-100 (1%) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifuged at 5000×g for 30 min at 4°C to remove the cellular debris. The protein content of the supernatant was determined by Bradford method (Bradford, 1976), with bovine serum albumin as the standard. The SOD activity was used to detect the inhibited level of auto-oxidation of epinephrine (Misra and Fridovich, 1972). Fifty mg of the protein was added to 50 mM phosphate buffer (pH 10.2) containing 0.1 mM EDTA and 0.4 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink colored product, which can be measured at 480 nm using a UV/VIS spectrophotometer in kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm. The SOD activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of auto-oxidation of epinephrine.

Glutathione Peroxidase (GPx) Activity

Fifty μg of the protein was added to 25 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.25 unit of glutathione reductase, and 0.1 mM NADPH. After incubation for 10 min at 37 °C, H_2O_2 was added to the reaction mixture at a final

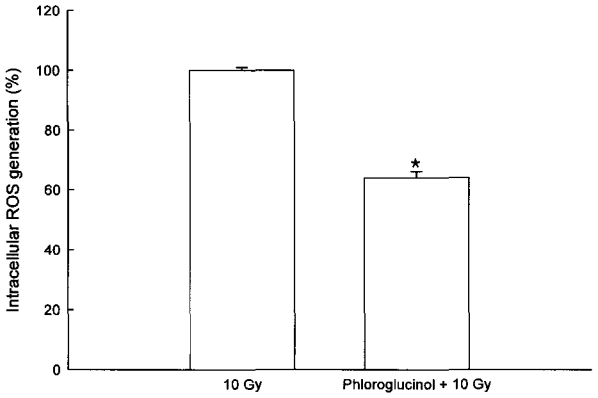

Fig. 2. Effect of phloroglucinol on ROS generated by γ-ray radiation. The intracellular reactive oxygen species generated by radiation was detected by DCF-DA method. The measurements were made in triplicate and values are expressed as means ± S.E. *significantly different from control ($p < 0.05$).

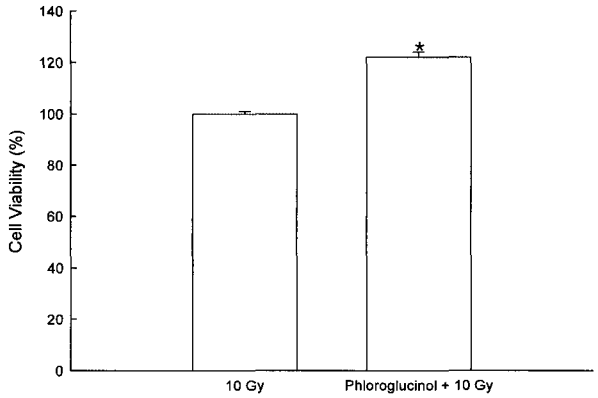

Fig. 3. Protective effect of phloroglucinol on γ-ray radiation induced oxidative damage of V79-4 cells. The viability of V79-4 cells on radiation was determined by MTT assay. The measurements were made in triplicate and values are expressed as means ± S.E. *significantly different from control ($p < 0.05$).

concentration of 1 mM. The absorbance was monitored at 340 nm for 5 min. The GPx activity was measured as the rate of NADPH oxidation by change in absorbance at 340 nm (Paglia and Valentine, 1967). The GPx activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 mM NADPH.

Statistical Analysis

All the measurements were made in triplicate and all values were represented as means ± S.E. The results

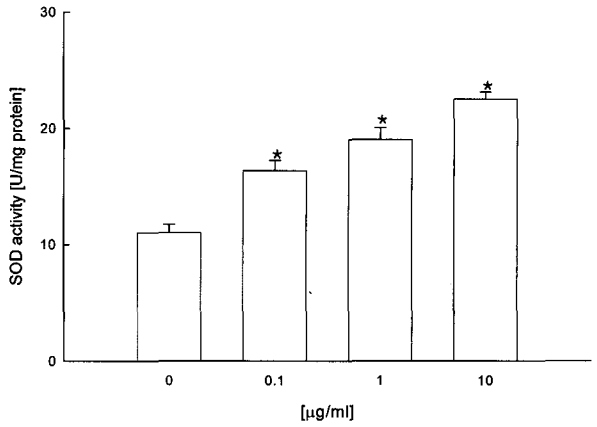

Fig. 4. Effect of phloroglucinol on the SOD activity. The enzyme activity is expressed as average enzyme unit per mg protein ± S.E. *significantly different from control ($p < 0.05$).

were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ were considered significantly.

Results and Discussion

It is reported that irradiation produces a marked accumulation of ROS and results in cell death (Kang *et al.*, 2003b; Lynch *et al.*, 2003). We examined whether phloroglucinol showed the ROS scavenging effect and the protective effect on γ-radiation at 10 Gy. The ROS scavenging effect by phloroglucinol was determined after 24 h of γ-radiation at 10 Gy. As shown in Fig. 2, 10 μg/ml of phloroglucinol showed the ROS scavenging activity of 64% in γ-radiation. The cell survival was determined after 4 days of γ-radiation. As shown in Fig. 3, phloroglucinol increased the cell survival of 22% in γ-radiation. These results suggest that phloroglucinol protects the cell damage induced by oxidative stress. In order to investigate whether the radical scavenging activity of phloroglucinol was mediated by antioxidant enzyme, the SOD and GPx activity in phloroglucinol treated V79-4 cells were measured; in the case of SOD activity at concentration of 0.1, 1, and 10 μg/ml of phloroglucinol, the activity was 16, 19, and 22, as compared to 11 U/mg protein of the control (Fig. 4); in the case of GPx activity, it was 12, 18, and 18 U/mg protein at concentration of 0.1, 1, and 10 μg/ml, as compared to 11 U/mg protein of the control. In conclusion, phloroglucinol exerted ROS scavenging activity, promoted

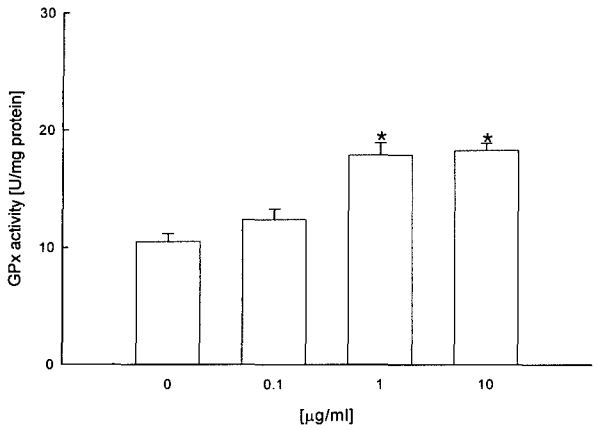

Fig. 5. Effect of phloroglucinol on the GPx activity. The enzyme activity is expressed as average enzyme unit per mg protein ± S.E. *significantly different from control ($p < 0.05$).

cell viability via activation of the SOD and GPx activity.

Acknowledgements

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