

# Cytoprotective Effect of Phloroglucinol on Oxidative Stress Induced Cell Damage via Catalase Activation

Kyoung Ah Kang,<sup>1</sup> Kyoung Hwa Lee,<sup>1</sup> Sungwook Chae,<sup>1</sup> Rui Zhang,<sup>1</sup> Myung Sun Jung,<sup>1</sup> Young Min Ham,<sup>2</sup> Jong Seok Baik,<sup>2</sup> Nam Ho Lee,<sup>2</sup> and Jin Won Hyun<sup>1\*</sup>

<sup>1</sup>Department of Biochemistry, College of Medicine and Applied Radiological Science Research Institute, Cheju National University, Jeju-si 690-756, Korea

<sup>2</sup>Department of Chemistry, College of Natural Sciences, Cheju National University, Jeju-si 690-756, Korea

**Abstract** We investigated the cytoprotective effect of phloroglucinol, which was isolated from *Ecklonia cava* (brown alga), against oxidative stress induced cell damage in Chinese hamster lung fibroblast (V79-4) cells. Phloroglucinol was found to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxy radical, intracellular reactive oxygen species (ROS), and thus prevented lipid peroxidation. As a result, phloroglucinol reduced H<sub>2</sub>O<sub>2</sub> induced apoptotic cells formation in V79-4 cells. In addition, phloroglucinol inhibited cell damage induced by serum starvation and radiation through scavenging ROS. Phloroglucinol increased the catalase activity and its protein expression. In addition, catalase inhibitor abolished the protective effect of phloroglucinol from H<sub>2</sub>O<sub>2</sub> induced cell damage. Furthermore, phloroglucinol increased phosphorylation of extracellular signal regulated kinase (ERK). Taken together, the results suggest that phloroglucinol protects V79-4 cells against oxidative damage by enhancing the cellular catalase activity and modulating ERK signal pathway. *J. Cell. Biochem.* 97: 609–620, 2006. © 2005 Wiley-Liss, Inc.

**Key words:** phloroglucinol; oxidative stress; catalase

*Ecklonia cava*, which is a brown alga belongs to the family Laminariaceae, is an 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., 1989a, 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 compounds of phloroglucinol unit, were identi-

fied to be responsible for the biological activities in *Ecklonia* species. Phlorotannin 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 was found to possess cytoprotective effect.

Reactive oxygen species (ROS) are associated with tissue damage and are the contributing factors for inflammation, aging, cancer, arteriosclerosis, hypertension, and diabetes [Laurindo et al., 1991; Nakazono et al., 1991; Parthasarathy et al., 1992; Palinski et al., 1995; Darley-Usmar and Halliwell, 1996; Cooke et al., 1997; Farinati et al., 1998]. For cytoprotection against ROS, cells have developed a variety of antioxidant defense mechanisms. Catalase is located at the peroxisome and converts hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into molecular oxygen and water. Catalase plays important roles in cellular protection by oxidative stress induced cell damages [Pietarinen et al., 1995; Doctrow et al., 2002; Cui et al., 2003; Banmeyer et al.,

Grant sponsor: Korea Science and Engineering Foundation (KOSEF) in the Ministry of Science and Technology (MOST), Korean Government.

\*Correspondence to: Jin Won Hyun, PhD, Department of Biochemistry, College of Medicine and Applied Radiological Science Research Institute, Cheju National University, Jeju-si 690-756, Korea. E-mail: jinwonh@cheju.ac.kr

Received 28 June 2005; Accepted 2 September 2005

DOI 10.1002/jcb.20668

© 2005 Wiley-Liss, Inc.

2004; Sun et al., 2005]. In addition, catalase regulates the cell growth via activation of the extracellular signal regulated kinase (ERK) pathway, leading to the acceleration of the cell growth inhibited by oxidative stress [Hachiya and Akashi, 2005].

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 catalase.

## MATERIALS AND METHODS

### Preparation of Phloroglucinol

The dried *E. 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 vacuo 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  $\text{CHCl}_3$ -MeOH gradient solvent (2/1  $\rightarrow$  0/1). The phloroglucinol (661 mg), the structural subunit of phlorotannin derivatives in brown algae, was obtained from earlier fractions. The phloroglucinol (1,3,5-trihydroxybenzene) was identified by spectroscopic comparison of the literature report as well as commercial authentic compound (Fig. 1) [Fukuyama et al., 1989b]. The purity of phloroglucinol assessed by HPLC was  $>90\%$ . Phloroglucinol was freshly dissolved in dimethyl sulfoxide (DMSO); the final concentration of which did not exceed 0.1%.

### Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, 2',7'-dichlorodihydrofluorescein diacetate

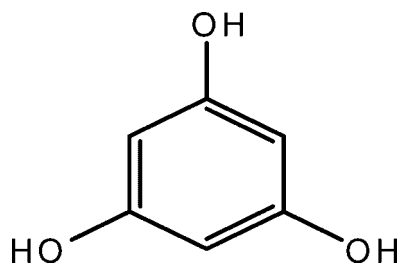


Fig. 1. Chemical structure of phloroglucinol.

(DCF-DA), and Hoechst 33342 were purchased from Sigma Chemical Company (St. Louis, MO). PeroXOquant<sup>TM</sup> quantitative peroxide assay kit was purchased from Pierce (Rockford, IL). Primary rabbit polyclonal anti-ERK 2 (42 kDa ERK) and anti-phospho-ERK1/2 (phosphorylated 44 kDa/42 kDa ERK) (Thr 202/Tyr 204) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The other chemicals and reagents were of analytical grade.

### 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%  $\text{CO}_2$  and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100  $\mu\text{g}/\text{ml}$ ) and penicillin (100 U/ml).

### DPPH Radical Scavenging Activity

Various concentrations of phloroglucinol were added to a  $1 \times 10^{-4}$  M solution of DPPH in methanol, and the reaction mixture was shaken vigorously. After 1 h, the amount of residual DPPH was determined at 520 nm using a spectrophotometer [Lo et al., 2004].

### $\text{H}_2\text{O}_2$ Scavenging Activity

This assay is based on the ability of phloroglucinol to scavenge the  $\text{H}_2\text{O}_2$  in 2,2'-azino-di(3-ethyl-benzthiazoline-6-sulphonic acid)-peroxidase medium (ABTS) [Muller, 1975]. Twenty microliters of phloroglucinol and 20  $\mu\text{l}$  of 1 mM  $\text{H}_2\text{O}_2$  were mixed with 20  $\mu\text{l}$  of 0.1 M phosphate buffer (pH 5.0) in a 96-well plate and incubated at 37°C for 5 min. Then 30  $\mu\text{l}$  of ABTS and 30  $\mu\text{l}$  of peroxidase (1 U/ml) were added in the 96-well plate and incubated at 37°C for 10 min. The absorbance was determined at 405 nm using a spectrophotometer.

### Hydroxy Radical Scavenging Activity

Hydroxy radicals generated by the Fenton reaction reacted with deoxyribose. The hydroxy radicals reacted with thiobarbituric acid and form thiobarbituric acid reactive substance (TBARS), which is chromogen with absorbance at 532 nm [Gandhi and Nair, 2004]. The reaction

mixture consisted of 0.1 mM ferric chloride, 0.1 mM ascorbic acid, 0.1 mM EDTA, 1.0 mM H<sub>2</sub>O<sub>2</sub>, and 3 mM of deoxyribose in the 20 mM of phosphate buffer pH 7.4. Phloroglucinol was added to the reaction mixture at various concentrations, and then incubated at 37°C for 1 h. One milliliter of the thiobarbituric acid was added at the end of the incubation period, boiling for 20 min, and cooled to room temperature. Five milliliters of *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample, and the mixture was shaken well. After centrifugation at 1,000g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

#### Intracellular ROS Measurement and Image Analysis

DCF-DA detects intracellular nitric oxide in addition to ROS [Rosenkranz et al., 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 V79-4 cells were seeded in a 96-well plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of phloroglucinol and 30 min later, 1 mM H<sub>2</sub>O<sub>2</sub> was added to the plate. The cells were incubated for an additional 30 min at 37°C. 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. For image analysis for production of intracellular ROS, the V79-4 cells were seeded in coverslip loaded 6-well plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with phloroglucinol and 30 min later, 1 mM H<sub>2</sub>O<sub>2</sub> was added to the plate. After changing media, 100 μM of DCF-DA was added in the well and was incubated for an additional 30 min at 37°C. After washing with PBS, stained cells were mounted onto microscope slide in the mounting medium (DAKO, Carpinteria, CA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

#### Lipid Peroxidation Inhibitory Activity

Lipid peroxidation was assayed by thiobarbituric acid reaction [Ohkawa et al., 1979]. The

V79-4 cells were seeded in a culture dish at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of phloroglucinol. One hour later, 1 mM H<sub>2</sub>O<sub>2</sub> was added to the plate, and was incubated for further 1 h. The cells were then washed with cold PBS, scraped, and homogenized in ice-cold 1.15% KCl. One hundred microliters of the cell lysates was mixed 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 made up to a final volume of 4 ml with distilled water and heated to 95°C for 2 h. After cooling to room temperature, 5 ml of *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample, and the mixture was shaken well. After centrifugation at 1,000g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

#### Cell Viability

The effect of phloroglucinol on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in the viable cells [Carmichael et al., 1987]. The V79-4 cells were seeded in a 96-well plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of phloroglucinol. One hour later, 1 mM H<sub>2</sub>O<sub>2</sub> was added to the plate and incubated at 37°C for an additional 24 h. Fifty microliters of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800g for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl DMSO and the absorbance at 540 nm was read on a scanning multi-well spectrophotometer. To determine the effect of phloroglucinol on the viability of V79-4 cells during serum starvation, cells in 10% fetal calf serum were seeded in a 96-well plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, cells were serum starved (0.1% fetal calf serum), and then treated with 10 μg/ml of phloroglucinol for 1 h. The plate was incubated at 37°C for further 24 h and the cell viability was measured using MTT test. 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  $1 \times 10^5$  cells/ml. Sixteen hours after plating, cells were treated with 10  $\mu\text{g}/\text{ml}$  of phloroglucinol for 1 h. Plates were irradiated at 5 Gy and the plate was incubated at 37°C for 24 h and the cell viability was measured using MTT test.

### Flow Cytometry Analysis

Flow cytometry was performed to determine the apoptotic sub  $G_1$  hypo-diploid cells [Nicoletti et al., 1991]. The V79-4 cells were placed in a 6-well plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with 10  $\mu\text{g}/\text{ml}$  of phloroglucinol. After a further incubation of 1 h, 1 mM  $\text{H}_2\text{O}_2$  was added to the culture. After 24 h, the cells were harvested, and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS, and then incubated for 30 min in the dark at 37°C in 1 ml of PBS containing 100  $\mu\text{g}$  propidium iodide and 100  $\mu\text{g}$  RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The proportion of sub  $G_1$  hypo-diploid cells was assessed by the histograms generated using the computer program, Cell Quest and Mod-Fit.

### Catalase Activity

The V79-4 cells were seeded at  $1 \times 10^5$  cells/ml, and 16 h 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 sonication 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 centrifugated at 5,000g 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. Fifty micrograms of protein was added to 50 mM phosphate buffer (pH 7) containing 100 mM (v/v)  $\text{H}_2\text{O}_2$ . The reaction mixture was incubated for 2 min at 37°C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of  $\text{H}_2\text{O}_2$  [Misra and Fridovich, 1972]. The catalase activity was expressed as units/mg protein and 1 U of enzyme activity was defined as the amount of enzyme required to breakdown of 1  $\mu\text{M}$   $\text{H}_2\text{O}_2$ .

### Measurement of $\text{H}_2\text{O}_2$

Level of  $\text{H}_2\text{O}_2$  in medium is determined by PeroXOquant<sup>TM</sup> quantitative peroxide assay kits (Pierce), which detect  $\text{H}_2\text{O}_2$  based on oxidation of ferrous to ferric ion in the presence of xylenol orange [Nourooz-Zadeh et al., 1994].

### Western Blot

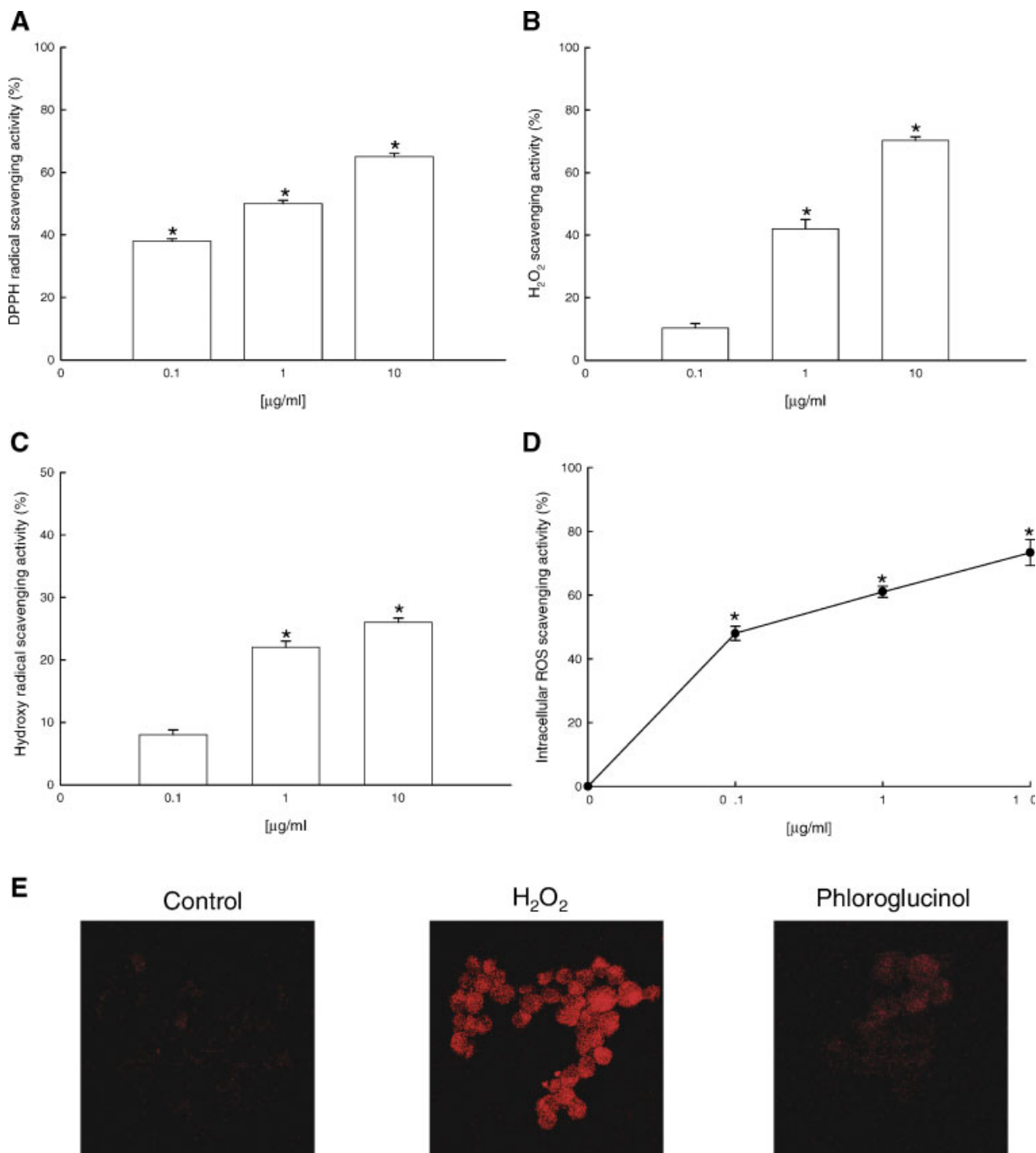
The V79-4 cells were placed in a plate at  $1 \times 10^5$  cells/ml. Sixteen hours after plating, the cells were treated with 10  $\mu\text{g}/\text{ml}$  of phloroglucinol. The cells were harvested at the indicated times, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100  $\mu\text{l}$  of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40  $\mu\text{g}$  of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), which were then incubated with primary rabbit monoclonal anti-ERK2, anti-phospho-ERK1/2, and primary sheep monoclonal anti-catalase antibodies. The membranes were further incubated with goat anti-rabbit or rabbit anti-sheep immunoglobulin G-horseradish peroxidase conjugates (Pierce), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

### Statistical Analysis

All the measurements were made in triplicate and all values were represented as means  $\pm$  SE. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference.  $P < 0.05$  were considered significantly.

## RESULTS

The radical scavenging effect of phloroglucinol on DPPH free radical,  $\text{H}_2\text{O}_2$ , and hydroxy radical scavenging activities was measured. Phloroglucinol showed the quenching effects on these ROS; in the case of DPPH radical (Fig. 2A), the scavenging activity was 38%, 50%, and 65% at concentration of 0.1, 1, and 10  $\mu\text{g}/\text{ml}$ , respectively; in the case of  $\text{H}_2\text{O}_2$  (Fig. 2B), the scavenging activity was 10%, 42%, and 70% at



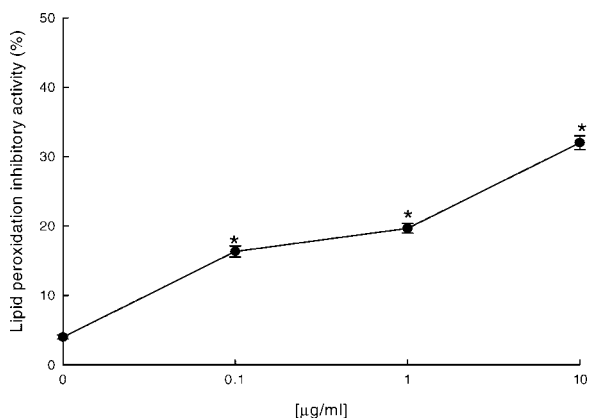
**Fig. 2.** Effect of phloroglucinol on scavenging reactive oxygen species (ROS). The amount of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (A), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (B), and hydroxy radical (C) was determined spectrophotometrically. The intracellular ROS generated was detected by 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) method (D) and by confocal microscopy (E). Representative confocal images illustrate the

increase in red fluorescence intensity of DCF produced by ROS in  $\text{H}_2\text{O}_2$  treated V79-4 cells as compared to control and the lowered fluorescence intensity in  $\text{H}_2\text{O}_2$  treated V79-4 cells in the presence of phloroglucinol (original magnification  $\times 400$ ). \*Significantly different from control ( $P < 0.05$ ). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

concentration of 0.1, 1, and 10  $\mu\text{g/ml}$ , respectively; in the case of hydroxy radical (Fig. 2C), the scavenging activity was 8%, 22%, and 26% at concentration of 0.1, 1, and 10  $\mu\text{g/ml}$ , respec-

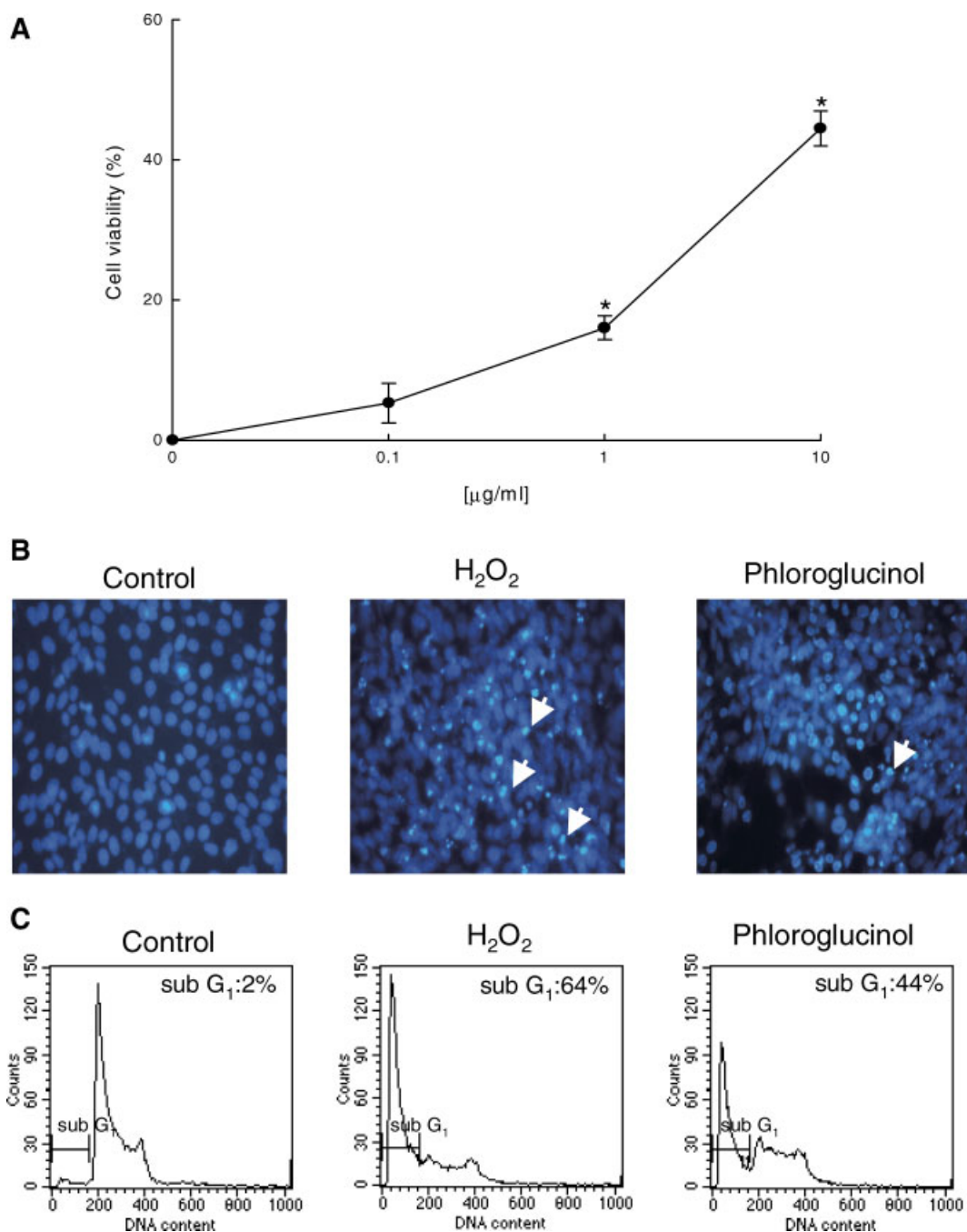
tively. In addition, the radical scavenging effect of phloroglucinol on the intracellular ROS was measured. The intracellular ROS scavenging activity of phloroglucinol was 28%, 61%, and

73% at concentrations of 0.1, 1, and 10  $\mu\text{g/ml}$ , respectively (Fig. 2D). As shown in Figure 2E, the fluorescence intensity of DCF-DA staining was enhanced in  $\text{H}_2\text{O}_2$  treated V79-4 cells. However, phloroglucinol at 10  $\mu\text{g/ml}$  reduced the red fluorescence intensity by  $\text{H}_2\text{O}_2$  treatment, reflecting a reduction of ROS generation. The ability of phloroglucinol to inhibit lipid peroxidation in  $\text{H}_2\text{O}_2$  treated V79-4 cells was also investigated. The generation of TBARS was inhibited in the presence of phloroglucinol. The inhibitory effect of phloroglucinol was 16%, 20%, and 32% at concentration of 0.1, 1, and 10  $\mu\text{g/ml}$ , respectively, when compared to 4% inhibition in untreated group (Fig. 3). The protective effect of phloroglucinol on cell survival in  $\text{H}_2\text{O}_2$  treated V79-4 cells was measured. Cells were treated with phloroglucinol at various concentrations for 1 h prior to the addition to  $\text{H}_2\text{O}_2$ . The cell viability was determined 24 h later by MTT assay. As shown in Figure 4A, treatment with phloroglucinol induced a dose dependent increase in the cell survival rate; 5% at 0.1  $\mu\text{g/ml}$ , 16% at 1  $\mu\text{g/ml}$ , and 45% at 10  $\mu\text{g/ml}$ . In order to study the cytoprotective effect of phloroglucinol on apoptosis induced by  $\text{H}_2\text{O}_2$ , nuclei of V79-4 cells were stained with Hoechst 33342 for microscopy and with propidium iodide for flow cytometric analysis. The microscopic pictures in Figure 4B showed that the control cells had intact nuclei, and the  $\text{H}_2\text{O}_2$  treated cells showed significant nuclear fragmentation, characteristic of apoptosis. However, when the cells were treated with phloroglucinol for 1 h prior to  $\text{H}_2\text{O}_2$  treatment, a dramatic decrease in nuclear fragmentation was observed. In addition



**Fig. 3.** Effect of phloroglucinol on inhibition of lipid peroxidation. Lipid peroxidation was assayed by measuring the amount of thiobarbituric acid reactive substance (TBARS). \*Significantly different from control ( $P < 0.05$ ).

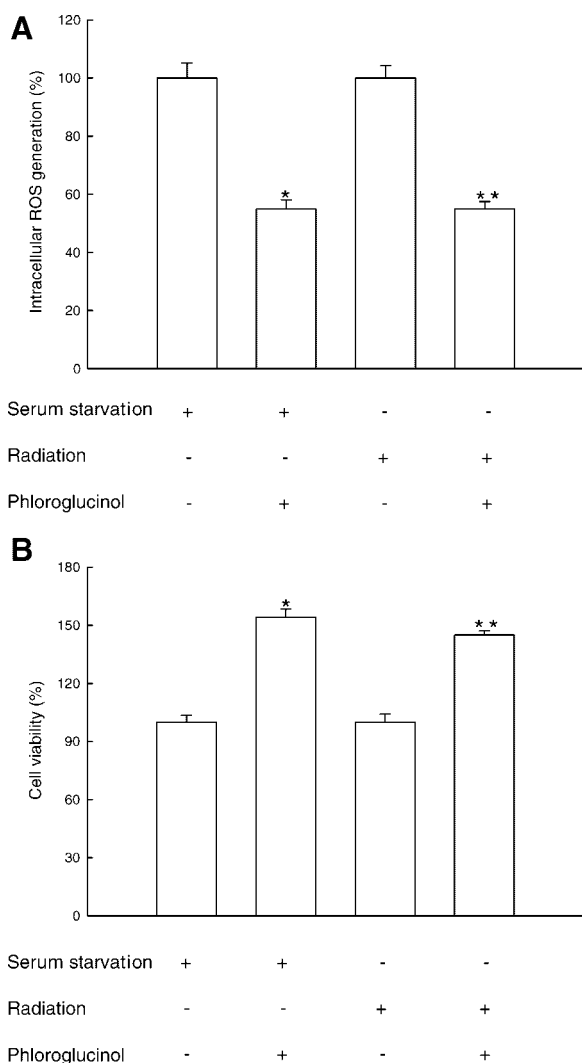
to the morphological evaluation, the protective effect of phloroglucinol against apoptosis was confirmed by flow cytometry. As shown in Figure 4C, an analysis of the DNA content in the  $\text{H}_2\text{O}_2$  treated cells revealed an increase of 64% of apoptotic sub  $G_1$  DNA content, as compared to 2% of apoptotic sub  $G_1$  DNA content in untreated cells. Treatment with 10  $\mu\text{g/ml}$  of phloroglucinol decreased the apoptotic sub  $G_1$  DNA content to 44%. It is reported that serum starvation or irradiation produces a marked accumulation of ROS and results in cell death [Lynch et al., 2003; Kang et al., 2003b]. We examined whether phloroglucinol showed the ROS scavenging effect and the protective effect on serum starvation or  $\gamma$ -radiation. The ROS scavenging effect by phloroglucinol was determined after 24 h of serum starvation or  $\gamma$ -radiation at 5 Gy. As shown in Figure 5A, 10  $\mu\text{g/ml}$  of phloroglucinol showed the ROS scavenging activity of 45% in serum starvation and 45% in  $\gamma$ -radiation. The cell survival was determined after 24 h of serum starvation or  $\gamma$ -radiation. As shown in Figure 5B, phloroglucinol increased the cell survival of 54% in serum starvation and 45% in  $\gamma$ -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 catalase activity in phloroglucinol treated V79-4 cells were measured. Phloroglucinol increased catalase activity (Fig. 6A); it was 18, 26, and 37 U/mg protein at concentration of 0.1, 1, and 10  $\mu\text{g/ml}$ , as compared to 15 U/mg protein of the control. To confirm the activation of catalase by phloroglucinol in terms of protein, the Western blot analysis was performed. As shown in Figure 6B, the protein expression of catalase by phloroglucinol was increased within 6 h. The 3-amino-1,2,4 triazol (ATZ) is known as a specific inhibitor of catalase [Margoliash et al., 1960]. To determine the effect of catalase inhibitor on protection of phloroglucinol from  $\text{H}_2\text{O}_2$  induced damage, V79-4 cells were pre-treated with 20 mM of ATZ for 1 h, followed for 30 min with phloroglucinol and exposed to 1 mM  $\text{H}_2\text{O}_2$  for 24 h. As shown in Figure 6C, ATZ treatment abolished the protection activity of phloroglucinol in  $\text{H}_2\text{O}_2$  damaged cells. It is reported that most polyphenolic compounds interact with commonly used cell culture media to generate  $\text{H}_2\text{O}_2$  [Long et al., 2000]. This generated low



**Fig. 4.** Protective effect of phloroglucinol on H<sub>2</sub>O<sub>2</sub> induced oxidative damage of V79-4 cells. The viability of V79-4 cells (A) was determined by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay. Apoptotic body formation (B) was observed under a fluorescent microscope after Hoechst 33342 staining and are indicated by arrows. Apoptotic sub G<sub>1</sub> DNA content (C) was detected by flow cytometry after propidium iodide staining. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

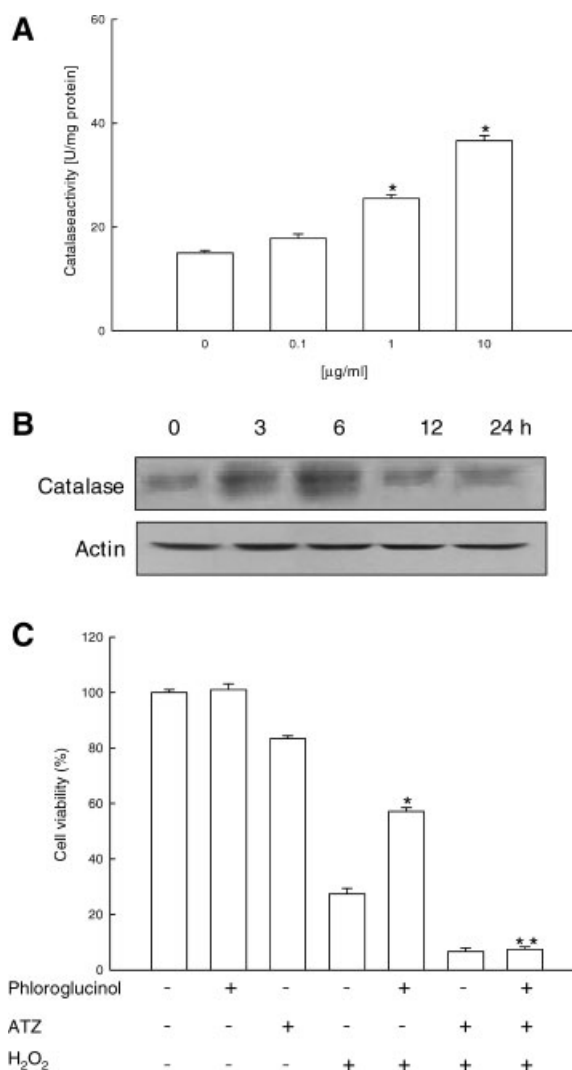
level of H<sub>2</sub>O<sub>2</sub> can trigger the rise in antioxidant enzymes. Whether phloroglucinol generates H<sub>2</sub>O<sub>2</sub> in media, phloroglucinol was added to cell culture media at a final concentration of 10 µg/ml and amount of H<sub>2</sub>O<sub>2</sub> was measured by the

ferrous iron oxidation-xylene orange assay. As shown in Table I, H<sub>2</sub>O<sub>2</sub> was little detected in phloroglucinol treated media (<1 µM of H<sub>2</sub>O<sub>2</sub>), suggesting the antioxidant activity in phloroglucinol treated cells were not increased



**Fig. 5.** Protective effect of phloroglucinol on serum starvation or  $\gamma$ -ray radiation induced oxidative damage of V79-4 cells. The intracellular ROS (A) generated by serum starvation or radiation was detected by DCF-DA method. The viability of V79-4 cells (B) on serum starvation or radiation was determined by MTT assay. The measurements were made in triplicate and values are expressed as means  $\pm$  SE. \*Significantly different from serum starved cells ( $P < 0.05$ ). \*\*Significantly different from irradiated cells ( $P < 0.05$ ).

by  $H_2O_2$  generated in phloroglucinol treated media. To better understand the protective mechanism of phloroglucinol on V79-4 cells, we examined the activation of the ERK protein by Western blot analysis with the phospho-ERK specific antibody. As shown in Figure 7A, within 6 h phloroglucinol activated phosphorylated ERK dramatically. However, there was no change in the total ERK protein level. To determine the effect of ERK inhibitor on protection of phloroglucinol from  $H_2O_2$  induced



**Fig. 6.** Effect of phloroglucinol on the catalase activity. The enzyme activity (A) is expressed as average enzyme unit per milligrams of protein  $\pm$  SE. \*Significantly different from control ( $P < 0.05$ ). Cell lysates were electrophoresed and the expression of catalase (B) is detected by its specific antibody. After treatment of 3-amino-1,2,4 triazol (ATZ), phloroglucinol or/and  $H_2O_2$ , the viability of V79-4 cells (C) was determined by MTT assay. \*Significantly different from  $H_2O_2$  treated cells ( $P < 0.05$ ). \*\*Significantly different from  $H_2O_2$  plus phloroglucinol treated cells ( $P < 0.05$ ).

damage, V79-4 cells were pre-treated for 30 min with U0126 (10 nM), specific inhibitor of ERK kinase, followed for 30 min with phloroglucinol, and exposed to 1 mM  $H_2O_2$  for 24 h. As shown in Figure 7B, U0126 treatment abolished the protection activity of phloroglucinol in  $H_2O_2$  damaged cells.

## DISCUSSION

Oxidative stress refers to the mismatched redox equilibrium between the production of



**TABLE I. Generation of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) in Cell-Culture Media**

H <sub>2</sub> O <sub>2</sub> present in DMEM media [ $\mu$ M]	
None	1.2 $\pm$ 0.4
DMSO <sup>a</sup>	1.1 $\pm$ 0.3
Phloroglucinol	0.8 $\pm$ 0.01

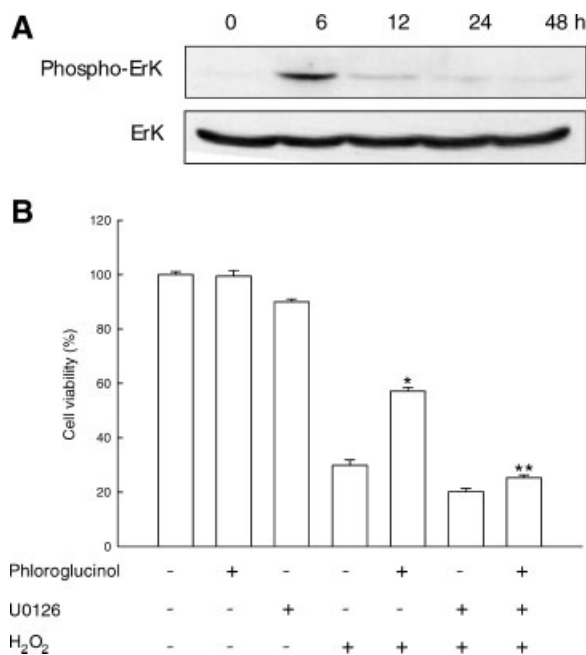
Phloroglucinol at a final concentration of 10  $\mu$ g/ml was added to DMEM culture media and incubated at room temperature for 1 h. H<sub>2</sub>O<sub>2</sub> was then measured by the ferrous iron oxidation–xylenol orange assay. Data are means  $\pm$  SE.

<sup>a</sup>Dimethyl sulfoxide (DMSO) was of the same concentration as used to dissolve phloroglucinol.

ROS and ability of the cells to defend against ROS. ROS such as superoxide anion, hydroxyl radicals, and H<sub>2</sub>O<sub>2</sub>, are unwanted and toxic by-products formed during aerobic metabolism. ROS can cause cell death via apoptosis and/or necrosis in many cell types, which can be blocked or delayed by various antioxidants and antioxidative proteins/enzymes [Carmody and Cotter, 2001; Kim et al., 2001; Jang and Surh, 2003].

Phlorotannins are marine algal polyphenols and mainly exist in brown algae [Shibata et al.,

2002]. They are commonly known to have defensive or protective functions against herbivores. Phlorotannin compounds such as phloroglucinol, eckol, 6,6'-bieckol, dieckol, phlorofucofuroeckol were identified to be responsible for the biological activities in *Ecklonia* species. Although some reports suggest that phlorotannins from algae exhibit the antioxidant effect on free radicals [Nakamura et al., 1996; Kang et al., 2003a; Kim et al., 2004], there are no reports on the cytoprotective effect against oxidative stress induced cell damage and its mechanism of phloroglucinol, isolated from *E. cava*. In our present study, it was observed that upon exposure to H<sub>2</sub>O<sub>2</sub>, phloroglucinol decreased ROS. Phloroglucinol has a polyphenol structure and polyphenols are electron-rich compounds and prone to enter into efficient electron-donation reactions with oxidizing agents to produce phenoxy radical (PhO $\cdot$ ) species as intermediates. Phenoxy radicals are stabilized by resonance delocalization of the unpaired electron to the *ortho* and *para* positions of the ring. In addition to the resonance stability, phenoxy radicals can also be stabilized by hydrogen bonding with an adjacent hydroxyl group. Phenoxy radicals also undergo dimerization (“phenol coupling”) to produce new CC or CO linkage [Larson, 1997]. This intrinsic stability of phenolic structures might be related to antioxidative activity of phloroglucinol. The cells exposed to H<sub>2</sub>O<sub>2</sub> exhibited distinct morphological features of apoptosis, such as nuclear fragmentation and an increase in sub G<sub>1</sub> DNA content. However, cells that were pretreated with phloroglucinol had significantly reduced percentage of apoptotic cells, as shown by morphological changes and reduction in sub G<sub>1</sub> DNA content. Our results are also consistent with the antioxidant activity of *N*-acetylcysteine, which also prevents H<sub>2</sub>O<sub>2</sub> induced apoptosis (data not shown), indicating that the inhibition of ROS formation may be important for cytoprotection against oxidative damage. Catalase plays a significant role in effective augmentation of antioxidant defense mechanisms in cells. Phloroglucinol increased catalase activity and its protein expression, suggesting that the scavenging of ROS may be related to the increased antioxidant activity. Therefore, the effects of phloroglucinol on cell viability might involve dual actions: direct action on oxygen radical scavenging, as shown by DPPH radical, H<sub>2</sub>O<sub>2</sub>, OH radical scavenging,



**Fig. 7.** Effect of phloroglucinol on extracellular signal regulated kinase (ERK) activity. Cell lysates were electrophoresed and proteins of phospho-ERK1/2 and ERK2 (**A**) were detected by their respective specific antibody. After treatment of U0126, phloroglucinol or/and H<sub>2</sub>O<sub>2</sub>, the viability of V79-4 cells (**B**) was determined by MTT assay. \*Significantly different from H<sub>2</sub>O<sub>2</sub> treated cells ( $P < 0.05$ ). \*\*Significantly different from H<sub>2</sub>O<sub>2</sub> plus phloroglucinol treated cells ( $P < 0.05$ ).

and indirect action through induction of catalase. Antioxidant enzymes would be potential target molecules mediating antiapoptotic function of ERK pathway against oxidative stress. We examined the activation of ERK, which is an important component of intracellular signaling cascades mediating oxidative survival. The phosphorylation of ERK can phosphorylate cytoplasmic and nuclear targets and participates in a wide range of cellular programs including proliferation, differentiation, and movement [Pages et al., 1991; Robinson and Cobb, 1997; Widmann et al., 1999; McCubrey et al., 2000]. The level of phosphorylated ERK in phloroglucinol treated cells was induced, and treatment of U0126, specific inhibitor of ERK kinase, suppressed the protection activity of phloroglucinol in H<sub>2</sub>O<sub>2</sub> damaged cells, suggesting that the protective effect of phloroglucinol on cells may also be involved in activating ERK pathway.

In addition, the ERK pathway is known to influence the expression of several genes, which are mostly involved in cell proliferation. The ERK signaling cascade has been implicated in nuclear factor kappa B (NF- $\kappa$ B) activation through phosphorylation of inhibitory I $\kappa$ B [Chen and Lin, 2001]. Recently, increasing evidence supports the role of NF- $\kappa$ B in regulating of antiapoptotic gene expression and promotion of cell survival. The transcriptional regulation of catalase is mediated partially by NF- $\kappa$ B. Sequence analysis of the mouse catalase gene revealed putative binding sites for NF- $\kappa$ B [Zhou et al., 2001]. Further studies are needed to elucidate that phloroglucinol might associate with ERK-NF $\kappa$ B-catalase signaling pathways.

In conclusion, phloroglucinol exerted ROS scavenging activity, promoted cell viability, inhibited H<sub>2</sub>O<sub>2</sub> induced apoptosis, activated ERK protein, and enhanced the catalase activity.

## REFERENCES

- Ahn MJ, Yoon KD, Min SY, Lee JS, Kim JH, Kim TG, Kim SH, Kim NG, Huh H, Kim J. 2004. Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. *Biol Pharm Bull* 27:544–547.
- Banmeyer I, Marchand C, Verhaeghe C, Vucic B, Rees JF, Knoops B. 2004. Overexpression of human peroxiredoxin 5 in subcellular compartments of Chinese hamster ovary cells: Effects on cytotoxicity and DNA damage caused by peroxides. *Free Radic Biol Med* 36:65–77.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. *Cancer Res* 47:936–941.
- Carmody RJ, Cotter TG. 2001. Signaling apoptosis: A radical approach. *Redox Rep* 6:77–90.
- Chen BC, Lin WW. 2001. PKC- and ERK-dependent activation of I kappaB kinase by lipopolysaccharide in macrophages: Enhancement by P2Y receptor-mediated CaMK activation. *Br J Pharmacol* 134:1055–1065.
- Cooke MS, Mistry N, Wood C, Herbert KE, Lunec J. 1997. Immunogenicity of DNA damaged by reactive oxygen species implications for anti-DNA antibodies in lupus. *Free Radic Biol Med* 22:151–159.
- Cui XY, Fu PF, Pan DN, Zhao Y, Zhao J, Zhao BC. 2003. The antioxidant effects of ribonuclease inhibitor. *Free Radic Res* 37:1079–1085.
- Darley-Usmar V, Halliwell B. 1996. Blood radicals: Reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharm Res* 13:649–662.
- Doctrow SR, Huffman K, Marcus CB, Tocco G, Malfroy E, Adinolfi CA, Kruk H, Baker K, Lazarowych N, Mascarenhas J, Malfroy B. 2002. Salen-manganese complexes as catalytic scavengers of hydrogen peroxide and cytoprotective agents: Structure–activity relationship studies. *J Med Chem* 45:4549–4558.
- Farinati F, Cardin R, Degan P, Ruge M, Mario FD, Bonvicini P, Naccarato R. 1998. Oxidative DNA damage accumulation in gastric carcinogenesis. *Gut* 42:351–356.
- Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Kido M, Mori H, Nakayama Y, Takahashi M. 1989a. Structure of an anti-plasmin inhibitor, eckol, isolated from the brown alga *Ecklonia kurome* Okamura and inhibitory activities of its derivatives on plasma plasmin inhibitors. *Chem Pharm Bull* 37:349–353.
- Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Mori H, Nakayama Y, Takahashi M. 1989b. Anti-plasmin inhibitor. V. Structures of novel dimeric eckols isolated from the brown alga *Ecklonia kurome* Okamura. *Chem Pharm Bull* 37:2438–2440.
- Fukuyama Y, Kodama M, Miura I, Kinzyo Z, Mori H, Nakayama Y, Takahashi M. 1990. Anti-plasmin inhibitor. VI. Structure of phlorofucofuroeckol A, a novel phlorotannin with both dibenzo-1,4-dioxin and dibenzofuran elements, from *Ecklonia kurome* Okamura. *Chem Pharm Bull* 38:133–135.
- Gandhi NM, Nair CK. 2004. Radiation protection by diethyldithiocarbamate: Protection of membrane and DNA in vitro and in vivo against gamma-radiation. *J Radiat Res* 45:175–180.
- Hachiya M, Akashi M. 2005. Catalase regulates cell growth in HL60 human promyelocytic cells: Evidence for growth regulation by H<sub>2</sub>O<sub>2</sub>. *Radiat Res* 163:271–282.
- Han ES, Kim JW, Eom MO, Kang IH, Kang HJ, Choi JS, Ha KW, Oh HY. 2000. Inhibitory effect of *Ecklonia stolonifera* on gene mutation on mouse lymphoma tk<sup>+/+</sup> locus in L5178Y-3.7.2.C cell and bone marrow micronuclei formation in ddY mice. *Environ Mutagen Carcinogen* 20:104–111.
- Jang HH, Surh YJ. 2003. Protective effects of resveratrol on  $\beta$ -amyloid induced oxidative PC12 cell death. *Free Radic Biol Med* 34:1100–1110.

- Kang K, Park Y, Hwang HJ, Kim SH, Lee JG, Shin HC. 2003a. Antioxidative properties of brown algae polyphenolics and their perspectives as chemopreventive agents against vascular risk factors. *Arch Pharm Res* 26:286–293.
- Kang S, Song J, Kang H, Kim S, Lee Y, Park D. 2003b. Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells. *Eur J Endocrinol* 148:147–155.
- Kang HS, Chung HY, Kim JY, Son BW, Jung HA, Choi JS. 2004. Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. *Arch Pharm Res* 27:194–198.
- Kim HJ, So YJ, Jang JH, Lee JS, Oh YJ, Surh YJ. 2001. Differential cell death induced by salsolinol with and without copper: Possible role of reactive oxygen species. *Mol Pharmacol* 60:440–449.
- Kim JA, Lee JM, Shin DB, Lee NH. 2004. The antioxidant activity and tyrosinase inhibitory activity of phlorotannins in *Ecklonia cava*. *Food Sci Biotech* 13:476–480.
- Larson RA. 1997. Phenolic and enolic antioxidants. In: Larson RA, editor. *Naturally occurring antioxidants*. New York: Lewis publishers. pp 83–87.
- Laurindo FR, da Luz PL, Uint L, Rocha TF, Jaeger RG, Lopes EA. 1991. Evidence for superoxide radical-dependent coronary vasospasm after angioplasty in intact dogs. *Circulation* 83:1705–1715.
- Lee JH, Oh HY, Choi JS. 1996. Preventive effect of *Ecklonia stolonifera* on the frequency of benzo(a)pyrene-induced chromosomal aberrations. *J Food Sci Nutr* 1:64–68.
- Lee JH, Kim ND, Choi JS, Kim YJ, Moon YH, Lim SY, Park KY. 1998. Inhibitory effects of the methanolic extract of an edible brown alga, *Ecklonia stolonifera* and its component, phloroglucinol on aflatoxin B1 mutagenicity in vitro (Ames test) and on benzo(a)pyrene or *N*-methyl *N*-nitrosourea clastogenicity in vivo (mouse micronucleus test). *Nat Prod Sci* 4:105–114.
- Lo SF, Nalawade SM, Mulabagal V, Matthew S, Chen CL, Kuo CL, Tsay HS. 2004. In vitro propagation by asymbiotic seed germination and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity studies of tissue culture raised plants of three medicinally important species of *Dendrobium*. *Biol Pharm Bull* 27:731–735.
- Long LH, Clement MV, Halliwell B. 2000. Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* 273:50–53.
- Lynch AM, Moore M, Craig S, Lonergan PE, Martin DS, Lynch MA. 2003. Analysis of interleukin-1 beta-induced cell signaling activation in rat hippocampus following exposure to gamma irradiation. *J Biol Chem* 278:51075–51084.
- Margoliash E, Novogrodsky A, Schejter A, Chejter A. 1960. Irreversible reaction of 3-amino-1,2,4-triazole and related inhibitors with the protein of catalase. *Biochem J* 74:339–348.
- McCubrey JA, May WS, Duronio V, Mufson A. 2000. Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* 14:9–21.
- Misra HP, Fridovich I. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 247:3170–3175.
- Muller HE. 1975. Detection of hydrogen peroxide produced by microorganisms on an ABTS peroxidase medium. *Zentralbl Bakteriol Mikrobiol Hyg* 250:151–154.
- Murray JI, Whitfield ML, Trinklein ND, Myers RM, Brown PO, Botstein D. 2004. Diverse and specific gene expression responses to stresses in cultured human cells. *Mol Biol Cell* 15:2361–2374.
- Nagayama K, Iwamura Y, Shibata T, Hirayama I, Nakamura T. 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *J Antimicrob Chemother* 50:889–893.
- Nakamura T, Nagayama K, Uchida K, Tanaka R. 1996. Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fisher Sci* 62:923–926.
- Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, Inoue M. 1991. Does superoxide underlie the pathogenesis of hypertension? *Proc Natl Acad Sci USA* 88:10045–10048.
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Meth* 139:271–279.
- Nourooz-Zadeh J, Sarmadi-Tajaddini J, Wolff SP. 1994. Measurement of plasma hydrogenperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Anal Biochem* 220:403–409.
- Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95:351–358.
- Pages G, Lenomand P, L'Allemania G, Chambard JC, Meloche S, Pouyssegur J. 1991. Mitogen activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc Natl Acad Sci USA* 90:8319–8323.
- Palinski W, Miller E, Witztum JL. 1995. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci USA* 92:821–825.
- Park DC, Ji CI, Kim SH, Jung KJ, Lee TG, Kim IS, Park YH, Kim SB. 2000. Characteristics of tyrosinase inhibitory extract from *Ecklonia stolonifera*. *J Fish Sci Tech* 3:195–199.
- Parthasarathy S, Steinberg D, Witztum JL. 1992. The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. *Annu Rev Med* 43:219–225.
- Pietarinen P, Raivio K, Devlin RB, Crapo JD, Chang LY, Kinnula VL. 1995. Catalase and glutathione reductase protection of human alveolar macrophages during oxidant exposure in vitro. *Am J Respir Cell Mol Biol* 13:434–441.
- Pryor WA, Stone K, Zang LY, Bermudez E. 1998. Fractionation of aqueous cigarette tar extracts: Fractions that contain the tar radical cause DNA damage. *Chem Res Toxicol* 11:441–448.
- Robinson MJ, Cobb MH. 1997. Mitogen activated protein kinase pathways. *Curr Opin Cell Biol* 9:180–186.
- Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ. 1992. A microplate assay for the

- detection of oxidative products using 2',7'-dichlorofluorescein-diacetate. *J Immunol Meth* 156:39–45.
- Shibata T, Yamaguchi K, Nagamura K, Kawaguchi S, Nagamura T. 2002. Inhibitory activity of brown algae phlorotannins against glycosidases from the viscera of the turban shell *Turbo cornutus*. *Eur J Phycol* 37:493–500.
- Sun C, Shan CY, Gao XD, Tan RX. 2005. Protection of PC12 cells from hydrogen peroxide-induced injury by EPS2, an exopolysaccharide from a marine filamentous fungus *Keissleriella* sp. YS4108. *J Biotechnol* 115:137–144.
- Widmann C, Gibson S, Jarpe B, Johnson GL. 1999. Mitogen activated protein kinase: Conservation of a three kinase module from yeast to human. *Physiol Rev* 79:143–180.
- Zhou LZ, Johnson AP, Rando TA. 2001. NF- $\kappa$ B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. *Free Radic Biol Med* 31:4079–4086.