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Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation

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ABSTRACT

In the present study, three kinds of phlorotannins, marine algal polyphenol, were isolated from a brown alga *Ecklonia cava*, and their inhibitory effect on melanogenesis as well as the protective effect against photo-oxidative stress induced by UV-B radiation was investigated. The effect on melanogenesis was evaluated via the inhibitory effects of tyrosinase and melanin synthesis. Among the phlorotannins, dieckol showed higher effect than that of the other phlorotannins in the both assays; especially the value of dieckol in the tyrosinase inhibition assay was relatively higher than that of a commercial tyrosinase inhibitor (kojic acid). The UV-B protection effect was evaluated via DCFH-DA, MTT, comet assays, and morphological changes in fibroblast. Intracellular ROS induced by UV-B radiation was reduced by the addition of phlorotannins and cell viability was dose-dependently increased. Moreover, dieckol demonstrated strong protective properties against UV-B radiation-induced DNA damage via damaged tail intensity and morphological changes in fibroblast. Hence, these results indicated that dieckol isolated from *E. cava* has potential whitening effects and prominent protective effects on UV-B radiation-induced cell damages, which might be used in pharmaceutical and cosmeceutical industries.

1. Introduction

Cosmetics are commercially available products that are used to improve the appearance of the skin. In recent years, the number of women concerning about whiter skin complexion, especially in Asia has increased dramatically (Tengamnuay et al., 2006). Melanin is the major pigment responsible for the color of human skin. It may be overproduced with chronic sun exposure, melasma, or other hyperpigmentation diseases. Therefore, a number of depigmenting agents have been developed in cases of undesirable skin discoloration. Tyrosinase, a copper-containing monooxygenase, is a key enzyme that catalyzes melanin synthesis in melanocytes. It is catalyzes the hydroxylation of tyrosine into dihydroxyphenylalanine (DOPA) and other intermediates (Sturm et al., 2001; Kajiwara et al., 2006; Wang et al., 2006). Thus, inhibition of tyrosinase activity or its production can prevent melanogenesis.

Skin is the preferred target of oxidative stress as continuously exposed to ultraviolet (UV) radiation from sunlight and environmental oxidizing pollutants (Thiele et al., 1997). It is well established that overexposure to UV radiation provokes acute sunburn reaction, which clinically manifests itself as erythema. Chronically irradiated skins by UV radiation are associated with abnormal cutaneous reactions such as epidermal hyperplasia, accelerated breakdown of collagen, and inflammatory responses (Longstreth et al., 1998; Marrot et al., 2001; Tanaka et al., 2007). UV radiation has a strong oxidative component, and photo-oxidative stress has been directly linked to the onset of skin photodamage (Fuchs, 1998; Caddeo et al., 2008). Hence, regular intake of dietary antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for preventing UV-induced damages.

Marine algae produce a great variety of secondary metabolites possessing many different skeletal types and biological activities (Heo et al., 2005; Heo and Jeon, 2008; Sabry et al., 2005). Although these marine algae expose to the adverse environmental conditions such as light and high oxygen concentrations that lead to the formation of free radicals, and other strong oxidizing agents, they do not affect any serious photodynamic damage *in vivo*. This fact suggests that marine algae like photosynthesizing plants have antioxidative mechanisms and compounds which act as antioxidant agents (Dykens et al., 1992; Jimenez-Escrig et al., 2001). Further, it is well established that brown algae contain phenolic

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compounds with strong antioxidant activity. Marine algal polyphenols, known as phlorotannins, which have only been found to exist within brown algae, are restricted to polymers of phloroglucinol (1,3,5-trihydroxybenzene). It has been reported that phlorotannins have several biological functions including anti-plasmin inhibitors, anti-allergic, antibacterial and antioxidant activities (Nakayama et al., 1989; Nakamura et al., 1996; Nagayama et al., 2002; Sugiura et al., 2006).

Ecklonia cava, a kind of brown alga, is plentifully produced in Jeju Island in Korea, and is utilized as food ingredient, animal feed, fertilizer and medicine. In addition, *E. cava* has a variety of compounds including carotenoid, fucoidans, and phlorotannins showing different biological activities. In a previous study (Ahn et al., 2007), we isolated three kinds of phlorotannins from *E. cava*, including phloroglucinol, eckol and dieckol, which showed potential antioxidant activity. Accordingly, the present study aimed to isolate a marine natural phlorotannins from *E. cava* and evaluated the effects of the phlorotannins on melanogenesis and their photoprotective effect on the cell damage induced by UV-B radiation.

2. Materials and methods

2.1. Materials

The marine alga *E. cava* was collected along the coast of Jeju Island, Korea, between October 2007 and March 2008. The samples were washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface. Then carefully rinsed with fresh water, and maintained in a medical refrigerator at −20 °C. Thereafter, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

2.2. Extraction and isolation

The phlorotannins were isolated as previously described by Ahn et al. (2007) with slight modifications. Briefly, the dried *E. cava* powder (500 g) was extracted three times with 80% MeOH and then was filtered. The filtrate was evaporated at 40 °C to obtain the methanol extract. After, the extract was suspended on distilled water, and partitioned with ethyl acetate. The ethyl acetate fraction 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 diethyl ether fraction was further purified by sephadex LH-20 column chromatography using stepwise gradient chloroform/methanol (2/1 → 0/1) solvents system. The phloroglucinol, eckol, and dieckol were purified by high performance liquid chromatography (HPLC) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 × 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV range: 230 nm, flow rate: 0.8 ml/min). Finally, the purified compounds were identified by comparing their ^{1}H and ^{13}C NMR data to the literature report. The chemical structures of the three phlorotannins are indicated in Fig. 1.

2.3. Inhibitory effect of mushroom tyrosinase

Tyrosinase inhibitory activity was performed according to the method of Vanni et al. (1990) with slight modifications. The reaction mixture contains 140 μl of 0.1 M phosphate buffer (pH 6.5), 40 μl of 1.5 mM L-tyrosine and 10 μl of samples. Then, 10 μl of mushroom tyrosinase (2100 units/ml) solution was added and the reaction was incubated at 37 °C for 12 min. After incubation, the amount of dopachrome produced in the reaction mixture was determined as the optical density at 490 nm in a microplate reader.

2.4. Inhibitory effect of melanin synthesis

Melanin contents were measured according to the method described by Tsuboi et al. (1998) with slight modifications. The B16F10 cells were placed in 6-well plates at a concentration of 3×10^5 cells/ml, and 24 h after plating the cells were treated with various concentrations of the compounds. After 24 h, the medium was removed and cells were washed twice with PBS. Later, the cell pellets containing a known number of cells (usually around 1×10^6) were dissolved in 1 ml of 1 N NaOH at 60 °C for 30 min and centrifuged for 10 min at 10,000 rpm. The optical densities (OD) of the supernatants were measured at 490 nm using an ELISA reader.

2.5. Cell culture

Human fibroblast were kindly supplied by Surface Science Laboratory of Center for Anti-aging Molecular Science (CAMS) of Department of Chemistry and School of Molecular Science of Korea Advanced Institute of Science and Technology (KAIST), maintained at 37 °C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml).

2.6. UV-B irradiation

Cells were exposed to UV-B range at a dose rate of 10–100 mJ/cm^2 (UV Lamp, VL-6LM, Vilber Lourmat, France) and 50 mJ/cm^2 dose was identified as the optimum irradiation dose. Therefore, the 50 mJ/cm^2 dose of UV-B was used for further experiments.

2.7. Intracellular reactive oxygen species (ROS) measurement

The DCFH-DA method was used to detect the levels of intracellular 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 fibroblast were seeded in 96-well plates at a concentration of 1×10^5 cells/ml. Sixteen hours after plating, the cells were exposed to UV-B (50 mJ/cm^2) and the compound was treated with various concentrations. The cells were incubated for an additional 24 h at 37 °C under a humidified atmosphere with 5% CO_2. Finally, DCFH-DA (5 μg/ml) was introduced to the cells, and 2′,7′-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin–Elmer LS-5B spectrofluorometer. In each assay we used two types of controls; one is negative control (that is non-irradiated cells) and positive control (that is UV-B irradiated cells).

2.8. Assessment of cell viability

Cell viability was estimated via MTT assay, which is a test of metabolic competence predicated upon the assessment of mitochondrial performance. It is colorimetric assay, which is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). The fibroblast cells were seeded in 96-well plate at a concentration of 1×10^5 cells/ml. After 16 h, the cells were exposed to UV-B (50 mJ/cm^2) with the compound at difference concentrations, and then the cells were incubated for 24 h at 37 °C. MTT stock solution (2 mg/ml) was then applied to the wells, to a total reaction volume of 200 μl. After 4 h of the incubation, the plates were centrifuged for 5 min at 800×g, and