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Inhibitory effect of diphlorethohydroxycarmalol on melanogenesis and its protective effect against UV-B radiation-induced cell damage

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ABSTRACT

In this study, potential inhibitory effect of 21 species of marine algae on melanogenesis was assessed via tyrosinase inhibitory effect. The *Ishige okamurae* extract tested herein evidenced profound tyrosinase inhibitory effect, compared to that exhibited by other marine algae extracts. Thus, *I. okamurae* was selected for use in further experiments, and was partitioned with different organic solvents. Profound tyrosinase inhibitory effect was detected in the ethyl acetate fraction, and the active compound was identified as the carmalol derivative, diphlorethohydroxycarmalol (DPHC), which evidenced higher levels of activity than that of commercial whitening agent. Intracellular reactive oxygen species (ROS) induced by ultraviolet (UV)-B radiation was reduced by the addition of DPHC and cell viability was dose-dependently increased. Moreover, DPHC demonstrated strong protective properties against UV-B radiation via damaged DNA tail length and morphological changes in fibroblast. Hence, these results indicate that DPHC isolated from *I. okamurae* 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

Ultraviolet (UV) radiation on the surface of the earth is certainly increasing as a result of the depletion of stratospheric ozone. Generally, UV radiation in solar light at low dose affects growth and metabolic activity in organism, especially photoautophic organisms, which need light for synthesis of biomass and fixation of energy. However, high-intensity or accumulated UV radiation induces overproduction of reactive oxygen species (ROS) that interact with proteins, lipids, and DNA thus altering cellular functions (He and Häder, 2002; Wang et al., 2008). This observation has stimulated research into the role of natural antioxidants that can mitigate photobiologic damage. Our interest in this field has focus on phlorotannins, a marine algal polyphenols that have been recently used in biological systems for their antioxidant activities (Ahn et al., 2007; Heo and Jeon, 2009). It now appears that important actions can be attributed to some phlorotannins, and evidence indicates that they may reduce UV radiation-mediated cutaneous disease such as epidermal hyperplasia, carcinogenesis, and melanogenesis (Pavia et al., 1997; Swanson and Druehl, 2002).

Phlorotannins are organic polymers of phloroglucinol (1,3,5-trihydroxybenzene), unique to the brown algae. They represent an interesting class of active polyphenolic compounds in the protection of UV light-induced skin damage due to their wide spectrum of activities including antioxidant, anti-diabetes, anti-HIV, and anti-allergic activities (Kakegawa et al., 1992; Heo et al., 2008; Artan et al., 2008; Heo et al., 2009). As mentioned in the previous reports with the phlorotannins, higher plants are naturally exposed to solar radiation and therefore to relatively high doses of UV radiation; thus they have developed a number of defense mechanisms against UV-induced damage, such as the capability to absorb UV radiation by accumulation of phenolic compounds in their superficial layers (Swanson and Druehl, 2002).

The objectives of the current study were to isolate diphlorethohydroxycarmalol (DPHC) from *Ishige okamurae* based

Abbreviations: DPHC, diphlorethohydroxycarmalol; UV, ultraviolet; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide; PI, propidium iodide; OD, optical densities; DMSO, dimethylsulfoxide; PBS, phosphate buffer saline; LMPA, low melting point agarose; NMPA, normal melting point agarose.

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on the results of NMR and other analytical data, and to evaluate its inhibitory effect of melanogenesis and protective effects against UV-B radiation-induced cell damage. In order to select a proper sample having higher activity, we assessed the tyrosinase inhibitory effect of 80% methanolic extracts from 21 species of marine algae; as a result, *I. okamurae* was selected for further experiments, owing to its higher tyrosinase inhibitory activity.

2. Materials and methods

2.1. Materials

The marine algae were 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. Mushroom tyrosinase, L-tyrosine, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT), Hoechst 33342, and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). And other chemicals used were 99% or greater purity.

2.2. Extraction procedure of 80% methanolic extracts from marine algae

The marine algae samples were pulverized into powder using a grinder. The algal powder (1 g) was extracted with 80% methanol (100 ml) at room temperature for 24 h and filtrated. After filtration, the methanolic extracts were evaporated to dryness under vacuum. Concentration of the tested extracts was adjusted to 100 μg/ml. This extracts were used for further biological study.

2.3. Isolation and structural identification of active compounds

The algal powder (500 g) was extracted three times with 80% methanol and filtered. The filtrate was then evaporated at 40 °C to obtain the methanol extract, which was dissolved in water, then partitioned with *n*-hexane, chloroform, ethyl acetate, and butanol. Since the ethyl acetate fraction exhibited higher tyrosinase inhibitory effects than that of other fractions, the active compound was extracted from the ethyl acetate fraction using a silica gel and Sephadex LH-20 column chromatography. The active compound was finally purified by HPLC, and the structure of the compound was identified by comparing the NMR spectral data and HRESI-MS analysis with those in existing literature (Li and Glombitza, 1991; Heo and Jeon, 2009).

2.4. Inhibitory effect of mushroom tyrosinase

Tyrosinase inhibitory activity was performed according to the method of Vanni et al. (1990) with minor 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.

2.5. Inhibitory effect of melanin synthesis

Melanin contents were measured according to the method of Tsuboi et al. (1998) with a slightly modification. The B16F10 cells were placed in six-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. And 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.

2.6. Cell culture

The Laboratory of Surface Science of Korea Advanced Institute of Science and Technology (KAIST) kindly provided the human fibroblast cell line. Briefly, primary human dermal fibroblasts cells were obtained from foreskin of healthy volunteers, age 20–30 years. The skin was minced, followed by incubation with collagenase (1 mg/ml in DMEM medium) for 1–2 h at 37 °C. Then, collagenase was removed by washing with Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Rockville, MD). The isolated cells were allowed to attach on plastic plates and cultured at 37 °C in an incubator with humidified atmosphere of 5% CO_2. Cells were cultured in DMEM medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 unit/ml). After six and eight passages the fibroblasts were used for experiments.

2.7. UV-B irradiation

UV-B radiation was carried out using a VL-6LM UV lamp (Vilber Lourmat, Torcy, France), which emits most of its energy within the UV-B range (280–315 nm). Fibroblast cells (1×10^5 cells/ml) were irradiated in complete media with different doses of UV-B (10–100 mJ/cm^2) to find optimum dose. The dose of UV-B irradiation was monitored using a model IL-1700 Research Radiometer (International Light, Peabody, USA) with a radiometer sensor for UV-B (SED240). Depending on our findings, finally, 50 mJ/cm^2 irradiation was set in this study.

2.8. 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.

2.9. Assessment of cell viability

Cell viability was then estimated via an 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 800g, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide (DMSO), and the absorbance was measured via ELISA at a wavelength of 540 nm. Relative cell viability was evaluated in accordance with the quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2.10. Determination of UV-B induced DNA damage by comet assay

Comet assay was performed to determine the irradiative DNA damage (Singh, 2000). The number of cultured cells was adjusted as 1×10^5 cells/ml and the cells were incubated with various concentrations of the compound for 30 min at 37 °C. After preincubation, the cells were centrifuged at 300*g* for 5 min and washed using phosphate buffer saline (PBS). Then, the cells were resuspended in PBS and were exposed to UV-B (50 mJ/cm^2). The non-irradiated control cells were resuspended only in PBS without UV-B radiation. The cells were washed with 1 ml of PBS and centrifuged. The cell suspension was mixed with 75 μl of 0.5% low melting point agarose (LMPA), and added to the slides precoated with 1.0% normal melting point agarose (NMPA). After keeping them for 10 min at 4 °C, the slides were covered with another 75 μl of 0.5% LMPA and kept for 40 min at 4 °C for solidification of the agarose. And the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine and 1% Triton X-100) for 1 h at 4 °C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4 °C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4 °C, and then treated with ethanol for another 5 min before staining with 50 μl of ethidium bromide (20 μg/ml). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

2.11. Microscopic analysis for death cells

The type of cell death induced by UV-B was determined by fluorescent microscopy after staining with Hoechst 33342 and propidium iodide (PI), as described by Naito (2001). The fibroblasts were placed in 24-well plates at a concentration of 1×10^5 cells/ml. Sixteen hours after plating, the cells were exposed to UV-B