Effect of Phlorotannins Isolated From *Eisenia bicyclis* on Melanogenesis in Mouse B16 Melanoma Cells

Yuki Ohno¹, Shiori Kondo², Kiho Tajima², Toshiyuki Shibata³, and Tomohiro Itoh¹

**Abstract**
Phlorotannins isolated from brown algae, such as *Eisenia bicyclis*, have positive physiological effects, including anti-cancer, anti-inflammatory, and anti-Alzheimer’s disease. Although phlorotannins have been shown to inhibit tyrosinase, an enzyme essential for melanogenesis, their effect on melanogenesis remains unexplored. Thus, we isolated phlorotannins from *E. bicyclis* and examined their effects on α-melanocyte-stimulating hormone (α-MSH)-induced melanogenesis in murine B16 melanoma cells. Both fucofuroeckol-A (FF-A) and phlorofucofuroeckol-A (PFF-A) suppressed α-MSH-induced melanogenesis. Neither inhibited human tyrosinase (TYR) activity, but both inhibited tyrosinase-related protein-2 activity. FF-A downregulated the expression of microphthalmia-associated transcription factor and TYR, which subsequently suppressed melanin production. These results suggest that phlorotannins could be beneficial as melanin control drugs for hyperpigmentation disorders.

**Keywords**
*Eisenia bicyclis*, phenolics, pholorotannins, fucofuroeckol-A, phlorofucofuroeckol-A, melanogenesis, B16 melanoma cells

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The epidermis is composed of multiple layers that serve as protection against external factors such as pathogens and UV radiation (UVR).¹ Melanocytes, located in the basal layer of the epidermis, play a key role in the defense against UVR by producing melanin through a complex process called melanogenesis.¹² Although the melanin pigment is crucial in the protection of human skin, excessive melanin accumulation can induce skin disorders, including freckles and melasma.³,⁴ Thus, effective approaches to inhibit melanin synthesis are required.

Upon UVR exposure, the melanogenesis signaling pathway is initiated by several melanogenic factors, including α-melanocyte-stimulating hormone (α-MSH) and endothelin-1.⁵⁶ When α-MSH is cleaved from pro-opiomelanocortin, it increases the levels of intracellular cyclic adenosine monophosphate (cAMP) via the melanocortin 1 receptor. This activates the cAMP-protein kinase A (PKA) pathway and subsequently the downstream molecule, microphthalmia-associated transcription factor (MITF), which regulates the mRNA expression of melanogenesis enzymes such as tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1) and 2 (TRP-2).⁷ TYR acts as a rate-limiting enzyme and regulates melanogenesis by catalyzing hydroxylation.⁸ Therefore, most skin whitening agents, such as hydroquinone, kojic acid, and ascorbic acid, target TYR. However, allergic and toxic effects on the skin have been reported when these agents are used in high doses and for a long time.⁹-¹¹ Accordingly, many researchers are currently focused on investigating natural skin whitening compounds with fewer side effects.

*Eisenia bicyclis* is a perennial brown alga that inhabits the coastal areas of Japan and Korea. It contains various compounds such as polysaccharides, tripeptides, and phlorotannins.¹²-¹⁴ Phlorotannins are responsible for its anti-cancer,¹⁵ anti-inflammatory,¹⁶ anti-Alzheimer’s disease,¹⁷ and anti-tyrosinase¹⁸ activities. However, the effects of phlorotannins isolated from *E. bicyclis* on melanogenesis remain unknown. Thus, this study investigated the anti-melanogenesis effects of extracts derived from *E. bicyclis*.

¹Laboratory for Molecular Chemistry of Aquatic Materials, Department of Life Sciences, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie, Japan
²Department of Life Sciences and Chemistry, Faculty of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie, Japan
³Laboratory for Quality in Marine Products, Department of Life Sciences, Graduate School of Bioresources, Mie University, Kurimamachiya, Tsu 514-8507, Japan.

**Corresponding Author:**
Tomohiro Itoh, Laboratory for Molecular Chemistry of Aquatic Materials, Department of Life Sciences, Graduate School of Bioresources, Mie University, Kurimamachiya, Tsu 514-8507, Japan.
Email: titoh@bio.mie-u.ac.jp
Results and Discussion

Regardless of age or sex, melanin control drugs are commonly used to prevent skin disorders such as freckles and melasma. However, the long-term use of whitening agents such as hydroquinone, kojic acid, and ascorbic acid can lead to skin diseases. Hence, many researchers are currently focused on searching for safe and effective natural compounds that can serve as substitutes for melanin control drugs. Recently, brown algae have attracted considerable attention of the cosmetic industry because they contain various biologically active compounds. Although the tyrosinase inhibitory effect of phlorotannins isolated from brown algae has been reported, their impact on the regulatory factors involved in melanin synthesis remains unclear. In the present study, we evaluated the inhibitory activity of phlorotannins isolated from E. bicyclis on melanogenesis and investigated the mechanisms underpinning these functions in melanoma cells.

To determine the cytotoxicity of the prepared crude phlorotannins (C-Phlo) and purified compounds, we performed a cell proliferation assay using WST-1 reagents. C-Phlo had no dramatic influence on cell viability in the concentration range of 10–100 µg/mL (Figure 1(A)). Moreover, among the 6 phlorotannins, only fucofuroeckol-A (FF-A) had an impact on cell viability (79.3%) when used at a concentration of 50 µM compared with the control Figure 1(B). Thus, phlorotannin concentrations of up to 50 µM were chosen for the subsequent experiments.

![Figure 1](image1.png)

**Figure 1.** The effect of crude phlorotannins and 6 purified compounds on B16-4A5 cell viability. Cells (2.0 × 10⁵ cells/mL) were treated with either (A) crude phlorotannins (C-Phlo, 10–100 µg/mL) or (B) six purified compounds (25 or 50 µM). Cell viability was determined using a WST-1 cell proliferation assay. Data represent the mean ± SE (n = 12).

![Figure 2](image2.png)

**Figure 2.** The effect of crude phlorotannins (C-Phlo) and six compounds on melanin content in B16-4A5 cells. Cells (5.0 × 10⁴ cells/mL) were pretreated with either (A) C-Phlo (10, 100 µg/mL) or (B) six compounds (25 or 50 µM) for 2 hours and melanin content was measured after incubation with α-MSH (2 µM) for 72 hours. Arbutin (200 µM) was used as a positive control. Data represent the mean ± SE and statistical analysis was conducted using the Tukey-Kramer test (n = 12, P < 0.01).
Although melanin is necessary to protect skin cells from UVR damage, excessive melanin production and accumulation induce hyperpigmentation. Thus, as a next step, we examined whether C-Phlo extracted from *E. bicyclis* suppresses α-MSH-induced melanogenesis. As shown in Figure 2(A), C-Phlo significantly decreased melanin production in a dose-dependent manner. To confirm whether the phlorotannins contained in C-Phlo decreased melanin content, we repeated the experiment using the purified phlorotannins. All six phlorotannins exhibited anti-melanogenesis activity, and the effect was similar to that of arbutin treatment (Figure 2(B)).

Although melanogenesis is a complicated process and is regulated by various factors, targeting TYR, the rate-limiting enzyme, is an easy strategy for inhibiting hyperpigmentation. It has been reported that phlorotannins such as phloroglucinol, eckol, and dieckol inhibit melanin production by targeting TYR. In addition, it has been reported that chlorofucofu-rocokeol-A (PFF-A), which is less effective, also acts as a TYR inhibitor. Therefore, among the six phlorotannins isolated from C-Phlo, we focused on PFF-A (for which the anti-melanogenesis mechanism has not been elucidated) and FF-A (which has a similar structure to PFF-A and has not yet been reported) for subsequent experiments. Although mushroom tyrosinase (mTyr) has been used for TYR activity assays in many reports, we did not consider it to be ideal for our experiments because the structural characteristics of mTyr and TYR are different. Therefore, we evaluated the inhibitory effects of C-Phlo, FF-A, and PFF-A by using both mTyr and TYR. C-Phlo significantly attenuated mTyr activity in a dose-dependent manner (Figure 3(A)). Similarly, FF-A suppressed mTyr activity by approximately 20%, although the difference was not statistically significant (Figure 3(B)).

![Figure 3](image-url)

**Figure 3.** The effect of (A) crude phlorotannins (C-Phlo) or (B) FF-A and PFF-A, on tyrosinase activity. L-DOPA (15%) was incubated with C-Phlo (10, 100 µg/mL), FF-A, or PFF-A (10, 50 µM) at 37 °C for 10 minutes. After incubation, either mushroom tyrosinase (mTyr: 2,400 units/mg) or crude enzyme solution derived from HMV-II cells (TYR) was added at 37 °C for 15 minutes. Data represent the mean ± SE and statistical analysis was conducted using the Tukey-Kramer test (*n* = 12, *P* < 0.01).
hand, PFF-A did not suppress either mTyr or TYR activity (Figure 3(B)). Although Shim et al. and Manandhar et al. had previously shown that FF-A and PFF-A notably inhibit mTyr,23,24 our analysis showed different results. We thus hypothesized that FF-A and PFF-A might exert their anti-melanogenesis activity by targeting TRP-2 activity and we therefore performed inhibition analyses. Compared to the cells stimulated with α-MSH (positive control), TRP-2 activity was significantly downregulated, to a similar extent, by either FF-A or PFF-A (Figure 4).

To elucidate the molecular mechanism underlying the anti-melanogenesis effect of FF-A and PFF-A, we examined the mRNA expression of crucial melanogenic genes in B16 cells treated with FF-A and PFF-A, including Mitf, Tyr, Trp-1, and Trp-2, using real-time qPCR. The results showed that FF-A significantly downregulated the mRNA levels of Mitf and Tyr in the α-MSH-induced B16 cells (Figure 5). In addition, the protein expression levels of Mitf and Tyr in cells treated with FF-A also decreased (Figure 6). On the other hand, PFF-A-treated cells did not show any change in mRNA and protein expression compared to the untreated cells (Figures 5 and 6).

During melanogenesis, α-MSH stimulation results in the activation of the cAMP/PKA/CREB response element-binding protein (CREB) signaling pathway. CREB binds to and activates the Mitf promoter via the cAMP response element. Seo et al. have previously reported that Leathesia difformis extracts exert their anti-melanogenesis activity via the cAMP/PKA/CREB signaling pathway located upstream of Mitf.25 Therefore, we examined the levels of CREB to gain further insights into the mechanism underlying the suppression of melanogenesis in cells treated with FF-A. However, FF-A did not attenuate CREB phosphorylation after α-MSH stimulation (Figure 7). Since the translocation of p-CREB to the nucleus is observed in melanogenesis in B16 cells treated with forskolin,26 we next further analyzed the levels of p-CREB translocation to the nucleus. However, FFA did not inhibit the nuclear translocation of p-CREB (Supplemental Figure 1). Hence, we speculate that FF-A may inhibit either the transcriptional activity or other signaling pathways (such as PAK4, MAP kinases, and etc.) leading to MITF expression. With regard to the inhibitory mechanisms of melanogenesis by FFA, we need further study and would like to show our results in a later report.

In conclusion, melanogenesis is attenuated by FF-A through the suppression of Mitf and Tyr expression in α-MSH-induced B16 cells. However, since Mitf expression is regulated by the CREB transcription factor, further studies are needed to determine the mechanisms of this suppression. In contrast to FF-A, PFF-A did not influence either mRNA or protein expression; however, it reduced melanin production by targeting TRP-2 activity (Figure 8). Therefore, FF-A and PFF-A are useful botanical sources of anti-melanogenesis agents.

Materials and Methods

Antibodies

Antibodies against MITF (#MAB3747) were purchased from Millipore (Burlington, MA, USA), those against TYR (ab52493) and TRP-2 (ab74073) from Abcam (Cambridge, UK), those against TRP-1 sc-166857 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), those against C-Phlo1, and phosphorylated-CREB (Ser 133, #9198) from Cell Signaling Technology (Beverly, MA, USA), and those against β-actin (A1978) from Sigma-Aldrich (St. Louis, MO, USA). ECL anti-rabbit IgG horseradish peroxidase and ECL anti-mouse IgG horseradish peroxidase were purchased from GE Healthcare (Chicago, IL, USA).

Extraction and Purification

The brown alga Eisenia bicyclis (Kjellman) Setchell was obtained from the coasts of the Itoshima Peninsula (33°63′N, 130°18′E) in Fukuoka, Japan. The air-dried alga was pulverized using an absolute mill (Osaka Chemical, Osaka, Japan) and stored at −30 °C until further use. The preparation of crude phlorotannins (C-Phlo) from algal powder was performed according to the protocol described by Nagayama et al.27 Each phlorotannin was fractionated on a Wakogel C-300HG column (1.5 cm i.d. ×150 cm, Wako Pure Chemical Ind., Osaka, Japan) with chloroform-methanol-water (80:20:2, v/v) as the eluent.28 Further purification of the phlorotannins was conducted using a preparative HPLC system equipped with an Inertsil ODS-3
column (10 mm I.D. × 250 mm, GL Science, Tokyo, Japan), according to a previously described method. Identification of the isolated phlorotannins was carried out using an LC/MS system (6120 Quadrupole LC/MS with 1260 Series HPLC System, Agilent, CA, USA). Elution was performed at a flow rate of 1.0 mL/min using a linear gradient from 20% to 100% methanol for 20 minutes, and maintained for 20 minutes. Each phlorotannin was monitored by electrospray ionization mass spectrometric analysis in the negative mode. The purity of each phlorotannin at 98% or higher was confirmed by 3 dimensional HPLC (SPD-M10AV, Shimadzu, Kyoto, Japan) with an Inertsil ODS-3 column (4.6 mm I.D. × 250 mm, GL Science). The structural formulae of the isolated phlorotannins are shown in Figure 9.

Cell Culture

The mouse melanoma cell line B16 and the human malignant melanoma cell line HMV-II were purchased from the Riken BioResource Research Center (Ibaraki, Japan). The B16 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GE Healthcare, Chicago, IL, USA), and the HMV-II cells in Ham’s F-12 nutrient medium (Sigma-Aldrich, St. Louis, MO, USA), both supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 50 U/mL penicillin-50 µg/mL streptomycin (Thermo Scientific, MA, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

Cell Viability

Cell viability was assessed using a WST-1 cell proliferation assay. B16 cells were seeded in a 96-well plate at a density of 2.0 × 10⁵ cells/mL and pre-cultured for 24 hours. The cells were treated with 10, 25, 50, and 100 µg/mL of C-Phlo, or 25 and 50 µM of each purified phlorotannin, at 37 °C for 24 hours. After incubation, 100 µL of WST-1 (1/10 dilution in DMEM, Takara Bio, Ohtsu, Shiga, Japan) was added to each well for an additional 1 hours. The absorbance was measured at a wavelength of 470 nm using a microplate reader (Varioscan Lux™, Thermo Scientific, MA, USA).

Measurement of Melanin Content

B16 cells were seeded in a 12-well culture plate at a density of 5.0 × 10⁴ cells/mL and cultured for 24 hours. Cells were then treated...
with 10, 25, 50, or 100 µg/mL of C-Phlo; 25 or 50 µM of each phlorotannin; or 200 µM of arbutin as a positive control, for 2 hours. After treatment, cells were stimulated with 2 µM α-MSH (Sigma-Aldrich) for 72 hours. Cells were then washed twice with PBS and lysed with 100 µL of 1 n NaOH at 50 °C for 1 hours. Absorbance was measured at a wavelength of 475 nm using a microplate reader (Varioscan Lux™). The melanin content was calculated from a standard curve prepared using synthetic melanin, and normalized by the protein concentration of each cell, which was determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) as a standard.

Tyrosinase and Tyrosinase-Related Protein-2 Activity

Fully confluent HMV-II cells were washed twice with PBS and centrifuged at 145 × g for 3 minutes. Cell pellets (1.0 × 10⁷ cells) were suspended in 500 µL of RIPA buffer and disrupted using a sonicator (Ultrasonic cell disruptor, Wakenyaku, Kyoto, Japan) on ice. The suspension was further centrifuged at 13,500 × g for 15 minutes at 4 °C to prepare the crude enzyme solution. To measure tyrosinase activity, 50 µL of C-Phlo, FF-A, or PFF-A (final concentration: 10⁻¹⁰⁻¹⁰0 µM) was added to 25 µL of 15% L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich). To measure TRP-2 activity, the same volume of dopachrome was used as a substrate instead of L-DOPA. Dopachrome was prepared by oxidizing L-DOPA with Ag₂O in the presence of 0.1 mM urea in a 96-well plate at 37 °C for 10 minutes. After incubation, either a crude enzyme solution or 1 mg of mushroom tyrosinase (Sigma-Aldrich, 2400 units/mg) in 1 ml of 0.1 M phosphate buffer was added to each well at 37 °C for 15 minutes. The absorbance was measured using a microplate reader, and tyrosinase activity was calculated using the following formula:

$$\text{Tyrosinase TRP-2 activity (\%) = \frac{\text{Absorbance at 475 nm of cells treated with extract}}{\text{Absorbance at 475 nm of untreated cells}} \times 100}$$

RNA Isolation and Quantitative Real-Time PCR

To determine the expression levels of Mitf, Tyr, Trp-1, and Trp-2, total RNA was prepared using TRIzol™ reagent (Life Technologies) and quantified using a NanoDrop Lite (Thermo Fisher Scientific). We prepared cDNA from total RNA samples using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Subsequently, quantitative real-time PCR
was performed using the StepOne™ Real Time PCR System (Applied Biosystems) with Power SYBR® Green PCR master mix (Applied Biosystems) under the following cycling conditions: 45 cycles at 95 °C for 15 s and at 60 °C for 1 minutes. The primer sequences were as follows: Gapdh forward, 5’-GCACCGTCAAGGCTGAGAAC-3’ and reverse, 5’-TGTT-GAAGACGCCAGTGGA-3’; Mitf forward, 5’-ACTATGGCC CAAGGCAGAGCAAC-3’ and reverse, 5’-TCAGAGGCCTACA AGCCAAGGTAAATG-3’; tyrinase forward, 5’-CAAGGTAC AGGGATCCGCAAC-3’ and reverse, 5’-GGTGCAATGGCT TCTGGGTAA-3’; Trp-1-forward, 5’-TGATGCCGCTTTTGATC GAATG-3’ and reverse, 5’-GTTGGTAACCTGGAGGCC AAGATATG-3’; Trp-2-forward, 5’-AACCGCAGAGCAACCT TGCTAC-3’ and reverse, 5’-CTCCAGATTCCATG ACCAC-3’. Gapdh was used as an internal standard.

**Western Blot Analysis**

Whole cell lysates were obtained using RIPA buffer containing protease inhibitors (Roche Diagnostics, GmbH, Mannheim, Germany) and a phosphatase inhibitor (Sigma-Aldrich). Protein concentration was measured using a DC Protein Assay Kit (Bio-Rad). Equal amounts of protein were resuspended in lysis buffer [125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol] and denatured at 98 °C for 5 minutes. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking non-specific binding sites for 1 hours with 5% (w/v) nonfat milk (Becton, Dickinson and Company, Sparks, MD, USA) in Tris-buffered saline containing 0.1% (w/v) Tween 20 (FUJIFILM Wako Pure Chemical Co., Osaka, Japan, TBS-T), the PVDF membrane was incubated overnight at 4 °C with various primary antibodies. The membranes were then washed 3 times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at 25 °C. Immunolabeled proteins were detected using an ECL Prime Chemiluminescence Kit (GE Healthcare, Piscataway, NJ, USA) and a Davinch-Chemi imager™ (CAS-400SM, Davinch-K, Seoul, Korea).

**Statistical Analysis**

All data are expressed as the mean ± standard error (SE). Statistical comparisons between different treatments were assessed using the Tukey-Kramer test, and differences were considered significant when P values were less than 0.01.

**Declaration of Conflicting Interests**

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ORCID IDs
Toshiyuki Shibata https://orcid.org/0000-0001-5639-8511
Tomohiro Itoh https://orcid.org/0000-0003-3647-0955

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