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# Dihydromyricetin from *Ampelopsis grossedentata* inhibits melanogenesis through down-regulation of MAPK, PKA and PKC signaling pathways

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ABSTRACT

The inhibitory effects of dihydromyricetin purified from *Ampelopsis grossedentata* on melanogenesis and its antioxidant characteristics were investigated. Assays of tyrosinase activities and melanin content in B16F10 mouse melanoma cells were carried out spectrophotometrically, and the expression of melanogenesis-related proteins was determined by Western blotting. The possible signaling pathways involved in dihydromyricetin-mediated depigmentation were also examined using specific protein kinase regulators. The results revealed that dihydromyricetin effectively suppresses intracellular tyrosinase activity and decreases melanin amount in cells. Dihydromyricetin also exhibits antioxidant properties and effectively decreases intracellular reactive oxygen species (ROS) and reactive species (RS) levels. Our results indicated that dihydromyricetin inhibits melanogenesis through its antioxidant properties and by downregulating protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK) signaling pathways. The present study indicates that dihydromyricetin has the potential to be developed into a depigmentation skin care product.



## 1. Introduction

Melanin is the pigment responsible for giving characteristic color to the skin, hair and eyes, and it plays an important protective role against ultraviolet (UV) light-induced skin damage. It was reported that overproduction and accumulation of melanin pigment could create several skin problems, such as freckles, melisma, age spots, and post-inflammatory pigmentation [1]. Thus, the inhibition of melanin production has been the focus of medicinal and cosmetic treatments for skin depigmenting and lightening. Melanogenesis is regulated by tyrosinase and is also affected by factors such as UV rays and α-melanocyte-stimulating hormone (α-MSH) [2]. In the melanin production pathway, tyrosinase (monophenol monooxygenase, EC 1.14.18.1) participates in the hydroxylation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA). L-DOPA is further oxidized to its corresponding *o*-quinone [3]. In the past, several tyrosinase inhibitors, such as arbutin [4], azelaic acid [5] and kojic acid [6], were used in skin whitening products to prevent or treat skin pigmentation [7]. However, it was reported that chemical skin depigmenting agents can have significant side effects, including genotoxicity caused by arbutin [8], skin irritation or erythema caused by azelaic acid [9] and pigmented contact dermatitis caused by kojic acid [10]. Therefore, the search for a safe and effective skin depigmenting agent is still ongoing in the field of cosmetic research and development.

It has been reported that phosphorylation of mitogen-activated protein kinases (MAPK) and signaling cascades of extracellular

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responsive kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 regulate melanin production [11,12]. Therefore, some skin whitening agents can inhibit MITF transcriptional activity through downregulation of MAPK-mediated MITF phosphorylation. On the other hand, α-MSH binds to its receptor, increasing cyclic adenine monophosphate (cAMP) levels, and can then activate cAMP-dependent protein kinase (PKA), which is usually used to induce the phosphorylation of cAMP response element-binding protein (CREB) and increase MITF protein levels [13]. Importantly, topical application of a selective protein kinase C (PKC) inhibitor, bisindolylmaleimide, could reduce skin pigmentation [14].

When free radicals are improperly processed during melanogenesis, hydrogen peroxide is produced, causing the production of more damaging hydroxyl radicals and other reactive oxygen species [15], which place melanocytes under high-grade oxidative stress. The contribution of ROS to accelerate melanin production has been studied by using antioxidants such as *N*-acetyl cysteine to abolish UVB-induced melanogenesis [16]. It has also been found that nitric oxide (NO) produced by UV-irradiated keratinocytes stimulates melanin production by increasing the expression of tyrosinase and tyrosinase-related protein 1(TRP-1) [17,18]. The inhibitors of ROS generation and the ROS scavengers may down-regulate UV-induced melanogenesis [19]. In addition, it has been reported that antioxidants or compounds with redox properties can inhibit or delay hyperpigmentation [20] and that reduced glutathione (GSH), an important biological reductant, is involved in regulation of melanin synthesis [21]. Hence, inhibitors of melanogenesis, ROS scavengers and antioxidants have been increasingly used in cosmetics to prevent undesirable skin hyperpigmentation [22].

*Ampelopsis grossedentata* is a medicinal plant widely distributed in mountainous areas of southern China. The dried leaves and stems of *A. grossedentata*, also called vine tea, have been used as herbal medicine or health tea in Asia. It has been reported that *A. grossedentata* possesses a number of pharmacological activities, including anti-inflammatory, antibacterial, antioxidant, hypoglycemic and antithrombotic activities [23]. Dihydromyricetin, also known as ampelopsin, is the major bioactive component from the stems and leaves of *A. grossedentata* [24,25].

Dihydromyricetin displays many pharmacological activities, such as anti-inflammatory, hepatoprotective, antimicrobial and antioxidative properties [26–29]. The high in vivo antioxidant activity of dihydromyricetin encouraged our investigation into its possible anti-melanogenesis activity in melanoma cells.

The aim of the present study was to investigate the effects of dihydromyricetin on melanogenesis in B16F10 murine melanoma cells. Moreover, we examined the inhibitory effect and associated antioxidant characteristics of dihydromyricetin on melanin production by assessing its free radical scavenging activities. Furthermore, the possible signaling pathways involving dihydromyricetin-induced inhibition of melanogenesis were also studied.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All other chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO, USA). The microBCA kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The antibodies were from Santa Cruz Biotech (Santa Cruz, CA, USA), and the ECL reagent was from Millipore (MA, USA). Protein kinase regulators, including GF109203X (classical PKC inhibitor), H89 (cAMP-dependent protein kinase inhibitor), U0126 (MEK 1-inhibitor), SB203580 (p38 MAPK-inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor; JNK inhibitor), and PD98059 (MEK 1/2-inhibitor) were from Tocris (Ellisville, Missouri, USA).

### 2.2. Plant material, extraction and isolation of dihydromyricetin

Dried stems and leaves of *A. grossedentata* were refluxed with ethanol/$H_2O$ (1:1) for 1 h. The ethanol/$H_2O$ extract was concentrated under reduced pressure to evaporate ethanol, and then a large amount of yellow precipitate was collected by filtration from the resulting solution after cooling. The yellow precipitate was further purified on a Sephadex LH-20 column using MeOH as an eluting solvent, and recrystallization in water was subsequently carried out to afford dihydromyricetin. Recrystallizations were carried out five times in water to purify dihydromyricetin based on different solubility in boiled water and cold water. The purity of dihydromyricetin was over 98%, as analyzed by HPLC and NMR spectrum. Melting points were determined on a Yanaco MP-I3 micro melting point apparatus and are uncorrected. Optical rotation was obtained on a JASCO DIP-370 digital polarimeter. UV spectrum was measured on a Hitachi U-3310 spectrophotometer. IR spectrum was recorded on a Nicolet Avatar 320 FT-IR spectrometer. $^1H$, $^{13}C$, and 2D NMR spectra were recorded on a Varian VNMRS 600 MHz spectrometer. EIMS was obtained on Finnigan MAT GCQ spectrometer. Sephadex LH-20 (Amersham Biosciences, Sweden) was used for column chromatography. The optical rotations of dihydromyricetin were measured on a JASCO P1010 digital polarimeter. The UV spectra were recorded on a Hewlett Packard 8453 UV–VIS spectrometer. The IR spectra were obtained on a Perkin Elmer GX FT-IR spectrophotometer. The 1D and 2D NMR experiments were recorded on a Brüker AVANCE 300 MHz spectrometer operating at 300 MHz for protons and 75 MHz for carbon. The mass spectra were recorded on a VG 7070 mass spectrometer operating at 70 eV or on a VG Quattro triple quadrupole mass spectrometer for the electrospray mass spectra [30].

### 2.3. Cell culture and cell viability assay

The B16F10 cells (ATCC CRL-6475, BCRC60031) were obtained from the Bioresource Collection and Research Center (BCRC), Taiwan. The cells were cultured in DMEM with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 IU/50 μg/mL) in a humidified atmosphere containing 5% $CO_2$ and at an air temperature of 37 °C. The cell viability assay was performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The cells were exposed to various concentrations of dihydromyricetin (1, 25, 50 μM) for 24 h, and then the MTT solution was added to the wells. The absorbance of the wells at 570 nm was read using a microplate reader [31].

### 2.4. Assay of mushroom tyrosinase activity

The tyrosinase inhibition experiments were carried out as previously described [32]. The aqueous enzyme solution of mushroom tyrosinase (10 μL; 20 units/1 μL) was added to a 96-well microplate, for a total volume of a 200 μL mixture containing 5 mM L-DOPA, which was dissolved in 50 mM phosphate buffered saline (PBS) (pH 6.8) and dihydromyricetin (1, 25, 50 μM). The assay mixture was incubated at 37 °C for 30 min, and the absorbance of dopachrome at 490 nm was measured.

### 2.5. Measurement of intracellular tyrosinase activity and melanin content

The cells were treated with α-MSH (100 nM) for 24 h and then with various concentrations of dihydromyricetin (1, 25, 50 μM) or arbutin (2 mM) for an additional 24 h. After treatment, the cells were homogenized with a 50 mM PBS (pH 7.5) buffer containing 1.0% Triton X-100 and 0.1 mM PMSF. The cell extracts (100 μL) were