Inhibition of Melanogenesis and Oxidation by Protocatechuic Acid from *Origanum vulgare* (Oregano)

Tzung-Han Chou,[†] Hsiou-Yu Ding,[‡] Rong-Jyh Lin,[§] Jing-Yao Liang,[†,⊥] and Chia-Hua Liang*[,†]

Department of Cosmetic Science and Institute of Cosmetic Science, Chia Nan University of Pharmacy and Science, Tainan 717, Taiwan, Republic of China, Department of Parasitology, Faculty of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China, and Department of Urology, Kuo General Hospital, Tainan 700, Taiwan, Republic of China

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Antioxidant and antimelanogenesis activities of protocatechuic acid (**1**) from *Origanum vulgare* (oregano) were investigated. The antioxidative capacity of **1** was confirmed from its free-radical-scavenging activities, inhibition of lipid peroxidation, and suppression of reactive oxygen species in H_2O_2-induced BNLCL2 cells. The inhibition by **1** of tyrosinase and DOPA oxidase activity and melanin production was possibly related to the down-regulation of melanocortin-1 receptor, microphthalmia-associated transcription factor, tyrosinase, tyrosinase-related proteins-2, and tyrosinase-related proteins-1 expression in α-melanocyte-stimulating hormone-induced B16 cells. After a gel containing **1** was applied to mice, the values of *L** slightly increased, and *a** and erythema-melanin levels of skin were reduced by comparing the values of untreated control groups, indicating **1** can reduce melanin production. These results suggest that **1** may act as an effective quencher of oxidative attackers with antimelanogenesis properties.

Skin pigmentation is essential for protecting human skin against radiation. The overproduction and accumulation of melanin, however, may result in various skin dermatological disorders including melasma, freckles, age spots, and sites of actinic damage or other hyperpigmentation.[1] Genetic background is the most important factor for skin pigmentation. Other nongenetic factors such as hormonal changes, chronic inflammation, aging, and ultraviolet (UV) light also affect skin pigmentation by stimulating the expression of three major enzymes, tyrosinase, tyrosinase-related proteins-1 (TRP-1), and tyrosinase-related proteins-2 (TRP-2), involved in melanogenesis. Previous studies have reported that α-melanocyte-stimulating hormone (α-MSH), also known as the hormone melanocortin-1 (MC1), stimulates adenylate cyclase, producing cyclic adenosine monophosphate (cAMP) through the G protein-coupled melanocortin-1 receptor (MC1R), and subsequent upregulation of microphthalmia-associated transcription factor (MITF).[2] Binding of MITF to the gene promoters of melanogenic enzymes induces melanogenic gene expression. Newly synthesized tyrosinase protein undergoes maturation and activation through multiple mechanisms including copper binding, glycosylation, and phosphorylation.[3]

Melanin synthesis starts with the hydroxylation of L-tyrosine to DOPA and of DOPA to DOPA-quinone. These two reactions are catalyzed by tyrosinase enzymatic activity and constitute major regulatory points common to melanogenic pathways.[2] The conjugation of DOPA-quinone with cysteine or glutathione yields 5-*S*-cysteinyl-DOPA and glutathionyl-DOPA, which are progressively transformed into reddish-yellow pheomelanins through a series of reactions. The spontaneous oxidation of DOPA-quinone in the absence of thiol compounds yields DOPA-chrome, a semistable intermediate for which the decarboxylative rearrangement produces 5,6-dihydroxyindole (DHI). Oxidation and polymerization of DHI units yields eumelanin. However, in the presence of TRP-2/DOPA-chrome tautomerase (TRP-2/DCT), DOPA-chrome is converted into 5,6-dihydroxyindole-2-carboxylic acid (DHICA). TRP-1/DHICA oxidase catalyzes the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid. These two closely related structures, TRP-2/DCT and TRP-1, act to produce unstable quinones that undergo further polymerization, finally yielding brownish-black eumelanins.[4] In an effort to inhibit the activity of tyrosinase, many different types of tyrosinase inhibitors for preventing hyperpigmenation have been developed via either synthesis or isolation from natural sources.[5]

Protocatechuic acid (**1**) is a major benzoic acid derivative found in vegetables, nuts, brown rice, fruits, and herbal medicines and has a strong antioxidative effect.[6] Alpinia protocatechuic acid protects against oxidative damage in vitro and reduces oxidative stress in vivo. Several investigations have demonstrated that **1** prevents lipopolysaccharide-induced rat hepatic damage, inhibits oxidative LDL induced by either copper ion or nitric oxide donors, and suppresses MPP+-induced mitochondrial dysfunction, neurotoxicity, and apoptotic cell death in PC12 cells.[6−8] A recent study was performed to estimate the effectiveness of inflammatory leukocyte-derived oxidative stress potentiated by **1** in mouse skin via a tyrosinase bioactivation pathway.[9] The tyrosinase-derived reactive quinone intermediates of **1** have been found to bind to nucleophilic residues of proteins, the sulfhydryl groups and conjugates of which are recognized as haptens. These intermediates are involved partially in the alteration of cellular immune functions including the migration of oxygen radical-generating leukocytes to inflamed regions.[10] Inhibitory effects of **1** against tyrosinase activity in black rice bran were demonstrated in a preliminary manner using L-tyrosine as the substrate.[11] Antioxidants such as L-ascorbic acid derivatives are used as melanogenesis inhibitory agents, and reduced glutathione (GSH), which acts as a quencher of oxidative insult, is also involved significantly in the inhibition of melanogenesis.[12,13] Numerous melanocytotoxic phenol/catechol derivatives have been shown to be good substrates of either tyrosinase or peroxidase, and the inhibition of peroxidase may play a role in the mechanisms by which these derivatives act, as well as those of depigmenting agents, such as glucocorticoids.[14] Although some studies have demonstrated that the inhibition and scavenging of reactive species are considered to be involved in the down-regulation of hyperpigmentation or inhibition of UV-induced melanogenesis, the depigmenting effect of **1** on the skin is still not clear. Therefore, the purpose of the present study was to identify the activity and action mechanism of **1**, a constituent of *Origanum vulgare* L. (Lamiaceae) (oregano), on melanogenesis and oxidation.

* To whom correspondence should be addressed. Tel: 886-6-2664911-2441. Fax: 886-6-2667324. E-mail: tinna_ling@mail.chna.edu.tw.
[†] Department of Cosmetic Science, Chia Nan University of Pharmacy and Science.
[‡] Institute of Cosmetic Science, Chia Nan University of Pharmacy and Science.
[§] Kaohsiung Medical University.
[⊥] Kuo General Hospital.

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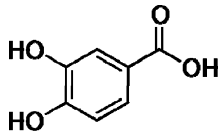

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Results and Discussion

Purification of Protocatechuic Acid (1). The ethanol extract of aerial parts of *O. vulgare* was partitioned against ethyl acetate, and the ethyl acetate-soluble fraction was purified via column chromatography to generate **1**. The structure of **1** was identified on the basis of spectroscopic data (UV, IR, NMR, and MS) analysis.

Effect of Protocatechuic Acid (1) on Free-Radical-Scavenging Capacity. Free-radical-scavenging capacity was evaluated by measuring the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities of **1**. The DPPH radical is one of the most commonly used substrates for the rapid evaluation of antioxidant activity, because of its stability (in radical from) and the simplicity of the assay. The DPPH$^{\bullet}$ assay was employed to study the ability of the investigated **1** to donate a donor of hydrogen atoms or electrons in the transformation of the DPPH radical into its reduced form, DPPH$^{\bullet}$-H. Figure 1A presents the DPPH$^{\bullet}$ scavenging activity of **1** and ascorbic acid (a positive control). In increasing doses of 1, 5, 10, 20, and 100 μg/mL of **1** and ascorbic acid, the values of DPPH$^{\bullet}$ scavenging activity were 43.0%, 68.4%, 70.4%, 74.6%, and 82.7% for **1** and 0.0%, 62.5%, 71.3%, 81.4%, and 87.6% for ascorbic acid, respectively. The EC_{50} values for **1** and ascorbic acid were 2.1 and 4.2 μg/mL, revealing that **1** affected the DPPH$^{\bullet}$ scavenging activity significantly ($p < 0.05$). The preformed radical monocation of ABTS$^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of the hydrogen-donating antioxidants. This decolorization assay is widely used to determine the hydrogen-donating ability of materials.[15] Figure 1B presents the ABTS$^{\bullet+}$ scavenging activity of **1** and ascorbic acid. Compound **1** had a higher ABTS$^{\bullet+}$ scavenging efficiency ($EC_{50} = 4.0$ μg/mL) than ascorbic acid ($EC_{50} = 4.9$ μg/mL). Therefore, protocatechuic acid exhibited clearly DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical-scavenging capabilities, which contribute to its electron-transfer/hydrogen-donating capacity. Also, in the Trolox equivalent antioxidant capacity (TEAC) method, which is based on the ability of several substances to scavenge the ABTS$^{\bullet+}$ cation radical, compound **1** was compared with a standard antioxidant (Trolox), as revealed by a dose−response curve. The antioxidant capacity measured of **1**, measured as a TEAC value, was 1350 mg of Trolox/g. These results demonstrate that the free-radical-scavenging capacity of **1** exceeded that of ascorbic acid.

Numerous studies have found that the reducing capacity of a compound may serve as a useful index of its potential antioxidant acitivity.[16] Accordingly, the reducing power of protocatechuic acid (**1**) was determined to partially elucidate its antioxidant mechanisms. As presented in Figure 1C, unlike ascorbic acid (a positive control), **1** had almost no reducing power, suggesting that the latter cannot properly be regarded as contributing to its antioxidative effect. The chelation of ferrous ions by **1** was also estimated, in which ferrozine can quantitatively form complexes with Fe^{2+}. The presence of compounds that exhibit chelating activity inhibits the formation of complexes.[17] However, **1** did not chelate with ferrous ion.

Inhibition of Lipid Peroxidation by Protocatechuic Acid (1). The ability of **1** to protect against the peroxidation of lipids was investigated using the 2-thiobarbituric acid (TBA) assay and the Fe^{2+}/ascorbate system of induction. The inhibition of lipid peroxidation was evaluated by measuring the formation of malondialdehyde (MDA), using liposomes that comprised an unsaturated phospholipid as an oxidizable substrate.[18] As displayed in Figure 2A, the inhibition of lipid peroxidation by **1** (1, 5, 10, 20, and 100 μg/mL) exceeded that by ascorbic acid, and this activity was dose-dependent. Figure 2B plots the activity of **1** (20 and 100 μg/mL) in inhibiting lipid peroxidation in mouse liver and brain tissues in the thiobarbituric acid reactive substances (TBARS) assay. Following treatment with **1** (20 and 100 μg/mL), the amounts of MDA−TBA products in liver tissue and in brain tissue were ca. 26.0−7.0% and 28.5−14.9% of the respective levels in the untreated control (100%). Treatment by **1** in this TBARS system reduced the degree of formation of MDA−TBA complex more markedly than Trolox. Hence, **1**, with a stronger radical-scavenging capacity, can be used as an electron donor and act as both primary and secondary antioxidant by inhibiting the formation of lipid peroxyl radicals.

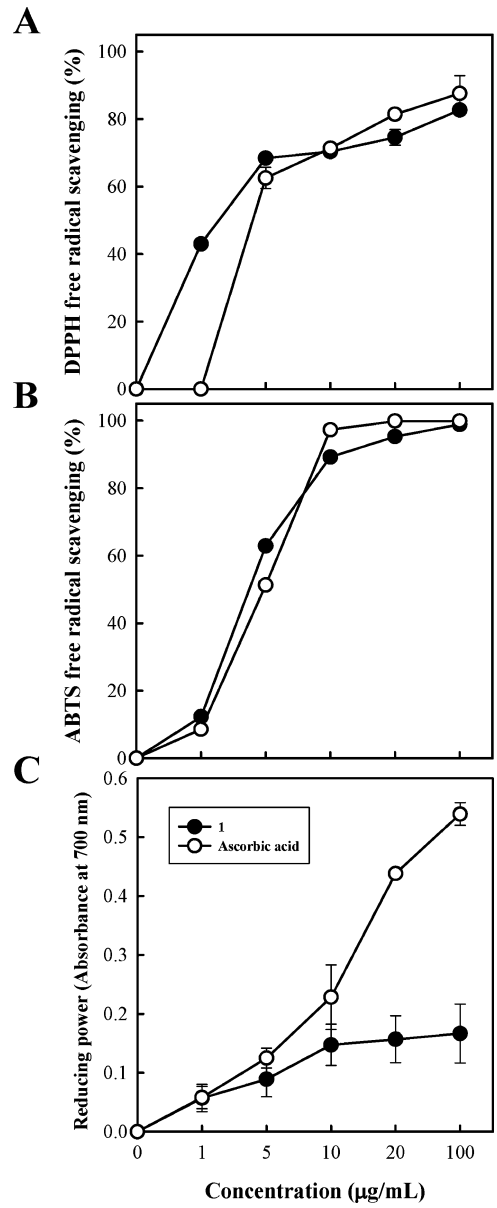

Figure 1. Free-radical-scavenging capacity of protocatechuic acid (**1**) and ascorbic acid (0, 1, 5, 10, 20, and 100 μg/mL) against DPPH$^{\bullet}$ (A) and ABTS$^{\bullet+}$ (B) radicals. (C) Reductive ability of **1** and ascorbic acid (0, 1, 5, 10, 20, and 100 μg/mL). Results are presented as mean ± SD ($n = 3$).

Inhibition of Cellular Reactive Oxygen Species (ROS) by Protocatechuic Acid (1). Radical scavengers are known to have a pro-oxidative effect, because of which they are converted to more reactive or stable radicals upon direct reaction with ROS, and these may contribute to the induction of secondary oxidative damage on target organs.[19] To estimate more accurately the direct scavenging

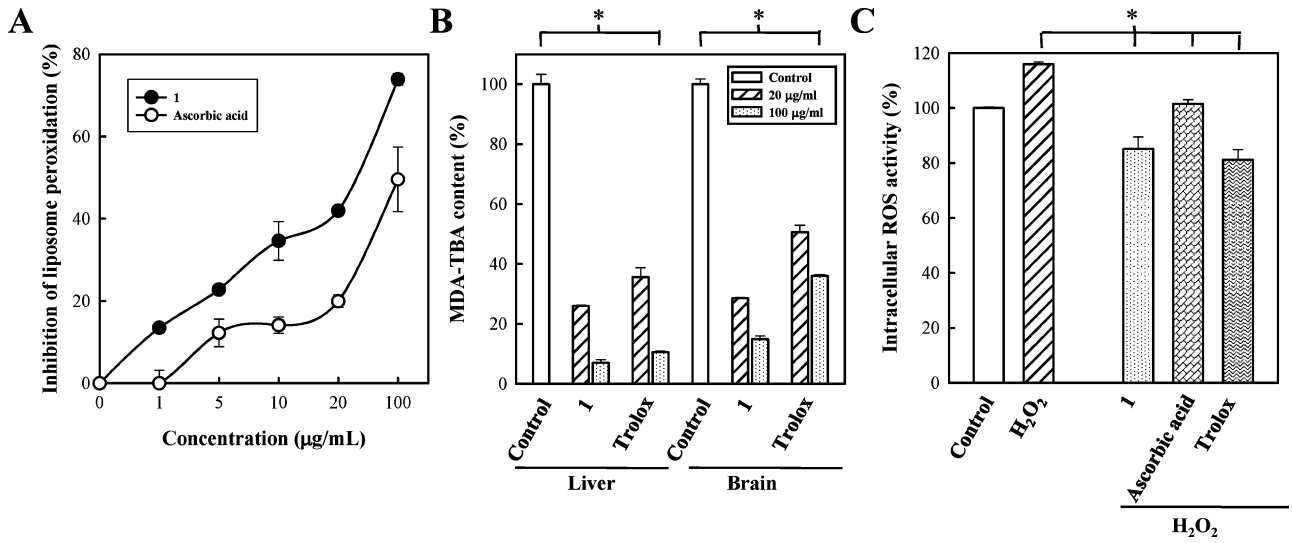

Figure 2. Inhibition of lipid peroxidation and TBARS activities and reduction in intracellular ROS level by protocatechuic acid (**1**). (A) Inhibition of lipid peroxidation in Fe^{2+}/ascorbate system using liposomes model of **1** and ascorbic acid (0, 1, 5, 10, 20, and 100 μg/mL). (B) After treatment of the mouse liver and brain homogenates with **1** and Trolox (20 and 100 μg/mL), the MDA−TBA content was determined by the TBARS assay. (C) Inhibition of cellular ROS production following preincubation with **1**, ascorbic acid, and Trolox (20 μg/mL), determined from intensity of DCFH-DA fluorescence in H_2O_2 (0.1 mM)-induced oxidative stimulation of BNLCL2 cells. Each value is presented as a mean ± SD from independent triplicate experiments.

effect of **1** on cellular radicals and verify the ability of this compound to scavenge free radicals in a cellular environment, the intracellular ROS of treated cells were investigated. The intracellular level of ROS was evaluated using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which is transformed to a fluorescent product, dichlorodihydrofluoroscine (DCF), upon treatment with H_2O_2.[20] In this work, a series of concentrations of H_2O_2 (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mM) was utilized to induce oxidative stress in BNLCL2 cells, and the cell viabilities were determined by a MTT assay. When BNLCL2 cells (1×10^5 cells/mL) were treated with H_2O_2 for 1 h, 0.001−0.5 mM H_2O_2 did not cause them to express significant cyotoxicity, but 1 mM H_2O_2 reduced cell viability by 14.1% (data not shown). In pilot studies, 0.005, 0.01, 0.05, 0.1, and 0.5 mM concentrations of H_2O_2 were used, and the assay at the lowest concentration (0.1 mM H_2O_2) neither changed the mitochondrial potential nor had a deleterious effect on cell metabolism (data not shown). Accordingly, an intracellular ROS test was performed using 0.1 mM H_2O_2. After the H_2O_2-induced oxidative stimulation of BNLCL2 cells, high accumulations of intracellular ROS with respect to the controls were observed (Figure 2C). When BNLCL2 cells were preincubated with **1**, ascorbic acid, and Trolox (20 μg/mL), the production of ROS was suppressed by **1** (85.1%) more than by ascorbic acid (101.5%) but to a similar extent to that by Trolox (81.2%), as determined by comparison with H_2O_2-treated cells (115.9%). An earlier investigation demonstrated that **1** has an antioxidative effect 10-fold more potent than that of α-tocopherol.[21] In the present study, **1** also exhibited antioxidative properties, suggesting its phenolic structure may support the scavenging of electrophiles, the inhibition of lipid peroxidation processes, and the inhibition of cellular oxygen species.

Effect of Protocatechuic Acid (1) on Cell Viability. Despite extensive research on the development of new antimelanogenesis agents, the use of currently available agents is rather limited, because of their high toxicity toward cells, along with their low stability against oxygen and water and poor skin penetration and insufficient efficacy.[22] In the development of alternative hypopigmentation compounds for application to foods or cosmetics, the issue of material safety is critical. For example, the use of hydroquinone, corticosteroids, and mercury-containing products in cosmetics has been banned by the Committee of the European Cosmetics Association and is now limited to formulations that are prescribed by dermatologists.[23,24] In this investigation, the cell viabilities of **1**, arbutin, and ascorbic acid (0, 5, 10, 20, 50, and 100 μg/mL) in melanoma B16 and skin fibroblast Hs68 cells were explored using a MTT assay. As shown in Figure 3A, treatment of B16 and Hs68 cells with various concentrations of **1** for 72 h revealed a cell viability that established the noncytotoxic nature of this compound. However, treatment of B16 and Hs68 cells with 50−100 μg/mL arbutin and ascorbic acid greatly reduced cell viability, especially in the case of treatment of Hs68 cells with ascorbic acid (Figure 3B). The pH value of a culture medium may be decreased with the addition of ascorbic acid, possibly resulting in a decrease of cell viability. Nakamura et al. (2000) established that treatment with a high dose of **1** (20 000 nmol) promotes tumor growth and causes oxidative stress in the female ICR mouse, although some studies have demonstrated that this compound is a strong antioxidant and cytotoxic agent[9,25] and that it induces apoptosis in colon and oral carcinoma cells.[26,27] Nevertheless, the present study demonstrated that that **1** at concentrations of 5−100 μg/mL interacts with B16 and Hs68 cells for 72 h without significant cytotoxicity and, therefore, appears to be safer than arbutin and ascorbic acid.

Inhibition of Tyrosinase Activity and Melanin Formation of Protocatechuic Acid (1). Following a comparison of the ability of **1** for cellular tyrosinase and DOPA oxidase activities and the melanin content in B16 cells with the corresponding values for arbutin and ascorbic acid, innocuous concentrations (10 and 20 μg/mL) of **1** were selected. In antimelanogenesis studies, α-MSH is usually used in the testing process. Melanin products are normally few in number if B16 cells are grown in the absence of α-MSH. In order to easily and clearly determine the influence of **1** on cellular melanin formation, α-MSH was utilized to induce melanin production during the cell testing procedure. As presented in Figure 4A, the percentage inhibitions of cellular tyrosinase activity in B16 cells treated with **1**, arbutin, and ascorbic acid (10 and 20 μg/mL) for 72 h in the presence of 1 μM α-MSH were approximately 22.2−32.9%, 33.1−48.3% and 29.8−63.7%, respectively, as determined by comparison with control cells. The inhibition of the oxidation of DOPA to DOPA-quinone in B16 cells in the presence of α-MSH (1 μM) and **1**, arbutin, and ascorbic acid (10 and 20 μg/mL) for 72 h was significantly increased, as the oxidation rates were 57.4−68.8%, 30.9−41.3%, and 41.7−49.1%, respectively, of

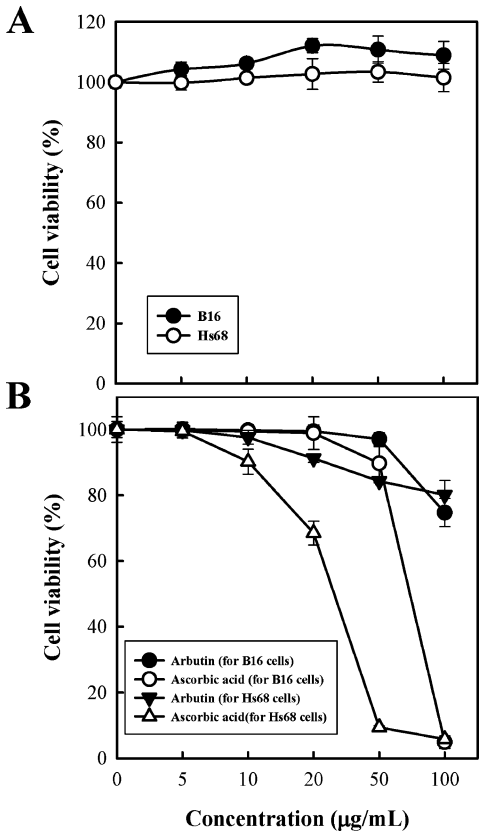

Figure 3. (A) Cell viability of B16 and Hs68 cells in the presence of protocatechuic acid (**1**). After treatment of B16 and Hs68 cells for 72 h with increasing doses (0, 5, 10, 20, 50, and 100 μg/mL) of **1**, cell viability was measured using a MTT assay. (B) Cell viability of B16 and Hs68 cells following treatment with arbutin and ascorbic acid. Cells were treated with 0, 5, 10, 20, 50, and 100 μg/mL of arbutin and ascorbic acid for 72 h, and the percentage cell viability was determined using a MTT assay. Each value is presented as a mean ± SD from independent triplicate experiments.

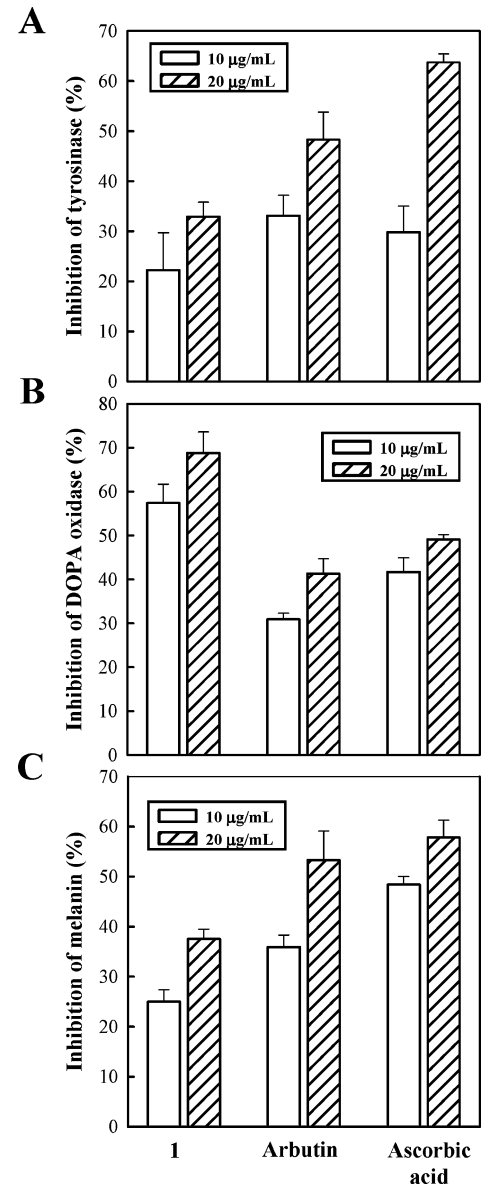

Figure 4. Inhibition of cellular tyrosinase activity (A), DOPA oxidase activity (B), and reduction in melanin content (C) in B16 cells by protocatechuic acid (**1**), arbutin, and ascorbic acid. Cells were treated with **1**, arbutin, and ascorbic acid (10 and 20 μg/mL) for 72 h, and the inhibition of cellular tyrosinase, DOPA oxidase, and melanin synthesis was determined. Each value is presented as a mean ± SD from independent triplicate experiments.

those in the control cells (Figure 4B). The treatment of B16 cells with **1** inhibited DOPA oxidase activity substantially more than either arbutin or ascorbic acid. B16 cells were exposed to **1** (10 and 20 μg/mL) in the presence of α-MSH (1 μM) for 72 h, and the extracellular melanin release was measured. These results suggested that protocatechuic acid (**1**) suppressed α-MSH-induced cellular melanin biosynthesis by inhibiting tyrosinase and DOPA oxidase activities. Some reports have suggested that antioxidants may prevent or delay pigmentation by different mechanisms, such as by scavenging ROS and reactive nitrogen species or by reducing the amount of *o*-quinones or other intermediates formed in melanin biosynthesis, delaying oxidative polymerization.[12−14] The scavenging ROS capacity of **1** has been verified in this study. Another antioxidant, α-tocopherol, is also found to inhibit the oxidative polymerization of phenylalanines such as DOPA during melanin formation.[13] It has been reported that free radicals may up-regulate the mRNA level for tyrosinase in melanin biosynthesis.[12] Therefore, the free-radical-scavenging effect of **1** may play an important role in inhibiting the melanogenic action. In Figure 4C, the addition of **1** to B16 cells reduced the melanin content in a dose-dependent manner. However, the efficacy of the inhibition of melanin synthesis by **1** was less than those of arbutin and ascorbic acid, possibly because these latter compounds have greater activities in reducing the viability of B16 cells.

Mechanism of Inhibition Melanogenesis by Protocatechuic Acid (1) in Vitro and in Vivo. To elucidate whether the inhibition of melanin biosynthesis by **1** was related to the levels of expression of melanogenesis-related proteins, such as MC1R, MITF, tyrosinase, and TRP-2 and TRP-1, B16 cells were exposed to α-MSH (1 μM) in the presence of **1** (20 μg/mL) for 72 h, and protein expression and nuclear morphology were examined by immunofluorescence staining. Treatment with **1** did not alter the nuclear morphology in B16 cells, as determined by DNA staining with Hoechst 33342 (blue) (Figure 5A). The results were supported by a cell viability assay (Figure 3A). The expression of MC1R and MITF is localized in the plasma membrane and in the nuclei of B16 cells, respectively. Tyrosinase, TRP-2, and TRP-1 expression in cells may be revealed by cytoplasmic localization. Immunofluorescence staining of cells with antibodies against MC1R, MITF, tyrosinase, TRP-2, and TRP-1 with FITC (green) indicated their localization within the cells and demonstrated that **1** down-regulated the expression of these proteins (Figure 5A). Similar results were obtained concerning the down-regulation of MC1R, MITF, tyrosinase, TRP-2, and TRP-1 expression by **1** (20 μg/mL) in B16 cells for 72 h, as determined

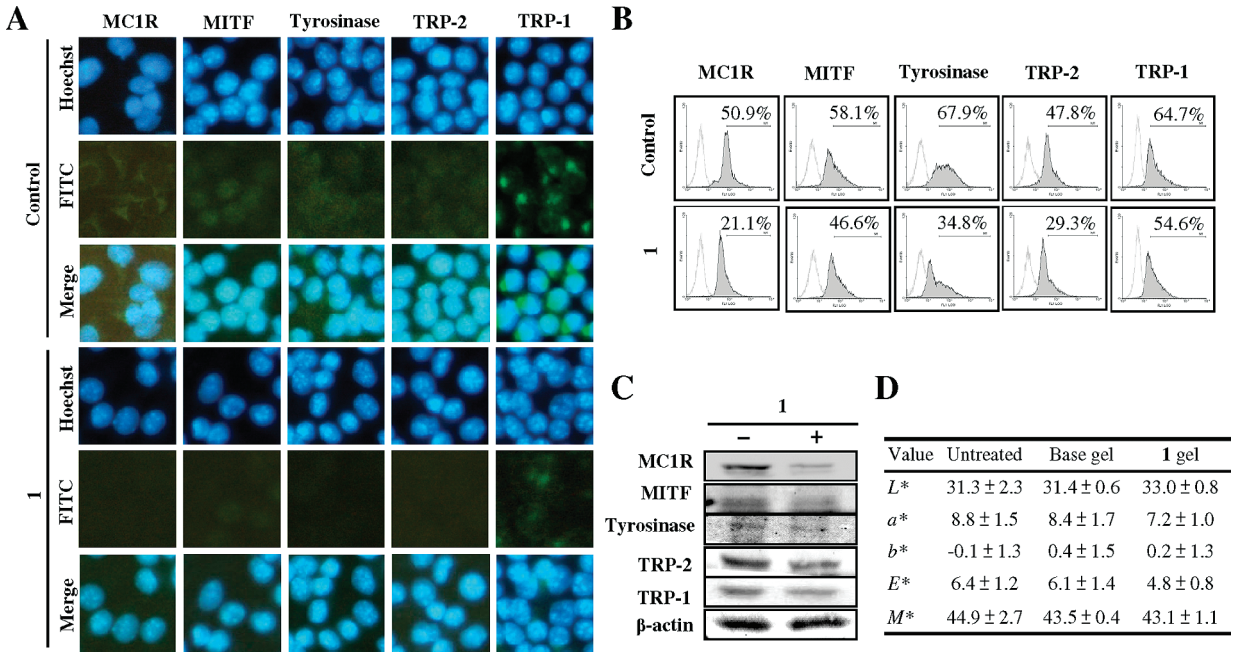

Figure 5. (A) Changes in expression of MC1R, MITF, tyrosinase, TRP-2, and TRP-1 in protocatechuic acid (**1**)-treated B16 cells. Cells were treated with or without 20 μg/mL of **1** for 72 h. Cell nuclei were visualized by staining DNA with Hoechst 33342 (blue) and by immunocytochemical staining of cells with antibodies against MC1R, MITF, tyrosinase, TRP-2, and TRP-1 with FITC (green) before observation under a fluorescent microscope (200×). (B) In a flow cytometry assay, clear histograms represent the isotype control (normal mouse IgG_1), and gray histograms represent the binding of MC1R, MITF, tyrosinase, TRP-2, TRP-1, and β-actin antibodies in control and **1** (20 μg/mL)-treated cells. (C) Western blotting of protein expressions in cells after treatment with **1** (20 μg/mL) for 72 h. (D) L^*, a^*, b^*, E^*, and M^* values of skin before and after 10 days of treatment with **1**-gel. Each animal was divided into two halves through the vertebra. To the right side was applied only base-gel, and to the left side was applied the gel containing **1** ($n = 12$).

using a flow cytometry assay. As shown in Figure 5B, the intensity of MC1R, MITF, tyrosinase, TRP-2, and TRP-1 expression was lower by 50.9% to 21.1%, 58.1% to 46.6%, 67.9% to 34.8%, 47.8% to 29.3%, and 64.7% to 54.6%, respectively, in the B16 cells than in the control cells ($n = 3$). Following inoculation with **1** (20 μg/mL), the protein expression of each of MC1R, MITF, tyrosinase, TRP-2, and TRP-1 in B16 cells was confirmed by the western blotting assay (Figure 5C). However, previous studies have shown that treatment with a high dose of **1** (20 000 nmol) modifies the inflammatory responses in the mouse skin and results in much higher tyrosinase expression than that in an albino mouse.[11] The present investigation found that **1** at low concentrations of 10 and 20 μg/mL inhibited cellular tyrosinase activity, DOPA oxidase activity, and melanin synthesis, and reduced the expression of melanogenesis-related proteins in B16 cells. Although the antioxidation and antimelanogenic effect of **1** was verified, the in vivo efficacy of this compound remains unclear. The modification by **1** of dermatological disorders of melanin formation in epidermis in vivo was determined using a facile and direct method, involving a skin reflectance spectrophotometer. The values L^*, a^*, and b^* and the erythema−melanin (E/M) levels were measured after 12 mice were treated with **1**-gel samples for 10 days, consistent with the growth cycle of mouse fur. In Figure 5D, treatment with **1**-gel slightly increased L^* and b^* and reduced a^* and E/M levels from their corresponding values for the base-gel group and the untreated control group. This suggests that the application of a gel containing protocatechuic acid (**1**) to mice reduces the formation of melanin by the skin. Further studies must be carried out to elucidate directly whether **1** applied topically can reduce human skin pigmentation under physiological conditions.

In summary, the contribution of the antioxidant activity of protocatechuic acid (**1**) to its electron-transfer/hydrogen-donation ability was verified by examining its radical-scavenging capacity and the inhibition of lipid peroxidation and cellular reactive oxygen species by this compound. The results of in vitro testing revealed that **1** inhibits tyrosinase and DOPA oxidase activities and reduces melanin synthesis. Such antimelanogenic phenomena may be associated with the reduction by **1** of protein levels of MC1R, MITF, tyrosinase, TRP-2, and TRP-1 in α-MSH-stimulated cells. The application of a gel containing **1** to mouse skin was found to reduce the production of melanin therein. Moreover, protocatechuic acid (**1**) of a low dosage was noncytotoxic and performed well in both melanogenesis inhibition and antioxidation.

Experimental Section

General Experimental Procedures. Melting points were determined using a Mel-Temp (50/60 cycles, 110−120 V-200 W) apparatus. The IR spectra were obtained using a ThermoMattson, IR 300 spectrometer. NMR spectra were recorded on a Varian Unity-INOVA spectrometer operated at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR with complete proton decoupling. The spectra were observed on CD_3OD, and chemical shifts were recorded as δ values by using tetramethylsilane as an internal standard. The electron impact mass spectra (EIMS) were determined by a Bruker Daltronics APEX II 30e mass spectrometer. A reversed-phase HPLC with the Prep-Phe L column (5 μm particle size, 50 mm × 250 mm) purchased from Shimadzu Corporation was used. A Shimadzu HPLC system (Tokyo, Japan), consisting of a LC-8A pump equipped with a multisolvent delivery system and a SPD-10A UV detector, was employed.

1,1-Diphenyl-2-picrylhydrazyl (DPPH$^\bullet$), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$), 2,5,7,8-tetramethylchroman carboxylic acid (Trolox), 2-thiobarbituric acid (TBA), 4-hydroxyphenyl β-D-glucopyranoside (arbutin), L-ascorbic acid, hydrogen peroxide (H_2O_2), L-3-(3,4-dihydroxyphenyl)alanine (DOPA), ethylene diaminetetraacetic acid disodium salt dehydrate (EDTA), trichloracetic acid (TCA), and α-melanocyte-stimulating hormone (α-MSH) were purchased from Sigma Chemical Co. (St. Louis, MO). 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (ferrozine) was purchased from Fluka (Buchs, Switzerland). Test compounds were dissolved at a concentration of 100 mg/mL in dimethyl sulfoxide (DMSO) stock solution. The stock solution was diluted to

the desired final concentrations with growth medium just before use. The final DMSO concentration did not exceed 0.1%.

Plant Material. Dried *Origanum vulgare* was purchased from a local Chinese drug store, in Kaohsiung, Taiwan, during September 2006. A specimen of the plant was verified by Prof. Hang-Ching Lin of the National Defense Medicinal Center, Taipei, Taiwan, where a voucher specimen (CNUPS No. 950902) has been deposited.

Extraction and Isolation. The dried and powdered aerial parts of *O. vulgare* (10.0 kg) were extracted with 95% ethanol (EtOH) (20 L × 4) at room temperature (32 °C) for 5 h. The combined EtOH extracts were concentrated under reduced pressure to a yield a black syrup (930.0 g), which was dissolved in H_2O−methanol (MeOH) (5:95) solution (0.8 L) and then partitioned (1:1) with *n*-hexane to give an *n*-hexane-soluble fraction (90.0 g). The H_2O−MeOH (5:95) layer was evaporated to remove residual MeOH, and then distilled H_2O (600 mL) was added. This aqueous solution was partitioned with ethyl acetate (EtOAc) to obtain EtOAc-soluble (195.0 g) and H_2O-soluble fractions (630.0 g). The EtOAc-soluble fraction (195.0 g) was subjected to passage over a Sephadex LH-20 column and eluted with MeOH to give four fractions (A−D). The second fraction, B, was subjected to chromatography over silica gel, eluted successively with *n*-hexane−EtOAc (7.5:2.5), *n*-hexane−EtOAc (5:5), *n*-hexane−EtOAc (2.5:7.5), EtOAc, *n*-hexane−EtOAc (7.5:2.5), EtOAc−MeOH (9:1), and MeOH, to afford eight subfractions (1−8). The fourth subfraction was applied to a preparative Lobar RP-8 column eluted with MeOH−H_2O (45:55) and then to a HPLC phenyl column, to yield protocatechuic acid (**1**) (180.8 mg, 0.0194%), using MeOH−H_2O (35:65) as eluent.

Protocatechuic Acid (1): white needles; mp 195−196 °C; this compound exhibited spectroscopic data (IR, NMR, MS) comparable with literature values.[28] The purity of **1** used in the present investigation was 99.4%.

Assay for Free-Radical-Scavenging Activity. For DPPH$^{\bullet}$ radical scavenging activity analysis, various concentrations of **1** (100 μL) were added to 900 μL of an ethanolic solution of DPPH$^{\bullet}$ (0.1 mM). The absorbance (*A*) at 517 nm was measured (Hitachi U-2001, Japan) after the solution had been allowed to stand in the dark for 30 min. Ascorbic acid was used as a standard antioxidant. Controls contained all the reagents except protocatechuic acid (**1**) or the positive control substance. DPPH$^{\bullet}$ scavenging activity (%) = $(1 - A_{sample}/A_{control}) \times 100$. Percentage inhibition was calculated, while EC_{50} values were estimated from the percent inhibition versus concentration plot. The data are presented as mean values ± standard deviation ($n = 3$). For ABTS$^{\bullet+}$ radical-scavenging activity analysis, ABTS$^{\bullet+}$ was dissolved in water to a 7 mM concentration. ABTS$^{\bullet+}$ radical cations were produced by reacting ABTS$^{\bullet+}$ stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The ABTS$^{\bullet+}$ radical cation solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. An aliquot of each agent (0.1 mL) was mixed with 2.9 mL of diluted ABTS$^{\bullet+}$ radical cation solution. After reaction at 30 °C for 20 min, the absorbance at 734 nm was measured. Trolox and ascorbic acid were taken as standard antioxidants. The concentration of antioxidants giving the same percentage change in absorbance of the ABTS$^{\bullet+}$ as that of 1 mg of Trolox was regarded as the Trolox equivalent antioxidant capacity.

Assay for Reducing Power. Briefly, 0.2 mL of **1** was mixed with sodium phosphate buffer (0.5 mL, 0.2 mM, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$) (2.5 mL, 1%, w/v). The mixture was incubated at 50 °C for 20 min. A portion (0.5 mL) of 10% TCA (10%, w/v) was added to the mixture, which was then centrifuged at 10000*g* for 10 min. The upper layer (0.25 mL) was mixed with deionized water (6.59 mL) and ferric chloride ($FeCl_3$) (9.1 mL, 0.1%, w/v). Ascorbic acid was used as a standard antioxidant. Absorbance at 700 nm was measured and directly used to express reducing power.

Assay for Metal Chelating Activity. The chelating activity of **1** for ferrous ions was measured by following the ferrozine method with minor modifications.[17] The reaction mixture contained 10 μL of **1** and 20 μL of $FeCl_2$ (2 mM). After 5 min, the reaction was initiated by the addition of 5 mM ferrozine (40 μL), and the total volume was adjusted to 1 mL with deionized water. Then, the mixture was shaken vigorously and incubated at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. EDTA was used as a positive control. The ability of agents to chelate ferrous ion was calculated using the following equation: chelating activity (%) = $(1 - A_{sample}/A_{control}) \times 100$.

Determination of Liposome Peroxidation. The extent of lipid peroxidation was determined by measuring the color of adduct produced in the reaction between TBA and MDA in the TBA assay, performed with small modifications.[18] For investigation, Fe^{2+}/ascorbate-induced lipid peroxidations were used. As a model system of biological membranes, the commercial preparation of liposomes, pH 5−7, was used. The liposomes, 225−250 nm in diameter, were obtained by dissolving the commercial preparation in demineralized water (1:10) in an ultrasonic bath. For the assay, 32 μL of suspension of liposomes was incubated, together with 11 μL of 10 mM $FeSO_4$, 11 μL of 10 mM ascorbic acid, and appropriate amounts of different concentrations (ranging from 0 to 100 μL/mL of stock solution) of **1** in 1.515 mL of 50 mM Na_2HPO_4−NaH_2PO_4 buffer, pH 7.4 (2.5 mL final solution) at 37 °C for 1 h. Liposome peroxidation was terminated by the reaction of 0.8 mL of 1% TBA and 10% TCA reagent and 106 μL of 0.1 M EDTA and by heating at 100 °C for 20 min. After cooling and centrifugation (2600*g* for 10 min), in order to precipitate proteins, the content of the MDA was determined by measuring the absorbance of adduct at 532 nm. All reactions were carried out as four replicates. Inhibition percent of liposome peroxidation was calculated as $(1 - A_{sample}/A_{control}) \times 100$.

Determination of Thiobarbituric Acid Reactive Substances (TBARS) Formation. Seven-week-old male Institute of Cancer Research (ICR) mice (National Animal Laboratory Center, Taipei, Taiwan) weighing 30−35 g were used. Tissue lipid peroxides produced were monitored by measuring concentrations of TBARS. The concentration of TBARS was measured according to the method described by Ashokkumar and Sudhandiran[29] with a slight modification. In brief, liver and brain tissues were homogenized with a Polytron in ice-cold Tris-HCl buffer (40 mM, pH 7.4) to produce a 1:1 (w/v) tissue homogenate, which was centrifuged at 3000*g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with **1** (0.2 mL) in the presence of $FeSO_4$ (10 μM, 0.1 mL) and ascorbic acid (0.1 mM, 0.1 mL) at 37 °C for 1 h. The reaction was stopped by the addition of TCA (28% w/v, 0.5 mL), followed by TBA (1% w/v, 0.38 mL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the MDA−TBA complex in the supernatant was measured by its absorbance at 532 nm. MDA was used as a standard. The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $(1 - A_{sample}/A_{control}) \times 100$.

Cell Culture. Murine normal embryonic liver BNLCL2 cells, murine melanoma B16, and human skin fibroblast Hs68 cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO, Grand Island, NY), which was supplemented with 10% fetal bovine serum (Hazelton Product, Denver, PA) and 1% penicillin−streptomycin at 37 °C in 5% CO_2.

Measurement of Cellular ROS Levels. Briefly, BNLCL2 cells (1×10^5 cells/mL) were exposed to 20 μg/mL of **1**, ascorbic acid, and Trolox for 24 h. After incubation, cells were detached with trypsin-EDTA and washed once with PBS. Treated and control cells were resuspended in 0.5 mL of PBS containing 10 μM DCFH-DA at 37 °C for 30 min and then incubated with 0.1 mM H_2O_2 (as inducer for ROS production) at 37 °C for 30 min. Fluorescence intensities of DCF were measured by excitation (*ex*) wavelength of 504 nm and emission (*em*) wavelength of 524 nm using a multi-detection fluorescence microplate reader (BioTek, Synergy2).

Cell Viability Assay. B16 and Hs68 cells (1×10^4 cells/well) were each seeded in 100 μL of 96-well plates for at least 24 h prior to use. The cells were treated with 10 and 20 μg/mL of **1**, arbutin, and ascorbic acid for 72 h. The cell survival rate was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the manufacturer's instructions (Promega, Madison, WI). After the cells had been incubated for the indicated times, they were incubated with MTT solution (0.5 mg/mL) for 4 h. The formazan precipitate was dissolved in 100 μL of DMSO, and the absorbance at 570 nm was measured using an automated microplate reader (BioTek, Synergy2). Values are expressed as the mean cell viability as a percentage of that of the vehicle DMSO (0.1% final volume)-treated cultures.

Cellular Tyrosinase Activity Assay. Tyrosinase activity in B16 cells was examined by measuring the rate of oxidation of DOPA. B16 cells (1×10^5 cells/mL) were plated in 1 mL in 24-well multidishes and incubated at least 24 h prior to use. The cells were treated with α-MSH

(1 μM) alone or with α-MSH plus 10 and 20 μg/mL of **1**, arbutin, and ascorbic acid for 72 h. They were washed with PBS and lysed in 900 μL of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and freeze−thawed by incubation at −80 °C for 30 min followed by 25 °C for 25 min and 37 °C for 5 min; 100 μL of 10 mM DOPA was added. Following incubation at 37 °C for 4 h, the absorbance of the agents was measured at 475 nm (BioTek, Synergy2).

Cellular DOPA Oxidase Activity. The DOPA oxidase activity of tyrosinase was measured using a colorimetric assay, which relies on the oxidation of DOPA to DOPA-quinone, which can be reacted with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) to form a pink product with peak absorbance at 508 nm.[30] Briefly, B16 cells (1×10^5 cells/mL) were plated in 1 mL in 24-well multidishes and incubated at least 24 h prior to use. The cells were treated with α-MSH (1 μM) alone or with α-MSH plus 10 and 20 μg/mL of **1**, arbutin, and ascorbic acid for 72 h. The cells were washed in PBS and lysed in 0.2 mL of 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100. They were then frozen at −80 °C for 30 min, followed by 25 °C for 25 min and 37 °C for 5 min. Then, 1.9 mL of prewarmed, freshly prepared substrate (6.3 mM MBTH, 1.1 mM DOPA, 48 mM sodium phosphate buffer, pH 7.1) containing 4% v/v *N*,*N*′-dimethylformamide was added. After incubation at 37 °C for 4 h, the absorbance of agents was measured at 508 nm (BioTek, Synergy2).

Measurement of Cellular Melanin. Melanin content was used as an index of melanogenesis, and the extracellular melanin release was measured as described previously[31] with slight modification. Briefly, B16 cells (1×10^5 cells/mL) were plated in 3 mL in six-well plates for 24 h. α-MSH (1 μM) was then added, and cells were treated with 10 and 20 μg/mL of **1**, arbutin, and ascorbic acid in phenol red-free DMEM for 72 h. Then 100 μL aliquots of medium were placed in 96-well plates, and optical density was measured at 405 nm (BioTek, Synergy2). Melanin production was expressed as a percentage of that of the vehicle DMSO (0.1% final volume)-treated control.

Assay of MC1R, MITF, Tyrosinase, TRP-1, and TRP-2 Expression. B16 cells (1×10^5 cells/mL) were treated with α-MSH (1 μM) alone (the control group) or with α-MSH plus a 20 μg/mL concentration of **1** for 72 h, and the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/0.1% sodium citrate, and stained for 45 min at 4 °C using mouse anti-MC1R, MITF, tyrosinase, and TRP-1 and TRP-2 antibodies (1:300) (Santa Cruz, CA) in PBS, containing 0.5% BSA (PBS−BSA) and 0.1% sodium azide (Sigma-Aldrich). The cells were then washed twice in cold PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:500) (Santa Cruz, CA) at 4 °C for 30 min. They were washed with cold PBS and fixed in 4% paraformaldehyde. The cell nuclei were stained using 0.1 μg/mL of Hoechst 33342 (Promega). Immunofluorescence-stained protein expressions in cells was viewed under an inverted fluorescent microscope (Nikon, TE2000-U, Japan). For evaluation of protein expression by flow cytometry, specific protein expression was analyzed using a FACScan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA) using WinMDI software. For western blotting, cells were harvested, washed with PBS, and incubated for 20 min at 4 °C in 1 mL of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 5 mM EGTA (ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cell lysates containing 40 μg of solubilized protein were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane. The membranes were blocked in 5% skim milk. Blots were incubated with the antibodies against MC1R, MITF, tyrosinase, TRP-1, TRP-2, and β-actin. The membranes were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Blotted antibodies were visualized by the chemiluminescence method (ECL kit, Amersham).

Animals and Treatment. Carbopol 940, a poly(acrylic acid) polymer, was a donated sample from Lubrizol Advanced Materials, Inc., Cleveland, OH. The gel samples that contained **1** were prepared by dispersing 1% Carbopol 940 in a mixture of water. Protocatechuic acid (**1**) with a concentration of 0.02% w/w was added with the Carbopol 940 power to a normal saline solution (0.9% aqueous NaCl), which was magnetically stirred for 6 h. The 0.02% was the effective concentration of **1** on the inhibition of cellular melanin synthesis. The dispersions were then neutralized (pH 7.4) and the viscosity increased by adding 0.01% triethanolamine. The melanin content after treatment with **1** was investigated in the C57BL/6J mouse model with minor modifications.[32] Five-week-old mice, weighing around 20−25 g, were obtained from the National Animal Laboratory Center, Taipei, Taiwan. Throughout the experiment, animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2 °C and with alternating day and night cycles of 12 h. The animals were acclimatized for seven days prior to the experiment. After their fur had been removed, the animals were given one day's rest, and then each animal was divided into two sides along the vertebra of the back: the right side was smeared only with 0.1 g of gel samples (control), while the left side was smeared with 0.1 g of **1**-gel samples (experimental). Twelve animals were smeared daily, and the color values following 10 days of treatment with the gel sample and the **1**-gel samples were determined. A colormeter (DermaScan High Frequency Ultrasound, DSM II Colormeter, Crypro LN2, DermaLab and Dermaspectrometer, Cortex Technology) was used to evaluate optical density. In its standard configuration, the instrument features CIE-Lab (WLED optimized), *E*/*M*, and red-green-blue (RGB). In this system, the color value (or gray scale) is represented by *L**. The term *a** denotes color along the red/green axis, and *b** is color on the yellow/blue axis. An image analysis program was used to quantify the pigment distribution in terms of *E*/*M* indices, based on an evaluation and comparison of RGB color components inside the image color blocks. The colormeter was adopted to determine *L**, *a**, *b**, and *E*/*M* values at three locations on each specimen before and after it was tested.

Statistical Analysis. The experimental results were presented as mean ± SD. Statistically significant differences were determined using independent and paired Student's *t*-test on unpaired and paired samples, respectively. A value of $p < 0.05$ was regarded as significant in all experiments. Data were analyzed and relevant figures plotted using SigmaPlot software, Version 8.0 (San Rafael, CA).

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References and Notes

(1) Kim, Y. J. *Biol. Pharm. Bull.* **2007**, *30*, 1052–1055.
(2) Cardinali, G.; Ceccarelli, S.; Kovacs, D.; Aspite, N.; Lotti, L. V.; Torrisi, M. R.; Picardo, M. *J. Invest. Dermatol.* **2005**, *125*, 1190–1199.
(3) Wichers, H. J.; Recourt, K.; Hendriks, M.; Ebbelaar, C. E.; Biancone, G.; Hoeberichts, F. A.; Mooibroek, H.; Soler-Rivas, C. *Appl. Microbiol. Biotechnol.* **2003**, *61*, 336–341.
(4) An, S. M.; Lee, S. I.; Choi, S. W.; Moon, S. W.; Boo, Y. C. *Br. J. Dermatol.* **2008**, *159*, 292–299.
(5) Yoon, N. Y.; Eom, T. K.; Kim, M. M.; Kim, S. K. *J. Agric. Food Chem.* **2009**, *57*, 4124–4129.
(6) Lin, W. L.; Hsieh, Y. J.; Chou, F. P.; Wang, C. J.; Cheng, M. T.; Tseng, T. H. *Arch. Toxicol.* **2003**, *77*, 42–47.
(7) Yan, J. J.; Jung, J. S.; Hong, Y. J.; Moon, Y. S.; Suh, H. W.; Kim, Y. H.; Yun-Choi, H. S.; Song, D. K. *Biol. Pharm. Bull.* **2004**, *27*, 2024–2027.
(8) Shui, G.; Bao, Y. M.; Bo, J.; An, L. J. *Eur. J. Pharmacol.* **2006**, *538*, 73–79.
(9) Nakamura, Y.; Torikai, K.; Ohto, Y.; Murakami, A.; Tanaka, T.; Ohigashi, H. *Carcinogenesis* **2000**, *21*, 1899–1907.
(10) Nakamura, Y.; Torikai, K.; Ohigashi, H. *Free Radical Biol. Med.* **2001**, *30*, 967–978.
(11) Miyazawa, M.; Oshima, T.; Koshio, K.; Itsuzaki, Y.; Anzai, J. *J. Agric. Food Chem.* **2003**, *51*, 6953–6956.
(12) Rout, S.; Banerjee, R. *Biores. Technol.* **2007**, *98*, 3159–3163.
(13) Hachinohe, M.; Matsumoto, H. *J. Chem. Ecol.* **2005**, *31*, 237–246.
(14) Kasraee, B. *Dermatology* **2002**, *205*, 329–339.
(15) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.
(16) Barreira, J. C.; Ferreira, I. C.; Oliveira, M. B.; Pereira, J. A. *Food Chem. Toxicol.* **2008**, *46*, 2230–2235.
(17) Decker, E. A.; Welch, B. *J. Agric. Food Chem.* **1990**, *38*, 674–677.
(18) Mimica-Dukic, N.; Bozin, B.; Sokovic, M.; Simin, N. *J. Agric. Food Chem.* **2004**, *52*, 2485–2489.
(19) Penna, C.; Mancardi, D.; Rastaldo, R.; Pagliaro, P. *Biochim. Biophys. Acta* **2009**, *1787*, 781–793.

(20) Nakajima, Y.; Inokuchi, Y.; Shimazawa, M.; Otsubo, K.; Ishibashi, T.; Hara, H. *J. Pharm. Pharmacol.* **2008**, *60*, 1365–1374.
(21) Ueda, J.; Saito, N.; Shimazu, Y.; Ozawa, T. *Arch. Biochem. Biophys.* **1996**, *333*, 377–384.
(22) Nakagawa, M.; Kawai, K.; Kawai, K. *Contact Dermatitis* **1995**, *32*, 9–13.
(23) Chen, Y. R.; Chiou, R. Y. Y.; Lin, T. Y.; Huang, C. P.; Tang, W. C.; Chen, S. T.; Lin, S. B. *J. Agric. Food Chem.* **2009**, *57*, 2200–2205.
(24) Chu, H. L.; Wang, B. S.; Duh, P. D. *J. Agric. Food Chem.* **2009**, *57*, 7072–7077.
(25) Tseng, T. H.; Hsu, J. D.; Lo, M. H.; Chu, C. Y.; Chou, F. P.; Huang, C. L.; Wang, C. J. *Cancer Lett.* **1998**, *126*, 199–207.
(26) Babich, H.; Sedletcaia, A.; Kenigsberg, B. *Pharmacol. Toxicol.* **2002**, *91*, 245–253.
(27) Kampa, M.; Alexaki, V. I.; Notas, G.; Nifli, A. P.; Nistikaki, A.; Hatzoglou, A.; Bakogeorgou, E.; Kouimtzoglou, E.; Blekas, G.; Boskou, D.; Gravanis, A.; Castanas, E. *Breast Cancer Res.* **2004**, *6*, R63–74.
(28) An, L. J.; Guan, S.; Shi, G. F.; Bao, Y. M.; Duan, Y. L.; Jiang, B. *Food Chem. Toxicol.* **2006**, *44*, 436–443.
(29) Ashokkumar, P.; Sudhandiran, G. *Biomed. Pharmacother.* **2008**, *62*, 590–597.
(30) Winder, A. J. *J. Biochem. Biophys. Methods* **1994**, *28*, 173–183.
(31) Jee, S. H.; Lee, S. Y.; Chiu, H. C.; Chang, C. C.; Chen, T. J. *Biochem. Biophys. Res. Commun.* **1994**, *199*, 1407–1412.
(32) Kasraee, B.; Tran, C.; Sorg, O.; Saurat, J. H. *Dermatology* **2005**, *210*, 30–34.

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