Melanogenesis Inhibition by Gallotannins from Chinese Galls in B16 Mouse Melanoma Cells

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Nowadays herbal medicines of skin-whitening cosmetics are popular with women. We attempted to find the whitening activity compounds present in many herbal medicines used for this purpose and discuss their mechanisms in melanin biosynthesis. The 70% acetone extracts of 10 kinds of herbs were investigated for their mushroom tyrosinase activity inhibition. Among these 10 extracts, Chinese galls showed inhibitory activity against tyrosinase, with a 50% inhibitory concentration (IC_{50}) value of 22 μg/ml. In a B16 mouse melanoma cell culture assay, Chinese galls dose-dependently inhibited melanin biosynthesis. Using ultraviolet A (UVA) or α-melanocyte-stimulating hormone (α-MSH) to stimulate B16 cells after Chinese gall treatment, the melanin biosynthesis of B16 cells was inhibited in a dose-dependent manner. The active compounds of Chinese galls were isolated by column chromatography, and the melanin biosynthesis inhibition in B16 melanoma cells was measured. Three gallotannins, 2,3,4,6-tetra-*O*-galloyl-D-glucopyranose, 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose, and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose, were isolated from Chinese gall extract, and their IC_{50} values of tyrosinase inhibition activity were 54, 30, and 15 μM, respectively. By the mushroom tyrosinase inhibition kinetics assay, the three gallotannins were all determined to be non-competitive inhibitors. These results indicated that Chinese galls inhibit melanin biosynthesis, associated with hyperpigmentation and can be used as skin-whitening cosmetics for skin care.

Key words Chinese gall; gallotannin; tyrosinase; melanin biosynthesis; non-competitive inhibitor

Pigmentation of the skin is produced by dermal melanocytes. Melanin biosynthesis is the rate-limiting step in the transformation of the amino acid, L-tyrosine, to L-3,4-dihydroxyphenylalaline (L-DOPA) by tyrosinase catalyses in melanocytes. The subsequent oxidation of L-DOPA then yields the semi-stable intermediate, L-dopaquinone, which is converted by dopachrome tautomerase and tyosinase to 5,6-indolequinone-2-carboxylic acid and finally eumelanin.[1] Then the melanin is spread throughout dermal cells and keratinocytes *via* melanosomes to darken the tissues. However, melanogenesis is regulated by several factors, such as ultraviolet radiation (UVR) and α-melanocyte-stimulating hormone (α-MSH).[2] Therefore, Chinese herbs with possible applications as skin-whitening cosmetic agents were first screened by mushroom tyrosinase inhibition, antioxidation (1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging), and UVR absorption models. L-DOPA was used as a common substrate of mushroom tyrosinase to screen the tyrosinase-inhibition activities of compounds. The dopachrome level of the above reaction indicates the tyrosinase activity.

Natural products, with non-toxic and environmentally friendly properties compared to artificial synthetic chemicals, are good resources for skin-whitening cosmetic agents. Proanthocyanidin oligomers and tannins have been reported to inhibit melanogenesis in melanoma cells.[3,4] At present, ten kinds of herbal medicines used as skin care agents in traditional medicine were evaluated by a melanogenesis assay. Among the tested samples, the tyrosinase inhibition of Chinese galls was strongest. Second, the depigmenting effects of Chinese galls were explored on melanin synthesis in murine B16 F_{10} melanoma (B16) cells and the active compounds were isolated by column chromatography.

The melanogenesis of B16 cells can be induced by ultraviolet A (UVA) and α-MSH; moreover, the proliferation of B16 cells is also an important factor.[2,5,6] Therefore, the viability of B16 cells was monitored after treatment of test samples. Under less-cytotoxic concentrations, the melanin production level of B16 cells was measured after co-treatment with inducers. The above B16 cell model was used to evaluate the depigmenting effects of Chinese galls.

Chinese galls (Galla Rhois, *Wu Bei Zi*) are the excrescence produced by parasitic aphids, mainly *Melaphis chinensis* (BELL) BAKERS (Aphididae), on leaves of *Rhus chinensis* MILL. or *R. punjabensis* STEW. var. *sinica* (DIELS) REHD. *et* WILS (Anacardiaceae).[7] Chinese galls are used as an astringent traditional Chinese medicine for their antidiarrheal, hemostatic, and antibacterial properties. They contain high levels (50—70%) of gallotannins.[8] The principle action of Chinese galls is through their astringent effects on proteins, coagulating them to from an insoluble protective membrane to stop bleeding, and this inhibits secretion by mucous membranes.[7] They are used in the treatment of chronic intestinal infections, hematochezia, protoptosis, skin infections, and bleeding wounds. Chinese gall extract is a commercial source of tannic acid preparations. Moreover, gallotannins are strong antioxidants which are abundant in Chinese galls.[9] Therefore, we suggested that the melanogenesis inhibitory natural products of Chinese galls should belong to the tannins type and could be isolated by a systematic chromatographic procedure.

MATERIALS AND METHODS

General Dimethyl sulfoxide (DMSO) (≥99.5%, D4540),

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mushroom tyrosinase (T3824), L-tyrosine (≥99.0%, T8566), DOPA (D9628), methyl salicylate (M2047), (+)-tocopherol (T3634), kojic acid (K3125), ZnO (99.9%, 205532), arbutin (≥98%, A4256), and other chemicals were purchased from Sigma Industry (St. Louis, MO, U.S.A.).

^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured by a Bruker DRX 500 instrument and chemical shifts were given in δ (ppm) values. Electron spray ionization (ESI)-MS were taken on a Waters ZQ-4000 mass spectrometer with direct injection of MeOH solution of a sample. Reversed-phase high-performance liquid chromatography (HPLC) was performed on a LiChrospher RP-18e column (4 mm×250 mm, 5 μm) (Merck, Darmstadt, Germany) using 0.05% trifluoroacetic acid–CH_3CN (85 : 15) as the mobile phase. The flow-rate was 1.0 ml/min with UV absorbance detection at 280 nm. Column temperature was maintained at 40 °C. The column chromatography was carried out on a Toyopearl HW-40 (F) column (Tosoh Corp., Tokyo, Japan) and Shimpack Prep-ODS column (20 mm i.d.×250 mm, 10 μm, Shimadzu, Kyoto, Japan). All other reagents and chemicals were of analytical grade.

Preparation of Traditional Chinese Medicines (TCMs) The TCMs were purchased from a traditional herbal medicine store in Taipei, Taiwan. The TCMs were identified by Assoc. Prof. H. C. Chang, National Laboratories of Food and Drugs, Department of Health, Executive Yuan, Taipei. Chinese galls (Galla Rhois, *Wu Bei Zi*) voucher specimen (No. CG-0001) has been deposited in the School of Pharmacy, College of Pharmacy, Taipei Medical University.

Assay of Phytochemical Contents The phytochemical contents of various TCMs, the polyphenol compounds of which differ in chemical structure, were determined using the following specific color reactions.

(1) Total Phenols The Folin–Ciocalteu method was used to determine the total phenols content.[8] The extracts and Folin–Ciocalteu reagent (Merck) were both diluted 10 times with water. Five hundred microliters of Folin–Ciocalteu diluted reagent and 400 μl of a sodium carbonate solution (7.5%) were added to 100 μl of the diluted extract. The mixed solution was kept in a heater at 50 °C for 5 min and then transferred to a cold-water bath. The absorbance of the mixture was read at 600 nm. Results are expressed in gallic acid equivalents (GAE) per amount of TCMs extracted. Calibration was achieved with a gallic acid aqueous solution (7.8—250 μg/ml).

(2) Proanthocyanidins The vanillin–HCl method was used to determine the proanthocyanidins content.[10] A 600-μl portion of a freshly prepared solution of vanillin (1 g/100 ml) in 80% H_2SO_4 was added to 300 μl of the aqueous extract (10 mg/ml). After 15 min of reaction, the absorbance was read at 530 nm. Results are expressed in (+)-catechin equivalents per amount of TCMs extracted. Calibration was achieved with a (+)-catechin aqueous solution (7.8—250 μg/ml).

Isolation of Gallotannins from Chinese Galls Dried Chinese galls (37.5 g) were homogenized in 70% aqueous acetone (500 ml×3), and the homogenate was filtered. The concentrated filtrate was evaporated and freeze-dried to yield the 70% acetone extract (19.8 g). A portion (18 g) of the 70% aqueous acetone extract was chromatographed over a Toyopearl HW-40 (F) column (2.5 cm i.d.×41 cm) with H_2O, H_2O–MeOH (50% MeOH→60% MeOH→70% MeOH→80% MeOH), and 70% acetone. The 70% MeOH eluent (3.2 g) was rechromatographed over a Shimpack Prep-ODS column (20 mm i.d.×250 mm) with 0.05% trifluoroacetic acid–CH_3CN (83 : 17) to yield 1,2,3,6-tetragalloyl-β-D-glucopyranose (**1**; 23.4 mg), 1,2,3,4,6-pentagalloyl-β-D-glucopyranose (**2**; 802.4 mg), and 2,3,4,6-tetragalloyl-D-glucopyranose (**3**; 3.6 mg). A part (3.05 g) of the 60% MeOH eluent was purified by preparative HPLC with a Shimpack Prep-ODS column (20 mm i.d.×250 mm) eluted with 0.05% trifluoroacetic acid–CH_3CN (89 : 11) to yield digallic acid (**4**; 72.7 mg) and methyl gallate (**5**; 5.0 mg). The 50% MeOH eluent (0.52 g) was purified by a Shimpack Prep-ODS column (20 mm i.d.×250 mm) eluted with 0.05% trifluoroacetic acid–CH_3CN (91 : 9) to yield gallic acid (**6**; 14.0 mg). All structures were estimated by ^{1}H- and ^{13}C-NMR, including 2D-NMR techniques, and also by comparison of those data with authentic compounds.[11,12] The purity of each compound was determined by HPLC and was shown to exceed 95%.

Measurement of the Ultraviolet Absorption The extracts of TCMs were dissolved in MeOH to a final concentration of 10 mg/ml. The absorbance of the test solution was read at 365 (UVA), 300 (UVB), and 254 nm (UVC). Methyl salicylate was used as a positive control of UVB and UVC absorption, and ZnO was used as a positive control of UVA absorption.[13]

DPPH-Scavenging Activity Test samples were initially dissolved in EtOH at a concentration of 10 mg/ml and then stored at −20 °C until use. In brief, 100-μl serial dilutions of the stock solution were prepared and were added to 100 μl of 50 mM DPPH in EtOH in 96-well microtiter plates. Finally, the optical density was measured at 530 nm, using an MRX microplate reader (Dynex Technologies, Guernsey, Channel Islands, U.K.).[10] For the blank, EtOH was used instead of the DPPH solution, and for the control, EtOH was used instead of the sample solution. EC_{50} values, *i.e.*, the amount of each sample required to scavenge 50% of the DPPH free radicals, were calculated from regression lines, where the abscissa represents the concentration of the tested compound. (+)-Tocopherol was used as a positive control for DPPH-scavenging activity.

Assay of Tyrosinase Activity Tyrosinase activity was determined as described previously with minor modifications.[14] Test samples were dissolved in 10% DMSO. Briefly, 250 μl of the aqueous solution of mushroom tyrosinase (135 U/ml) and 250 μl of the test sample solution were added to a quartz glass cuvette, and finally the mixture was supplemented with 250 μl of a 0.03% L-DOPA solution with 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 min in the dark. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 475 nm in a spectrophotometer (Shimadzu, UV-1601). The inhibitory activity was expressed as the concentration that inhibited 50% of the tyrosinase activity (IC_{50}), as determined by the optical density. Kojic acid was used as a positive control.[15] IC_{50} values of test sample inhibition patterns were used to analyze the kinetic of tyrosinase inhibition by using Lineweaver–Burk's plot at various L-DOPA concentrations.[16]

Determination of Melanogenesis in B16 Cells The B16 melanoma cell line was obtained from ATCC CRL-6322

(Manassas, VA, U.S.A.). B16 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, U.K.),

of 54, 30, and 15 μM, respectively (Table 2). Furthermore, the IC_{50} concentrations of the active gallotannins (**1**, **2**, **3**) were used to analyze the kinetics of tyrosinase inhibition. The mode of inhibition of tyrosinase was determined by a Lineweaver–Burk plot analysis as shown in Fig. 2. When various concentrations of L-DOPA were used as the substrate, the three active gallotannins all decreased the V_{max} value of tyrosinase but did not change the K_{max} value. Therefore, the active gallotannins (**1**, **2**, **3**) were all identified as being non-competitive tyrosinase inhibitors.

The inhibitory potency of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose was greater than these of the other compounds on the mushroom tyrosinase assay, and the active compound could also scavenge DPPH radicals, with an IC_{50} value of 3.06 μM (2.87 μg/ml). Therefore, the melanogenesis inhibition of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose was explored in B16 cells.

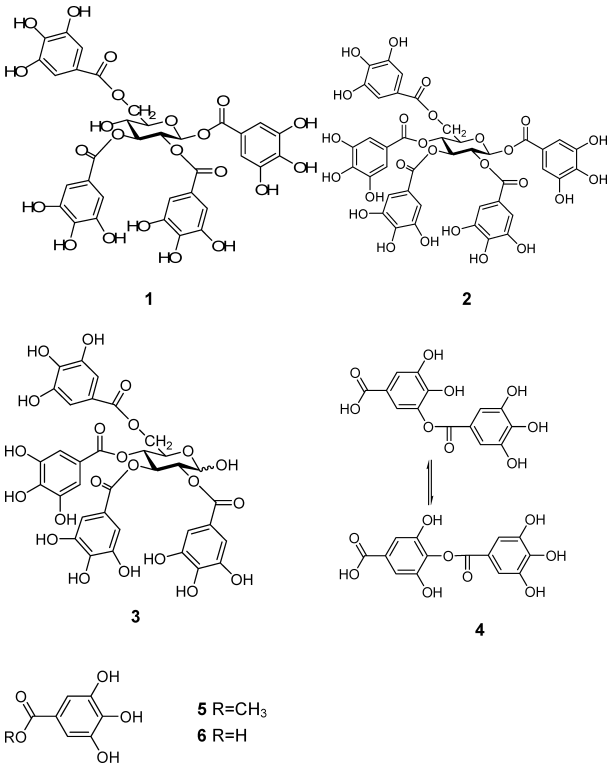

Fig. 1. Structures of Gallotannins from Chinese Galls: 1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (**1**), 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**2**), 2,3,4,6-Tetra-*O*-galloyl-D-glucose (**3**), Digallic Acid (**4**), Methyl Gallate (**5**), and Gallic Acid (**6**)

First, the cytotoxicity of test samples was measured by the MTT assay. In Fig. 3, the results showed that Chinese galls had little cytotoxicity on B16 cells at 25 μg/ml for 24 h of treatment, and the cytotoxicity percentage was 24.4%. Concentrations of Chinese galls of <25 μg/ml, those of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose of <50 μM, and those of arbutin of <500 μM did not exhibit cytotoxicity. The above concentrations of test samples were used in the inhibition experiments of melanin production in B16 cells. The melanin of B16 cells was induced by UVA irradiation for 2 d (1 h/d) after treatment with test samples. As shown in Fig. 4, Chinese galls and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose both inhibited the melanogenesis of B16 cells in dose-dependent manners in the groups with and without UVA irradiation; moreover the effect of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose was stronger than that of arbutin. In addition, the potencies of inhibition of the melanin content of B16 cells by Chinese galls or 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose did not significantly differ between the groups with and without UVA irradiation. However, the potencies of arbutin were influenced by UVA irradiation; arbutin did not decrease the melanin level of B16 cells under UVA exposure (Fig. 4). On the other hand, α-MSH was used as a chemical inducer to stimulate the melanogenesis of B16 cells. According to Fig. 5, the melanin content of B16 cells did not significantly decrease when B16 cells were co-treated with test samples and α-MSH. However, Chinese galls and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose still exhibited dose-dependent inhibition, and the inhibition percentage was higher than that of arbutin, a material of commercial whitening cosmetics.

According to the above results, we suggest that Chinese galls can be developed as a material for whitening cosmetics, and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose can be

Table 2. Tyrosinase Inhibition by Gallotannins from Chinese Galls

Compound	Inhibition (%)[a)]	IC_{50} (μM)
1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (**1**)	77.90	30
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**2**)	98.99	15
2,3,4,6-Tetra-*O*-galloyl-D-glucose (**3**)	69.79	54
Digallic acid (**4**)	35.90	>100
Methyl gallate (**5**)	27.48	>100
Gallic acid (**6**)	6.26	>100

a) The concentration of each sample was 100 μM.

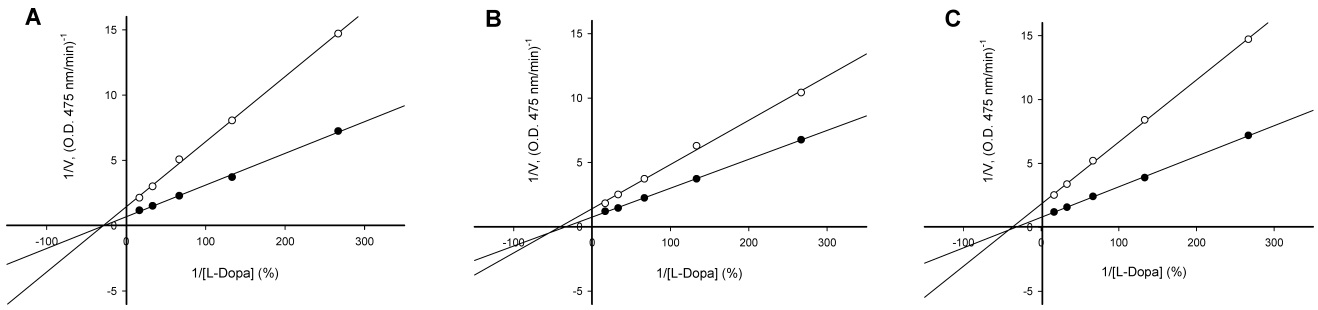

Fig. 2. Lineweaver–Burk Plots of Mushroom Tyrosinase

(A) With L-DOPA as a substrate, in the presence of 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose, 30 μM (○), and control (●). (B) With L-DOPA as a substrate, in the presence of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose, 15 μM (○), and control (●). (C) With L-DOPA as a substrate, in the presence of 2,3,4,6-tetra-*O*-galloyl-D-glucopyranose, 54 μM (○), and control (●). The measurements were taken at 475 nm.

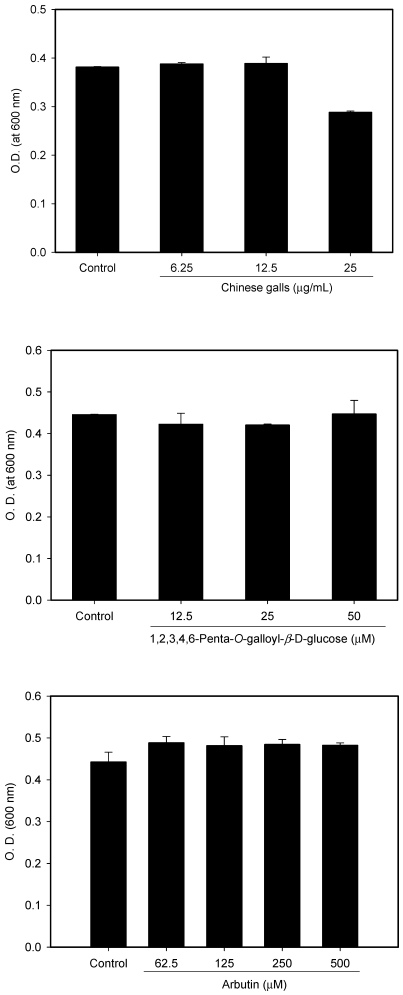

Fig. 3. Viability of B16 Cells after Treatment with Chinese Galls, 1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucose, and Arbutin for 24 h

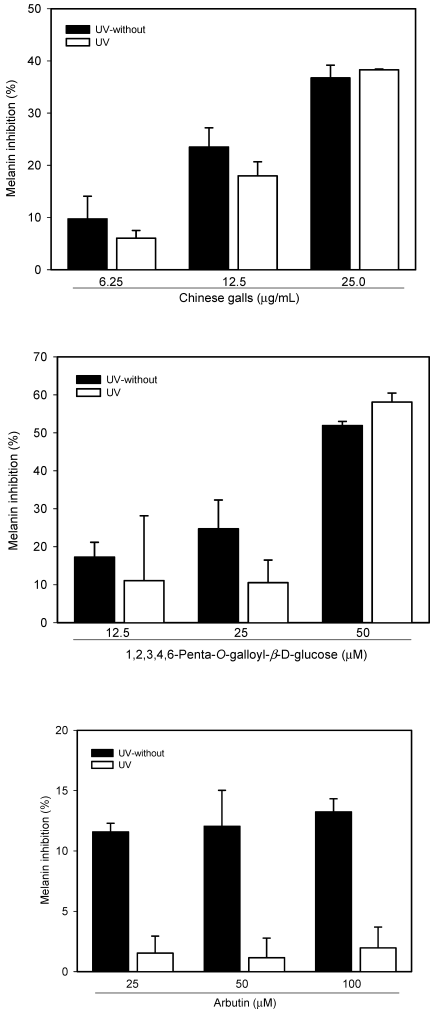

Fig. 4. B16 Cells after 2 d of Incubation with Chinese Galls, 1,2,3,4,6-Penta-*O*-galloyl-*β*-D-glucose, and Arbutin with or without UV Irradiation (1 h/d)

The power of the UVA irradiation was 2.4×10^5 mJ/cm^2.

used as a substance marker to assess the quality of Chinese galls.

Chinese galls contain large amounts of starches, fats, resins, and gallotannins.[5] The gallotannins in Chinese galls are formed by the galloylation of glucose. Penta-*O*-galloyl-*β*-D-glucopyranose usually forms the core and up to five other gallic acids are depsidically linked with galloyl groups in the core.[16] As this paper demonstrates, 6 kinds of gallotannin-related compounds were obtained from the 70% acetone extract of Chinese galls and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose was the major component with whitening activities.

In the melanogenesis pathway, tyrosinase is a rate-limiting, copper-containing enzyme. The catalyzed reactions of tyrosinase with O_2 include the following two steps: (1) L-tyrosine is hydroxylated to form L-DOPA and (2) L-DOPA is oxidized to L-dopaquinone.[2] In our studies, 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose more-efficiently inhibited tyrosinase to catalyze L-DOPA oxidation to L-dopaquinone than did the other gallotannins. However, gallic acid did not inhibit tyrosinase activity, and digallic acid (**4**), formed by the depsidic linkage of two gallic acids, showed only weak tyrosinase inhibition. Alkyl esters of gallic acid were more effective in inhibiting tyrosinase than was gallic acid.[19] In another report, when L-tyrosine was used as a substrate, gallic acid exhibited potent tyrosinase inhibitory activity, which may be attributed to its phenolic structure.[16] Therefore, we suggest that 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose is richer in galloyl groups and more-potently inhibited tyrosinase.

UVR is the most potent stimulus for growth and differentiation of melanocytes and generation of reactive oxygen species (ROS) and can stimulate keratinocytes to produce *α*-MSH.[6,10] *α*-MSH acts preferentially in human epidermal melanocytes to stimulate the synthesis of eumelanin.[10] However, UVA, one type of UVR can induce immediate pigment darkening[6] and was used as an inducer of melanogenesis of the B16 cell model. According to Figs. 4 and 5, neither Chinese galls nor 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose influenced the growth of B16 cells, but still inhibited melanogenesis with or without UVA irradiation, but when co-treated with *α*-MSH, they had no effect.

On the other hand, the rich polyphenol content of Chinese galls should exhibit antioxidative effects (Table 1). Therefore, the melanogenesis inhibition mechanism of Chinese galls and 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucopyranose might be derived from their antioxidation capability, because L-DOPA can be oxidized to L-dopaquinone by tyrosinase with

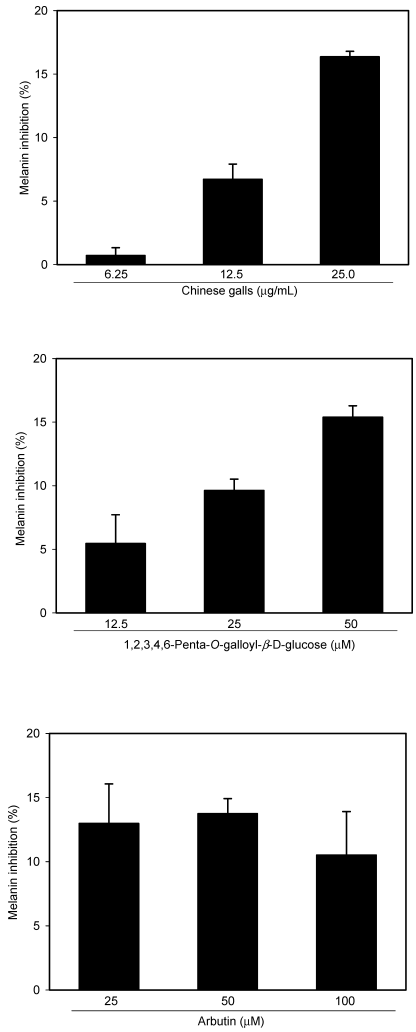

Fig. 5. Melanin Inhibition by Chinese Galls, 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranose, and Arbutin on α-MSH-Stimulated B16 Cells

O_2. In conclusion, we suggest that Chinese galls and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose inhibited the melanogenesis of B16 cells through inhibiting the proliferation of B16 cells and scavenging ROS generation by UVA irradiation.

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