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Inhibitory Effect of Dalbergioidin Isolated from the Trunk of *Lespedeza cyrtobotrya* on Melanin Biosynthesis

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Tyrosinase is a key enzyme for melanin biosynthesis, and hyperpigmentation disorders are associated with abnormal accumulation of melanin pigments, which can be reduced by treatment with depigmenting agents. The methanol extract of *Lespedeza cyrtobotrya* M_{IQ} showed inhibitory activity against mushroom tyrosinase. The active compound was purified from the methanol extract of *L. cyrtobotrya*, followed by several chromatographic methods, and identified as dalbergioidin (DBG) by spectroscopic methods. The results showed that DBG exhibited tyrosinase inhibitory activity with an IC_{50} of 20 μM. The kinetic analysis of tyrosinase inhibition revealed that DBG acted as a noncompetitive inhibitor. In addition, DBG showed a melanin biosynthesis inhibition zone in the culture plate of *Streptomyces bikiniensis* that has commonly been used as an indicator organism. Furthermore, 27 μM DBG decreased more than 50% of melanin contents on the pigmentation using the immortalized mouse melanocyte, melan-a cell.

Keywords: *Lespedeza cyrtobotrya*, dalbergioidin, tyrosinase, melan-a cell, melanin biosynthesis inhibitor, skin-whitening

Melanin is the pigment responsible for the color of human skin and hair. In mammalian melanocytes, melanins are synthesized in melanosomes that contain three major pigment enzymes: tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 [22, 29, 41]. Tyrosinase plays a critical role in the melanogenesis because it catalyzes the two rate-limiting melanogenesis steps; namely, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone. Thus, melanin production is mainly dependent on the expression and activation of tyrosinase [11, 12, 18].

Melanin is involved in localized hyperpigmentation such as melasma, chloasma, seborrheic keratosis, freckle, and lentigo. Melanin biosynthesis inhibitors are useful not only as skin-whitening agents in cosmetics but also as a remedy for disturbances in pigmentation [10, 14].

A number of melanogenesis inhibitors such as arbutin and kojic acid have been reported and are being used as cosmetic additives. However, no prominent curative effects can be expected. Recently, much attention has been focused on the application of natural products with potential use in cosmetics [2, 4–6, 9, 15, 18, 19, 26, 34, 36]. In our continuing search for new effective melanin biosynthesis inhibitors as skin-whitening agents from natural sources, methanol (MeOH) extracts of 2,000 Korean native plants were screened for their melanin biosynthesis inhibitory activity. Among them, we found that MeOH extract of *Lespedeza cyrtobotrya* inhibited melanin biosynthesis activity. An active component was isolated from the trunk, and was determined to be dalbergioidin (DBG).

Lespedeza cyrtobotrya is a deciduous tree of the leguminous family and distributed in forests at medium and low altitudes. It is a drought-enduring plant, and is held in high esteem as foliage, green manure crops, or honey resources and also for prevention of soil erosion [37]. A variety of compounds have been found in this plant; lespeol, xanthoangelol, haginin A, B, C, and D, genistein, daidzein, eriodictyol, isoliquiritigenin, and 3,9-dihydroxypterocarp-6a-en [23, 32, 33]. In the present study, the inhibitory effect of the compound obtained from *Lespedeza cyrtobotrya* on melanin biosynthesis was investigated.

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MATERIAL AND METHODS

Plant Materials

The methanol extract of *Lespedeza cyrtobotrya* was obtained from the Plant Extract Bank in KRIBB (Daejon, Korea).

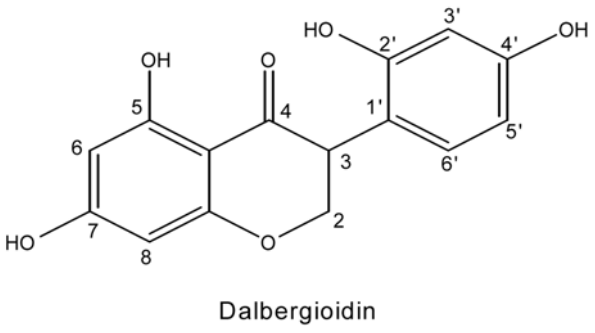
Extraction and Isolation

The methanol (MeOH) solution was evaporated to dryness (4 g). The MeOH extract was suspended in H_2O (1 l) and then partitioned successively with hexane, ethyl acetate (EtOAc), and butanol (BuOH). Among the solvent fractions, the EtOAc fraction showed the inhibitory activity of melanin synthesis in melan-a cell. Accordingly, the EtOAc fraction (1 g) was concentrated *in vacuo* and chromatographed on a C-18 reversed-phase HPLC column (YMC-ODS-AM 250×6 mm, 10 μm; Waters, Milford, MA, U.S.A.). The column was eluted with an isocratic mobile phase, which consisted of 30% aqueous acetonitrile, in order to obtain the compound I (5.7 mg). Absorbance was monitored at 220 nm with isocratic flow at 1.5 ml/min.

Compound I (Dalbergioidin): dark-brown powder; ESI-MS *m/z* 289.0 $(M+H)^+$, 311.0 $(M+Na)^+$, and 598.9 $(2M+Na)^+$; ^{1}H-NMR (CD_3OD, 300 MHz, ppm): 6.82 (d, *J*=4.8, H-6'), 6.32 (d, *J*=1.5, H-3'), 6.24 (dd, *J*=1.5, 4.9, H-5'), 5.85 (s, H-8), 5.85 (s, H-6), 4.43 (dd, *J*=6.3, 6.8, H-2), 3.31 (t, H-3); ^{13}C-NMR (CD3OD, 75 MHz, ppm): 131.0 (CH, C-6'), 107.8 (CH, C-5'), 159.2 (C-4'), 103.8 (CH, C-3'), 157.7 (C-2'), 114.0 (C-1'), 165.2 (C-8a), 104.3 (C-8), 168.9 (C-7), 96.2 (CH, C-6), 165.8 (C-5), 97.3 (C-4a), 49.9 (C-3), 71.5 (CH2, C-2). On the basis of ^{13}C-NMR and ESI-MS spectral data, the molecular formula of compound **I** was determined as $C_{15}H_{12}O_{16}$. According to the ^{1}H-NMR and library searching results, the compound was identified as dalbergioidin [3-(2,4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-4-one] [8, 13, 22] (Fig. 1).

Inhibitory Activities on Mushroom Tyrosinase

The reaction mixture for mushroom tyrosinase (E.C. 1.14.18.1, Sigma) activity consisted of 150 μl of 0.1 M phosphate buffer (pH 6.5), 3 μl of sample solution, 8 μl of mushroom tyrosinase (2,100 unit/ml, 0.05 M phosphate buffer at pH 6.5), and 36 μl of 1.5 mM L-tyrosine. Tyrosinase activity was determined by reading the optical density at 490 nm on a microplate reader (Bio-Rad 3550, Richnmond, CA, U.S.A.) after incubation for 20 min at 37°C. The inhibitory activity of the sample was expressed as the concentration that inhibits 50% of the enzyme activity (IC_{50}).



Dalbergioidin

Fig. 1. Chemical structure of dalbergioidin [3-(2,4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-4-one].

Inhibitory Effect of Melanin Production in *Streptomyces bikiniensis*

Inhibitory activity on melanin synthesis was determined by the paper-disc agar diffusion method [31]. A preserved culture of *S. bikiniensis* NRRL B-1049 was inoculated on a Papavizas' VDYA agar slant, which contained 200 ml of V-8 juice (Campbell Soup Co., Camden, NJ, U.S.A.), 2 g of glucose, 2 g of yeast extract (Difco), 1 g of $CaCO_3$, 20 g of agar (Difco), and 800 ml of distilled water, and the pH was adjusted to 7.2. After incubation for 2 weeks at 28°C, 2 ml of sterile water was added to the slant culture, and the spore mass formed on the aerial mycelium was scraped with an inoculating loop. The spore suspension thus obtained was transferred to sterile microtubes. Subsequently, 0.4 ml of the spore suspension of *S. bikiniensis* was added to the agar medium ISP No. 7 (40 ml) supplemented with 0.2% Bacto-yeast extract (Difco), and was uniformly spread over the agar surface with a glass hockey bar. After the agar surface was dried, a paper disc (8 mm diameter) soaked with the sample solution was placed on the agar plate. The plate was incubated for 48 h at 28°C; and the diameter zone (mm i.d.) of melanin formation was measured from the reverse side of the plates.

Kinetic Analysis of Tyrosinase Inhibition by Dalbergioidin

For the assay mixture, 1.5 mM L-tyrosine as a substrate, mushroom tyrosinase (2,100 units/ml), and 100 mM phosphate buffer (pH 6.5), with or without test sample, were added to a 96-well plate in a total volume of 200 μl. The initial rate of dopachrome formation from the reaction mixture was determined as the increase of absorbance at 490 nm per min (OD_{490}/min) using a microplate reader. The Michaelis constant (K_m) and maximal velocity (V_{max}) of tyrosinase were determined by Lineweaver-Burk plot using various concentrations of L-tyrosine as a substrate, as shown in our earlier study. The reaction kinetics required a modification of the Michaelis-Menten equation owing to competitive inhibition by DBG together with substrate inhibition by L-tyrosine. Results are means of three independent experiments.

Cell Cultures

The melanocyte cell line, melan-a, was kindly provided by D.C. Bennett (St. George's Hospital Medical School, London, U.K.). The melan-a line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), streptomycin-penicillin (100 μg/ml each), and 200 nM tetradeconyl phorbol acetate (TPA), a potent tumor promoter, at 37°C in 5% CO_2. Cells were subcultured every 3 days until a maximal passage number of 40. Confluent monolayers of melanocytes were harvested with a mixture of 0.05% trypsin and 0.53 mM EDTA (Gibco BRL, Grand Island, NY, U.S.A.).

Cell Viability Assay

Cell viability was determined using the crystal violet assay. After being incubated with the test substances for 24 h, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. Cells were stained for 5 min at room temperature and rinsed three times. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 540 nm.

Melanization Inhibition Assay on Melan-a Cell

Cells were seeded into a 24-well plate (Falcon, U.S.A.) at a density of 1×10^5 cells per well and allowed to attach overnight. The medium

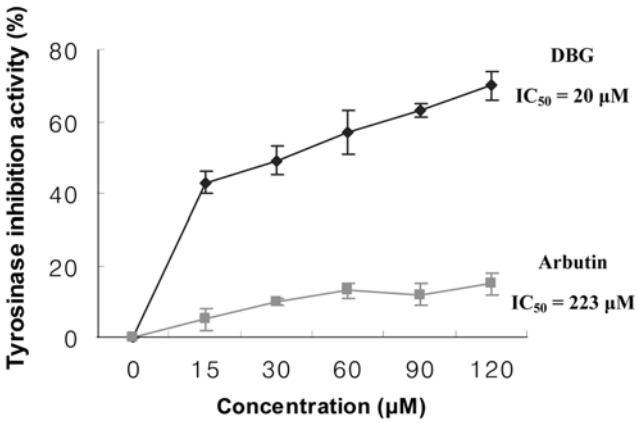


Fig. 2. Tyrosinase inhibitory activity of DBG.
Tyrosinase activity was measured using L-tyrosine as a substrate. Values are mean±SE of three determinations (n=3). The OD (optical density) value of the control was determined at 490 nm.

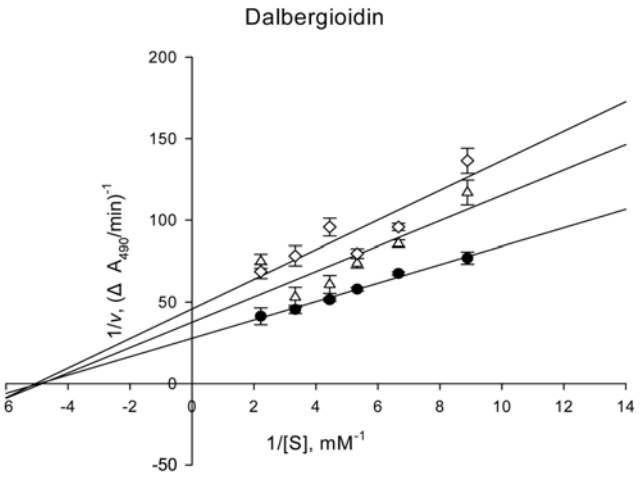


Fig. 3. Lineweaver-Burk plot of mushroom tyrosinase in the presence of DBG.
Data were obtained as mean values of 1/V, inverse of the increase of absorbance at 490 nm per min (ΔA_{490}/min), of three independent tests with different concentrations of L-tyrosine as a substrate. Inhibitors of the enzyme were DBG 0.87 mM (◇), 0.43 mM (△), and control (●). The modified Michaelis-Menten equation is $1/V_{max}=1/K_m$ $(1+[I]K_i)$. V=velocity of the reaction, S=L-tyrosine concentration, and K_i=inhibitor constant.

was replaced with fresh medium containing various concentrations of compounds. Cells were cultured for 72 h and further incubated for a day. After washing with phosphate-buffered saline (PBS), the cells were lysed with 250 μl of 0.85 N KOH and transferred to a 96-well plate. The melanin contents were determined by measuring the absorbance at 405 nm. Phenylthiourea (PTU) was used as a positive control [3].

Results

Effects of DBG on Tyrosinase Activity and *S. bikiniensis* Melanin Biosynthesis

We determined the inhibition rate of o-hydroxylation of tyrosine using L-tyrosine as a substrate. In addition, we monitored the inhibitory activity by spectrophotometric measurement of dopachrome formation. In addition, we determined the tyrosinase activity, and found that DBG clearly showed tyrosinase inhibitory activity in a concentration-dependent manner (Fig. 2) [16, 18]. The result showed that the IC_{50} of DBG was 20 μM, whereas that of the reference compound, arbutin, was 223 μM. We further examined the inhibitory effects of DBG on melanogenesis in *S. bikiniensis* by the paper-disc diffusion method using the inhibition of melanin production, and found that DBG potently inhibited melanin production in comparison with the known melanogenesis inhibitor arbutin (Table 1) [16, 18].

Table 1. Inhibitory activity on tyrosinase and melanin formation of *Streptomyces bikiniensis*.

Compound	Tyrosinase IC_{50} (μM)[a]	*S. bikiniensis* melanin biosynthesis inhibition zone (mm)[b]		
		30 μg[c]	20 μg	10 μg
DBG	20±3.5	42±4	35±1.5	23±3.3
Arbutin	223±19.6	0	0	0

[a]50% inhibitory concentration.
[b]*S. bikiniensis* was incubated with a paper disc soaked with the test samples at 28°C for 48 h. The inhibition zone of melanin formation was measured from the reverse side of the plate. The results were reproduced with three different cultures.
[c]Test sample concentration/paper disc.
Values are mean±SE of three determinations (n=3).

Lineweaver-Burk Plot of Mushroom Tyrosinase in the Presence of DBG

The mode of inhibition of the enzyme was determined by Lineweaver-Burk plot analysis. As shown in Fig. 3, the V_{max} (ΔA_{490}/min) for the dopa formation of the enzyme was 3.6×10^{-2}, and the K_m value for L-tyrosine was 0.2 mM. According to the Lineweaver-Burk plot of 1/V values with DBG at two different concentrations, 0.87 mM and 0.43 mM, DBG did not affect the K_m value of the enzyme, but it decreased the V_{max} values to 1.1×10^{-2} and 1.3×10^{-2}, respectively. Owing to the facts that V_{max} decreased more at higher DBG concentration and the K_m value was unchanged

Table 2. Kinetic parameters of mushroom tyrosinase.

Compounds	K_m (M)	V_{max} (ΔA_{490}/min)	K_i (M)
None	2.03×10^{-4}	0.036	–
Dalbergioidin	2.03×10^{-4}		
(4.34×10^{-7} M)		0.026	$1.78\pm0.31\times10^{-6}$
(8.68×10^{-7} M)		0.022	$1.34\pm0.38\times10^{-6}$

The kinetic parameters were obtained, with L-tyrosine as a substrate, by using the Lineweaver-Burk plot by the method of least squares.

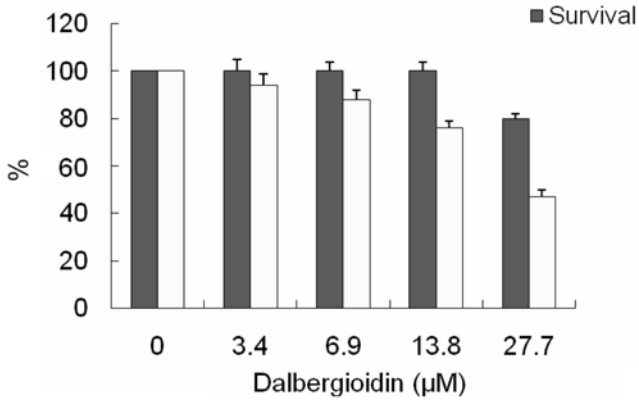


Fig. 4. Effects of DBG on melanin contents and cytotoxicity in melan-a cells.
Melan-a cells were treated with various concentrations of DBG for 60 h. Data are expressed as % of control and each column represents the mean± SE of three determinations (n=3).

at any DBG concentration, DBG acted as a noncompetitive inhibitor of tyrosinase in the presence of L-tyrosine (Table 2) [16, 18].

Effects of DBG on Melanin Synthesis of the Melan-a Cell Line

Desirable skin-whitening agents should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase and exhibit low cytotoxicity, and be nonmutagenic [7]. Therefore, DBG, PTU, and arbutin were examined for melanin biosynthesis of melan-a cells. In particular, arbutin from plant has been known as a tyrosinase inhibitor and also as a skin-whitening agent, and thus, it was used as a positive control [21]. Apparently, reduced pigmentation was observed in the cells treated with both DBG and PTU. The 50% inhibitory concentration of melanin biosynthesis was about 27 μM by DBG and 65.7 μM by PTU. However, arbutin had no effect on the melanin production of melan-a cells (Fig. 4 and Table 3) [18].

Table 3. Effects of DBG, PTU, and arbutin on cell growth and melanin production of melan-a cells.

Sample	Melanin synthesis IC_{50} (μM)	Cytotoxicity LD_{50} (μM)
DBG	27.7±2.1	140±15.3
PTU	65.7±0.7	644±7.2
Arbutin	122±5.3	>500

Melan-a cells were grown overnight to confluence in 24-well culture plates. Test samples were added to the plates and incubated for 3 days. The medium containing compound was renewed everyday and further incubated for a day. After 5 days of incubation, a microplate reader was used to determine the cell viability and melanin content in the melan-a cells. Phenylthiourea (PTU) and arbutin were used as positive controls. The results were reproduced with three different cultures.

Effects of DBG on Cell Proliferation

When selecting skin-lightening compounds for cosmetic formulations, one of the important points is that they should have minimal effects on melanocyte cell proliferation. Thus, proliferation of cells treated with DBG was evaluated with the crystal violet assay. DBG at 140 μM showed the 50% inhibitory activity on cell proliferation, suggesting that the inhibitory effect of DBG on melanin production was not due to its cytotoxicity (Table 3).

DISCUSSION

Melanin is an important skin pigment, which is caused by melanin storage in keratinocytes and the epidermis through the melanosomes in the melanocytes. In particular, melanosomes are unique intracytoplasmic organelles in melanocytes. Melanogenesis is mainly regulated by enzymes such as tyrosinase and dopachrome tautomerase. The starting material for the biosynthesis of melanin is the amino acid tyrosine, which is oxidized to dopaquinone catalyzed by the copper-containing enzyme tyrosinase. Therefore, tyrosinase is one of the key enzymes in melanin synthesis, and tyrosinase inhibitors can regulate the melanin synthesis by reducing tyrosinase production [1, 28].

In this study, in an effort to develop a safe and effective skin-whitening agent, we screened methanol extracts of 2,000 Korean native plants. Thus, we observed that the MeOH extract of *Lespedeza cyrtobotrya* had strong melanogenic inhibitory activity. The active compound was identified as dalbergiodin (DBG). DBG reduced the tyrosinase activity in a dose-dependent manner, and its IC_{50} was 20 μM. The potency was significantly higher than that of arbutin (223 μM), a well known inhibitor of tyrosinase. DBG at 27 μM concentration inhibited 50% of the melanin contents of melan-a cell compared with untreated control. The effective concentration of DBG was not cytotoxic to melan-a cells. This result suggests that the inhibitory effect of DBG on melanin production was not due to its cytotoxicity, and that DBG can be a safe skin-lightening agent without influencing melanocyte growth. In addition, DBG potently inhibited the melanin production of *S. bikiniensis* in a dose-dependent manner, and it also acted as a noncompetitive inhibitor of mushroom tyrosinase. As the only effect of a noncompetitive inhibitor is to decrease V_{max}, DBG can substantially reduce the melanin production by quantitatively interrupting dopachrome formation. Accordingly, we regard DBG as a promising skin-lightening agent, because it inhibited melanin synthesis more strongly than other skin-lightening agents available, such as arbutin.

In the 1980s, numerous whitening cosmetics and medicines, such as hydroquinones, kojic acid, and its derivatives, were developed. However, the clinical effects of these compounds

were unsatisfactory. For example, kojic acid has widely been used to prevent the enzymatic browning of food and also as a cosmetic whitening reagent. However, Tamotsu *et al*. [29] recently reported that kojic acid caused hepatocarcinogenesis. Therefore, kojic acid cannot be used as a food additive or cosmetic reagent. On the other hand, hydroquinones and their derivatives are widely used to treat hyperpigmentation in many countries; however, they are unstable, cytotoxic to melanocytes, and easily oxidized [24, 27, 30]. Therefore, a new tyrosinase inhibitor is greatly needed. Obviously, DBG effectively regulated melanogenesis without cell toxicity. Accordingly, it may be a good candidate as a potent inhibitor of tyrosinase. We are in a process to verify the efficacy of DBG and its derivatives on tyrosinase inhibitory activities. Additional studies of the inhibitory mechanism and preclinical animal tests for these compounds are in progress.

Acknowledgments

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