Inhibitory Effects of Kurarinol, Kuraridinol, and Trifolirhizin from *Sophora flavescent* on Tyrosinase and Melanin Synthesis

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Previously, it was reported that some prenylated flavonoids contained in the dichloromethane fraction of the ethanolic extract of *Sophora flavescent*, such as kurarinid, sophoraflavanone G, kurarinone, and kushenol F, are tyrosinase inhibitors; however, based on the level of these inhibitors in the extract, its inhibitory effect on tyrosinase activity was higher than expected. This has led us to further investigate other possible constituents that may contribute to the extract’s strong inhibitory activity. The results of this study indicate that kurarinol (1), kuraridinol (2), and trifolirhizin (3), from the ethyl acetate fraction of *Sophora* extract, can inhibit tyrosinase activity. Compared with kojic acid (16.22±1.71 μM), compounds 1—3 possessed potent tyrosinase inhibitory activity with IC50 values of 8.60±0.51, 0.88±0.06, and 506.77±4.94 μM, respectively. These three compounds were further tested for their inhibitory effects on melanogenesis. In cultured B16 melanoma cells, 1—3 markedly inhibited (>50%) melanin synthesis at 50 μM. This is the first study indicating that 1—3 exert varying degrees of inhibition on tyrosinase-dependent melanin biosynthesis, and therefore, are candidates as skin-whitening agents.

### Key words
- tyrosinase; *Sophora flavescent*; kurarinol; kuraridinol; trifolirhizin; melanoma B16 cell

Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine, in the presence of the enzyme tyrosinase. Tyrosinase, also known as polyphenol oxidase (PPO), is a multifunctional oxidase containing copper that is widely distributed in microorganisms, animal tissues, and plant materials, and is responsible for melanin biosynthesis. In the food industry, tyrosinase is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Controlling enzymatic browning is essential during the fruit pulp manufacturing process. Furthermore, many tyrosinase inhibitors are useful in cosmetics as skin-whitening agents, and also as remedies for pigmentation disturbances. For example, arbutin and kojic acid are widely utilized cosmetic agents in Northeast Asia. However, a recent study showed that kojic acid has serious side effects such as cytotoxicity, skin cancer, and dermatitis, and has been banned for cosmetic use in many countries. Considering this toxic effect, as well as the preventive potentials of the bioactive sources discovered thus far, such as in foods, herbs, and medicinal plants, a great deal of interest is beginning to focus on deriving tyrosinase inhibitors from natural products.

Melanin formation is considered to be deleterious to the color quality of fruits and vegetables; hence, inhibiting this enzyme would be useful for controlling the browning of plant-derived foods in the food industry. Furthermore, tyrosinase is one of the most important enzymes in the insect molting process, and investigating tyrosinase inhibitors may be important for finding alternative insect control agents.

The dried roots of *Sophora flavescent* were refluxed with MeOH for 3 h, and the ethanolic extract of *Sophora flavescent* was recently reported to be tyrosinase inhibitors, the extract’s inhibitory effect on tyrosinase activity was higher than expected based on the level of these inhibitors in the extract. This study evaluated the tyrosinase inhibitory activity of the ethyl acetate fraction of *S. flavescent*, and attempted to identify the active components. Furthermore, we examined the effects of the active components on melanin content within B16 melanoma cells.

### MATERIALS AND METHODS

**Plant Material** The *S. flavescent* roots were collected at Yeong-Cheon, Gyeongsangbuk-Do Province, Korea, in February 2005, and were authenticated by Prof. J. H. Lee at Dong Guk University, Kyeongju, Korea. A voucher specimen was deposited at the Division of Food Science and Biotechnology, Pukyong National University.

**Chemicals and Reagents** The L-tyrosine was purchased from Janssen Chimica (Geel Belgium), and the tyrosinase (EC 1.14.18.1), 3-isobutyl-1-methylxanthine (IBMX), and kojic acid were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Column chromatography was carried out using silica gel (Merck, 70—230 mesh). TLC was performed on a precoated Merck Kieselgel 60 F254 plate (0.25 mm). Fifty percent H2SO4 was used as a spray reagent. All the solvents for column chromatography were of reagent grade, and were acquired from commercial sources.

**Isolation of Active Compounds** The powdered roots (1 kg) of *S. flavescent* were refluxed with MeOH for 3 h (91×3). The total filtrate was concentrated to dryness in vacuo at 40°C to render the MeOH extract (230 g). The extract was then suspended in distilled H2O and partitioned with CH2Cl2 (28 g), EtOAc (23 g), n-BuOH (71 g), and H2O (104 g) in sequence. The EtOAc (23 g) fraction was chromatographed on a Si gel column (7×140 cm), using CH2Cl2:MeOH (25:1, gradient) as the solvent to yield 17 subfractions (Fr. 1 to Fr. 17). Fraction 15 (1.5 g) was sub-
ject to column chromatography over a Si gel column (3×100 cm) with CH₃Cl₂:MeOH (20 : 1, gradient) to obtain compounds 1 (kurarinol, 150 mg) and 2 (kuraridinol, 50 mg). Fraction 16 (15.4 g) was subjected to Si gel (hexane : EtOAc, 2 : 3, gradient), and to RP-18 column chromatography (70% MeOH), yielding compound 3 (triflorilizin, 700 mg).

**Tyrosinase Inhibitory Activity Assay** Tyrosinase activity, using L-tyrosine as a substrate, was spectrophotometrically determined by a previously described method, with slight modification. Ten microliters of each sample solution with different concentrations (1—500 μg/ml) and 20 μl of mushroom tyrosinase (1000 U/ml) in a 50 mM phosphate buffer (pH 6.5), were added to 170 μl of an assay mixture containing a 10 : 10 : 9 ratio of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5), and distilled water in a 96-well microplate. The samples dissolved in EtOH were diluted 30 times with H₂O prior to the experiments. After incubation of the reaction mixture at 25 °C for 30 min, the absorbance of the mixture was measured at 490 nm (ε=3.3×10³ M⁻¹ cm⁻¹) using a microplate reader (VERSA max, Molecular Device, CA, U.S.A.). The extent of inhibition from the samples was expressed as the concentration necessary for 50% inhibition (IC₅₀). One unit (U) of enzymatic activity was defined as the amount of enzyme needed to increase the absorbance at 280 nm by 0.001 per min, in a 3 ml reaction mixture containing L-tyrosine at pH 6.5 and 25 °C.

**Kinetic Analysis** The reaction mixture consisted of three different concentrations of the L-tyrosine substrate (0.5 to 2 mM) and mushroom tyrosinase, in a 50 mM potassium phosphate buffer. The samples of various concentrations were added to the reaction mixture, respectively. The Michaelis constant (Kₘ) and maximal velocity (Vₘₐₓ) of the tyrosinase were determined by Lineweaver–Burk plots. The reciprocal equation for the noncompetitive inhibition form was: 

\[1/V = K_v/V_{max} (1 + [I]/K_i) + 1/V_{max} (1 + [I]/K_i) \]

The inhibition constants (Kᵢ) of the noncompetitive inhibitors were calculated by the following equation: 

\[1/V_{max,app} = (1 + ([I]/K_i))/V_{max} \]

In the presence of any inhibitor concentration.

**Cell Culture** Melanoma B16 cells (CRL 6323) were obtained from ATCC (Manassas, U.S.A.), cultured in DMEM (13.4 mg/ml Dulbecco’s modified Eagle’s medium, 10 mM HEPES, 143 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, 24 mM NaHCO₃, pH 7.1) containing 10% FBS, and incubated at 37 °C under 5% CO₂ atmosphere. Melanin Quantification The B16 cells were seeded at a concentration of 10⁴ cells per well of 6-well culture plates, in DMEM (13.4 mg/ml Dulbecco’s modified Eagle’s medium, 10 mM HEPES, 143 U/ml benzylpenicillin potassium, 100 μg/ml streptomycin sulfate, 24 mM NaHCO₃, pH 7.1) containing 10% FBS, and incubated at 37 °C under 5% CO₂ atmosphere.

**RESULTS AND DISCUSSION**

In this study, we investigated tyrosinase inhibitory activity of the MeOH extract derived from *S. flavescens* root, along with its solvent soluble fractions as represented by CH₃Cl₂, EtOAc, and n-BuOH, and the water layer. The MeOH extract and the CH₃Cl₂, EtOAc, and n-BuOH fractions inhibited L-tyrosine oxidation catalyzed by tyrosinase in concentration-dependent manners, with IC₅₀ values of 50.47±0.72, 18.05±0.11, 15.09±0.11, and 73.86±4.42 μg/ml, respectively, and are compared to that of the positive control kójic acid (4.62±3.60 μg/ml). It is interesting to note that the EtOAc fraction had more potent inhibitory activity than the other fractions. The comparative inhibitory activities of the fractions against mushroom tyrosinase can be explained based on their compositional differences. Polar flavonoids and phenolic acids are of great interest for their tyrosinase inhibitory activity, and were expected to be present in the EtOAc fraction in large amounts. Although some prenylated flavonoids from the CH₃Cl₂ fraction of the ethanolic extract of *S. flavescens*, such as kurarinid, sophorafavanone G, kurarione, and kushenol F, were previously reported to be tyrosinase inhibitors, the possible tyrosinase-inhibitory effects, and the active principles inherent to the EtOAc fraction, had not been adequately investigated.

Therefore, we conducted further detailed phytochemical investigations of the EtOAc fraction via repeated chromatography over silica gel and RP-18 gel columns, which led to the isolation of three compounds. The structures of these compounds were determined by 1D (¹H, ¹³C-NMR) and 2D NMR (HMQC and HMBC) analyses, and by comparisons with published spectral data. The chemical structures of compounds 1—3 (Fig. 1) were identified as kurarinol (1), kuraridinol (2), and triflorilizin (3), respectively.

The inhibitory activities of these compounds toward mushroom tyrosinase were also investigated. Compounds 1 and 2 exhibited marked inhibitory activities on L-tyrosine oxidation catalyzed by tyrosinase, with IC₅₀ values of 8.60±0.51 and 0.88±0.06 μM, respectively, and which manifested in concentration-dependent manners. On the other hand, compound 3 showed moderate inhibitory activity with an IC₅₀ of 506.77±4.94 μM. Kuraridinol (2), in particular, demonstrated an inhibitory activity twenty times more potent than that of the positive control, kójic acid (IC₅₀ 16.22±1.71 μM). These differences may be explained by the specific binding properties between the enzyme and substrate. Kurarinol is reported to be a prenylated flavanone and an effective inhibitor of
alpha-glucosidase, beta-amylase, and cGMP phosphodiesterase-5, and also inhibits diacylglycerol acyltransferase activity.13,19,21,22) On the other hand, kuraridinol is a chalcone compound belonging to the prenylated flavonoids.

To the best of our knowledge, this is the first report on the inhibitory effects of these compounds against mushroom tyrosinase. The inhibition kinetics for compounds 1 and 2 were analyzed by Lineweaver–Burk plots, as shown in Fig. 2. The lines, which were obtained from the uninhibited enzyme and from the three different concentrations of 1 and 2, intersect to the left of the 1/1'-axis above the 1/S-axis. The results indicate that compounds 1 and 2 exhibited a noncompetitive-type of inhibition with respect to the substrate (L-tyrosine). The equilibrium constants for the inhibitor binding (K_i) of 1 and 2 were estimated to be 1.4×10^{-5} and 3.9×10^{-7} M, respectively (Table 1). This implies that 1 and 2 bind to a site adjacent to the active site in a manner that interferes with substrate binding.

It is well established that prenylated flavonoids containing the resorcinol moiety from S. flavescens inhibit mushroom tyrosinase.10—14) Examination of the structure–activity relationships of prenylated flavonoids has indicated substitutions of the lavandulyl or hydroxylavandulyl groups at the C-8 position, and 2',4'-dihydroxy groups on the B-ring. By comparing the tyrosinase inhibitory activity of 2 with that of 1, where both possess a B-ring 2',4'-dihydroxy moiety, a hydroxylavandulyl group at the C-8 position, and a methoxy group at C-5, revealed that kuraridinol (2) had a ten-fold higher activity than kurarinol (1), emphasizing that tyrosinase inhibitors with chalcone structure's have enhanced potential. In addition, kuraridin, which has a chalcone skeleton with similar structural features (A-ring, C-8 lavandulyl, C-5 hydroxyl, and B-ring 2',4'-dihydroxy groups), was recently demonstrated to have more potent tyrosinase inhibitory activity than sophoraflavone G and kurarinone, which have flavanone skeletons.12)

It was also recently reported that some chalcones possessing a 2',4'-substituted resorcinol moiety, act as potential tyrosinase inhibitors, and the position of the hydroxyl group attached to the chalcone ring is highly important for activity.23—25) Overall, these structure-related activity studies have added some understanding of the roles and contributions of different functional groups associated with tyrosinase inhibitors.25—28) Our results further support that 2',4'-dihydroxy chalcones, with substitutions of a lavandulyl or hydroxylavandulyl group at the C-8 position, and a methoxy or hydroxyl group at C-5, represent a new class of tyrosinase inhibitors, and are important for the inhibition of tyrosinase activity.

Skin-whitening agents that inhibit tyrosinase or other enzymes must first penetrate the melanocyte cell wall. Thus
IBMX-induced melanin production and cell growth

None of the compounds caused insignificant numbers of trypan blue-stained cells. Upon exposure to IBMX, the melanoma B16 cells were seeded at a density of 2.5 × 10^6 cells per well of 6-well culture plates, incubated in DMEM media for 24 h, and then treated with various concentrations of kurarinol (1) (solid box), kuraridinol (2) (open box) or trifolirhizin (3) (hatched box) in the presence of IBMX for 72 h. (A) Amounts of intracellular melanin within 1 × 10^7 cells were quantified using synthetic melanin as a standard. (B) Cell numbers were counted in a hemocytometer after staining with trypan blue. Values are mean ± S.E. from three independent experiments. # p < 0.01 versus medium alone-treated group. * p < 0.01 versus IBMX alone-treated group.

Fig. 3. Effects of Kurarinol (1), Kuraridinol (2) and Trifolirhizin (3) on IBMX-Induced Melanin Production and Cell Growth

The B16 cells were seeded at a density of 2.5 × 10^6 cells per well of 6-well culture plates, incubated in DMEM media for 24 h, and then treated with various concentrations of kurarinol (1) (solid box), kuraridinol (2) (open box) or trifolirhizin (3) (hatched box) in the presence of IBMX for 72 h. (A) Amounts of intracellular melanin within 1 × 10^7 cells were quantified using synthetic melanin as a standard. (B) Cell numbers were counted in a hemocytometer after staining with trypan blue. Values are mean ± S.E. from three independent experiments. # p < 0.01 versus medium alone-treated group. * p < 0.01 versus IBMX alone-treated group.

this study further tested compounds 1—3 in B16 melanoma cells in order to confirm their activities in melanocytes (Fig. 3). Here we found that the compounds showed inhibitory effects on melanogenesis in the melanoma B16 cell line that was stimulated with IBMX, an elevator of intracellular cAMP. The melanoma B16 cells, in a resting state, produced basal amounts (42—55 μg) of intracellular melanin per 1 × 10^7 cells, during incubation for 72 h (Fig. 3A). Upon exposure to IBMX alone, the cells significantly increased the production of intracellular melanin (370—403 μg) over the same period (Fig. 3A). Compound 1 showed dose-dependent inhibitory effects with 15% inhibition at 12.5 μM, 31% at 25 μM and 95% at 50 μM, showing an IC_{50} value of 29 μM on IBMX-induced production of intracellular melanin (Fig. 3A). Similarly, compound 2 decreased IBMX-induced production of intracellular melanin with 32% inhibition at 12.5 μM, 64% at 25 μM and 99% at 50 μM, showing an IC_{50} value of 17 μM (Fig. 3A). Compound 3 also exhibited 12% inhibition at 12.5 μM, 29% at 25 μM and 76% at 50 μM, showing an IC_{50} value of 36 μM (Fig. 3A). Growth of melanoma B16 cells was also measured using trypan blue exclusion. Upon exposure to IBMX alone, growth rate of the cells was significantly retarded but viability of the cells was not affected because of insignificant numbers of trypan blue-stained cells (Fig. 3B). None of compounds 1—3 had shown significant cytotoxic effects to IBMX-stimulated B16 cells (Fig. 3B). These results indicate that compounds 1—3 can inhibit cAMP-mediated melanin production because IBMX is an elevator of intracellular cAMP; cAMP is shown to be a key messenger in the regulation of skin pigmentation. Our data are consistent with others, where the inhibition of melanogenesis was characterized by the inhibition of tyrosinase.

In the present study, kurarinol, kuraridinol, and trifolirhizin showed significant dual inhibitory effects on tyrosinase and melanin synthesis. In particular, kuraridinol exhibited higher inhibitory activities on tyrosinase and melanin synthesis than kurarinol. In support of this, Kim et al. reported that as a chalcone compound, kurarinid had significant anti-tyrosinase activity.

In summary, the present study demonstrated that S. flavescentis and its components, kurarinol, kuraridinol, and trifolirhizin, have tyrosinase inhibitory effects. These compounds also inhibited melanin synthesis in B16 melanoma cells. The results suggest that kurarinol, kuraridinol, and trifolirhizin from S. flavescentis have potential roles as novel skin-whitening agents for ultraviolet-sensitive skin. However, in order to clarify their beneficial/harmful effects in vivo, this aspect should be investigated further.

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REFERENCES