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Molecular docking studies of a phlorotannin, dieckol isolated from *Ecklonia cava* with tyrosinase inhibitory activity

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ABSTRACT

In this study, the phlorotannin dieckol, which was isolated from the brown alga *Ecklonia cava*, was examined for its inhibitory effects on melanin synthesis. Tyrosinase inhibitors are important agents for cosmetic products. We therefore examined the inhibitory effects of dieckol on mushroom tyrosinase and melanin synthesis, and analyzed its binding modes using the crystal structure of *Bacillus megaterium* tyrosinase (PDB ID: 3NM8). Dieckol inhibited mushroom tyrosinase with an IC_{50} of 20 μM and was more effective as a cellular tyrosinase having melanin reducing activities than the commercial inhibitor, arbutin, in B16F10 melanoma cells, and without apparent cytotoxicity. It was found that dieckol behaved as a non-competitive inhibitor with L-tyrosine substrates. For further insight, we predicted the 3D structure of tyrosinase and used a docking algorithm to simulate binding between tyrosinase and dieckol. These molecular modeling studies were successful (calculated binding energy value: −126.12 kcal/mol), and indicated that dieckol interacts with His208, Met215, and Gly46. These results suggest that dieckol has great potential to be further developed as a pharmaceutical or cosmetic agent for use in dermatological disorders associated with melanin.

1. Introduction

Tyrosinase (monophenol monooxygenase; EC 1.14.18.1) is widely distributed in nature, and is a metalloenzyme oxidase that catalyzes two distinct reactions of melanin synthesis in which L-tyrosine is hydroxylated to 3,4-dihydroxyphenylalanine L-DOPA (monophenolase activity), and the latter is subsequently oxidated to dopaquinone (diphenolase activity).[1]

Melanin helps to protect skin from the damaging ultraviolet radiation of the sun. However, high concentrations of melanin in the skin result in hypopigmentation such as freckles and moles. Tyrosinase is the rate-limiting enzyme in melanin production, which occurs in melanocytes that are located within the basal epidermis.[2] Therefore, the inhibition of tyrosinase is one of the major strategies to treat hyperpigmentation.[3] Safe and effective tyrosinase inhibitors that act to minimize skin pigmentation abnormalities are desired. Despite a large number of tyrosinase inhibitors, only a few of these are used today, as many of them show side effects.[4,5] Well-known tyrosinase inhibitors such as kojic acid, hydroquinone, and 1-phenyl-2-thiourea (PTU) can cause adverse reactions such as dermatitis and skin irritation,[6] melanocyte destruction,[7] and skin cancer.[8] As a result, it is necessary to search for new candidates that show effective tyrosinase inhibition but are devoid of side effects; thus, metabolites biosynthesized by plants have become promising alternatives to synthetic analogs.[9,10]

Ecklonia cava, a kind of brown alga, is abundantly produced around Jeju Island, Korea, and is utilized as a food ingredient, animal feed, fertilizer, and medicine. In addition, *E. cava* contains a variety of compounds including carotenoids, fucoidan, and phlorotannins, which show different biological activities.[11–13] Very recently, some studies have explored the potential cosmetic activities of phlorotannins isolated from *E. cava*.[14] In our continuing investigation of phlorotannins isolated from *E. cava*, dieckol was chosen as a target compound for its tyrosinase inhibition activity and reduction of melanin synthesis.

The aim of this study was to evaluate the tyrosinase and melanin synthesis inhibitory activity of this phlorotannin to see if it can be applied in the cosmetic and pharmaceutical fields.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase, L-tyrosine [3-(4-hydroxyphenol)]-L-alanine(*S*)-2-amino-3-(4-hydroxyphenol) propionic acid], arbutin

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[2-hydroxymethyl-6-(4-hydroxyphenoxy)oxane-3,4,5-triol], and α-MSH (alpha-melanocyte stimulating hormone) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Extraction and isolation

The brown alga, *E. cava* was collected along the coast of Jeju Island, Korea, between February and March 2010. The sample was washed three times with tap water to remove the salt, epiphytes, and sand attached to the surface, then carefully rinsed with fresh water and maintained in a medical refrigerator at −20 °C. Thereafter, the frozen sample was lyophilized and homogenized with a grinder prior to extraction. The powdered *E. cava* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with EtOAc. The EtOAc extract was fractionated via silica column chromatography with stepwise elution of a $CHCl_3$–MeOH mixture (100:1–1:1) to generate separated active fractions. The combined active fraction was then further subjected to a Sephadex LH-20 column (GE Healthcare, USA) saturated with 80% MeOH, and finally purified via reversed-phase HPLC (ThermoFisher Scientific, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 150 × 20 mm, 4 μm; YMC Co.) by stepwise elution with methanol–water gradient (UV range: 230 nm, flow rate: 0.8 mL/min). The purified compound, dieckol was confirmed by comparing their LC/MS, ^{1}H NMR data to the literature report.[15]

Dieckol: LC/MS data (M^+, *m*/*z*: 742.0 Calcd for $C_{36}H_{22}O_{18}$). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9″), 9.51 (1H, s, OH-4″), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3″, 5″), 9.28 (1H, s, OH-2″), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7″), 9.15 (2H, s, OH-3′, 5′) 6.17 (1H, s, H-3″), 6.14 (1H, s, H-3), 6.02 (1H, d, *J* = 2.7 Hz, H-8), 5.98 (1H, d, *J* = 2.7 Hz, H-8″), 5.95 (1H, s, H-2‴, 6‴), 5.82 (1H, d, *J* = 2.7 Hz, H-6), 5.81 (1H, d, *J* = 2.7 Hz, H-6″), 5.80 (1H, t, *J* = 2.0 Hz, H-4′), 5.78 (2H, d, *J* = 2.0 Hz, H-2′, 6′).

The purity of dieckol (Fig. 1) was >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis. Dieckol was dissolved in DMSO and used for experiments adjusting the final concentration of DMSO in culture medium to <0.01%.

2.3. Cell culture

B16F10 cells (obtained from the Korea Cell Line Bank) were grown on Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbas, CA, USA) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cultures were maintained at 37 °C in a 5% CO_2 incubator. B16F10 cells were cultured in 24-well plates for melanin quantification and enzyme activity assays.

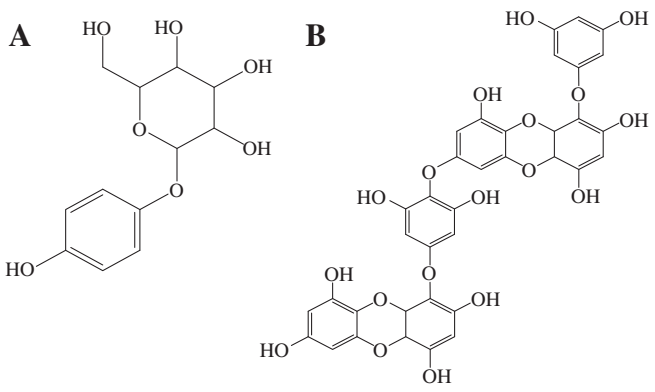

Figure 1. Chemical structures of arbutin (A) and dieckol (B).

2.4. Cell viability

Cell survival was quantified through a colorimetric MTT assay that measured the mitochondrial activity in viable cells. B16F10 cells were seeded (1×10^5 cells/mL) together with various concentrations of dieckol (40, 80, and 120 μM) and incubated for up to 72 h prior to MTT treatment. MTT stock solution (50 μL; 2 mg/mL in PBS) was added to each well to achieve a total reaction volume of 250 μL. After 4 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm.

2.5. Assay for the measurement of inhibitory effects on mushroom tyrosinase

Mushroom was used as the source of tyrosine for the entire study. To begin the assay,[16] a 20 μL of an aqueous mushroom tyrosinase solution (1000 units) was added to a 96-well microplate in a 200 μL assay mixture containing 1 mM L-tyrosinase solution and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25 °C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined at 492 nm using microplate reader.

2.6. Kinetic analysis of tyrosinase inhibition

Various concentrations of L-tyrosine (0.5–1.5 mM) as a substrate, 20 μL of aqueous mushroom tyrosinase solution (1500 units), and 50 mM potassium phosphate buffer (pH 6.5), with or without dieckol, were added to a 96-well plate in a total volume of 200 μL. Using a microplate reader, the initial rate of dopachrome formation from the reaction mixture was determined by the increase in absorbance at a wave length of 492 nm per minute. The Michaelis constant (K_m) of the tyrosinase activity were determined from a Lineweaver–Burk plot at various L-tyrosine concentrations. A modification of the Michaelis–Menten equation was required to describe the reaction kinetics due to competitive inhibition by the compounds together with substrate inhibition by L-tyrosine.[17]

2.7. Assay on cellular tyrosinase activity

Tyrosinase activity was estimated by measuring the rate of L-DOPA oxidation.[18] Arbutin was used as a positive control. α-MSH, which is known to stimulate melanogenesis, was used at 0.1 μM as the negative control, and its effect on melanogenesis was compared to that of the test sample. Cells were placed in 24-well dishes at a density of 1×10^5 cells/mL. B16F10 cells were incubated in the presence or absence of 0.1 μM α-MSH and they were then treated for 72 h with various concentrations of dieckol. The cells were washed and lysed in 100 μL of 50 mM sodium phosphate buffer (pH 6.5) containing 1% Triton X-100 and 0.1 mM PMSF (phenyl methyl sulfonyl fluoride), and then frozen −80 °C for 30 min. After being thawed and mixed, the cellular extracts were placed in a 96-well plate, and the absorbance at 492 nm was recorded every 10 min for 1 h at 37 °C using ELISA plate reader.

2.8. Determination of melanogenesis in B16F10 cells

The melanin contents were determined using a modification of the method to the literature report.[19] In the present study, the

amount of melanin was used as the index of melanogenesis. B16F10 cells (1×10^5 cells/mL) were transferred to 24-well dishes and incubated in the presence or absence of 0.1 μM α-MSH. The cells were then incubated for 72 h with various concentrations of dieckol. The samples were washed with PBS and then dissolved in 100 μL of 1 N NaOH. The samples were incubated at 60 °C for 1 h and mixed to solubilize the melanin. The amount of melanin contents were assessed by measuring the absorbance at 405 nm.

2.9. In silico docking of tyrosinase and new inhibitor candidate

Molecular docking is an application wherein molecular modeling techniques are used to predict how a protein (enzyme) interacts with small molecules (ligands).[20–22] The ability of a protein to interact with small molecules plays a major role in the dynamics of that protein, which may enhance or inhibit its biological function. In the present study, we performed docking of phlorotannin into the active site of the mushroom tyrosinase. The crystal structure of tyrosinase (PDB: 3NM8) was obtained from the Protein Data Bank (PDB, http://www.pdb.org). We performed the docking studies using CDOCKER in Accelrys Discovery Studio 3.0 (Accelrys, Inc). We describe the ligand structure of the tyrosinase inhibitor candidate in Figure 1. To prepare for the docking procedure, we performed the following steps: (1) conversion of the 2D structure into 3D structure; (2) calculation of charges; and (3) addition of hydrogen atoms using the CDOCKER docking program.

3. Results

3.1. Inhibitory activity of dieckol against mushroom tyrosinase

The inhibitory effect of dieckol against mushroom tyrosinase activity was examined by measuring the hydroxylation of L-tyrosine. Arbutin was selected as a positive control compound since its inhibitory activity was already reported.[23] As shown in Figure 2, dieckol inhibited tyrosinase activity in a dose-dependent manner (10–120 μM). In particular, dieckol showed 89.3% of tyrosinase inhibitory activity even at 120 μM, and its values were higher than those of the commercial whitening agent, arbutin.

3.2. Inhibitory type and inhibition constant of dieckol

Under the conditions employed in the present study, L-tyrosine oxidation was determined by the enzyme Lineweaver–Burk assay.

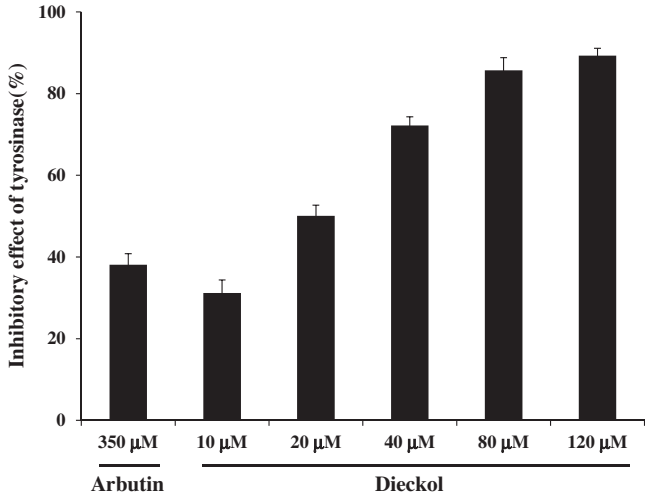

Figure 2. Inhibitory effect of dieckol isolated from *E. cava* against mushroom tyrosinase. L-Tyrosine was used as substrate, and arbutin was as positive control.

Dieckol behaved as a non-competitive inhibitor (Fig. 3), which means that it could combine with both the free enzyme and the enzyme-substrate complex. Thus, the interaction between dieckol and the enzyme was independent from the interaction between the substrate and the enzyme, and the inhibitor did not change substrate-enzyme affinity. With increasing concentrations, a series of lines was revealed with a common intercept on the 1/[L-tyrosine] axis, but with different slopes.

3.3. Cytotoxicity of dieckol in B16F10 melanoma cells

An MTT assay was used to investigate if the inhibitor would adversely induce B16F10 melanoma cell death. The samples were treated with various concentrations from 40 to 120 μM of dieckol. Arbutin was used as a positive control. α-MSH, which is known to stimulate melanogenesis, was used at 0.1 μM as the negative control, and its effect on melanogenesis was compared to that of the test sample. As shown in Figure 4, cells treated with all testing concentrations of dieckol exhibited more than 95% of cell viability for up to 72 h. In addition, arbutin and α-MSH exhibited no cytotoxic effects on B16F10 melanoma cells.[24]

3.4. Tyrosinase inhibitory activity of dieckol in B16F10 melanoma cells

We examined the effect of dieckol in reducing tyrosinase activity in B16F10 melanoma cells. As shown in Figure 5, dieckol had a similar inhibition effect on B16F10 melanoma cells as it did on mushroom tyrosinase. To determine the effect of dieckol on tyrosinase activity, the cells were then exposed to 0.1 μM α-MSH in the presence of 40, 80, and 120 μM dieckol or 350 μM arbutin, which is a representative tyrosinase inhibitor. As shown in Figure 5, cellular tyrosinase activity was significantly reduced in the dieckol-treated cells, and was below the levels found in the arbutin-treated cells.

3.5. Melanin synthesis by dieckol treatment in B16F10 melanoma cells

Melanin formation is the most important factor to determine mammalian skin color, and inhibition of melanin formation may result in reduced skin darkness. The inhibitory potency of dieckol against melanin synthesis was evaluated in melanoma B16F10 cells. As shown in Figure 6, the cellular melanin content of the 120 μM dieckol-treated cells was significantly reduced below the levels of the arbutin-treated cells, consistent with dieckol suppression of melanin synthesis in B16F10 cells by inhibition of tyrosinase activity.

3.6. In silico docking of tyrosinase

Tyrosinase is a copper-containing enzyme and is widely distributed in nature, and tyrosinase inhibitors that chelate copper or change the shape of the active site of the enzyme show competitive or non-competitive inhibition. Thus, the inhibition mechanism of phlorotannins such as dieckol might involve binding to the active site of mushroom tyrosinase.

We predicted the tertiary structure of tyrosinase and stimulated docking to dieckol. Using Discovery Studio 3.0 (DS), we searched for tyrosinase residues that would bind to arbutin and dieckol. The docking of the tyrosinase-ligand complexes were well-performed with arbutin and dieckol stably posed in the pocket of the tyrosinase by DS 3.0 (Fig. 7A–D). As for arbutin, the binding site predicted by the 2D program of DS 3.0 (Fig. 7E) was formed by the following residues: His208 (pi interaction bond), Gly216 (hydrogen bond), and Asn205 (hydrogen bond). In the case of dieckol conformation, the binding site predicted by the 2D program of DS 3.0

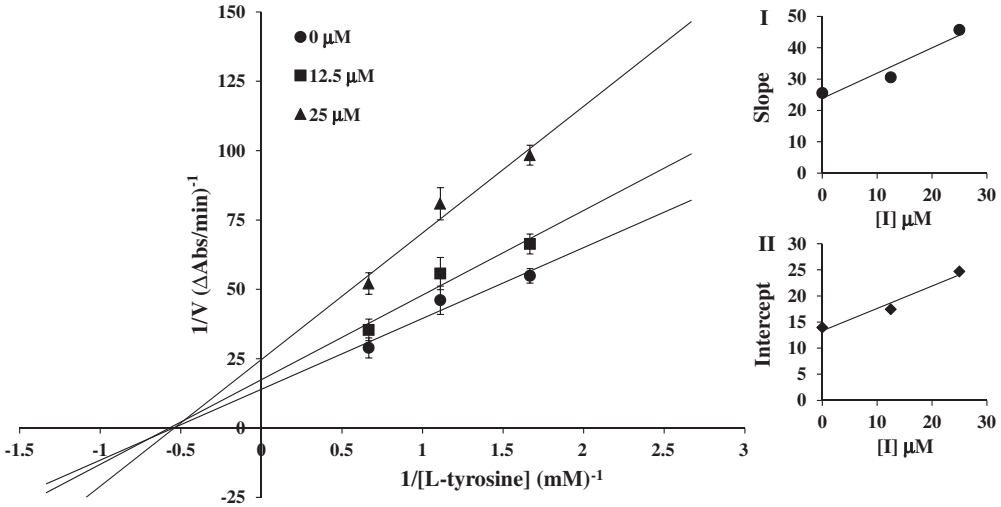

Figure 3. Lineweaver–Burk plots for inhibition of mushroom tyrosinase in presence of dieckol. Concentration of dieckol for curves were 0, 12.5, and 25 μM, respectively, with L-tyrosine as substrate. The insets represent the plot of the slope or the vertical intercepts versus dieckol concentration to determine inhibition constants.

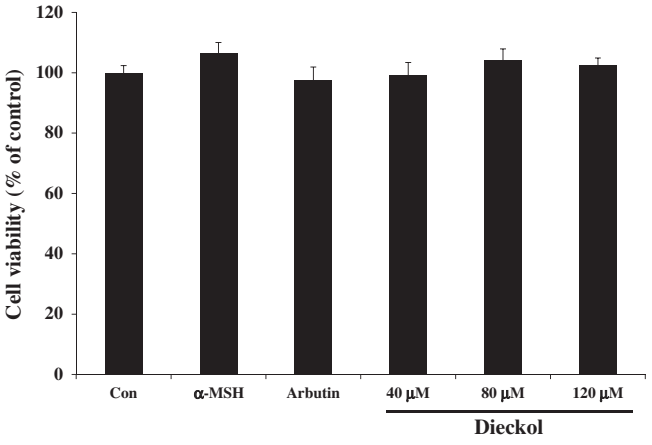

Figure 4. B16F10 cell viability with various concentrations of dieckol. Arbutin was used as melanin and tyrosinase inhibitor at 350 μM and α-MSH was a melanin stimulator at 0.1 μM.

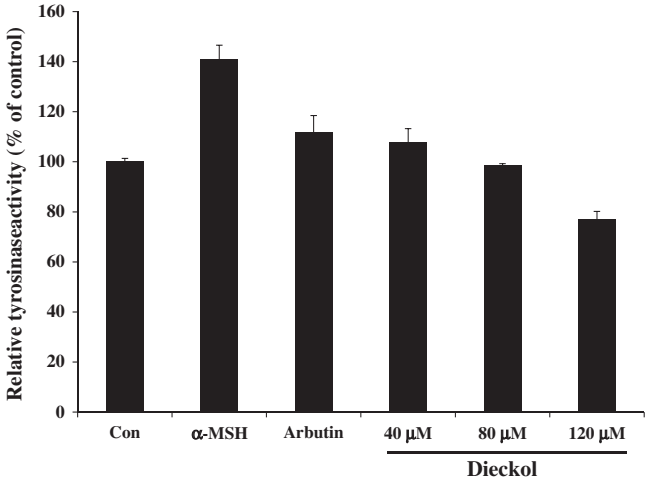

Figure 5. Effect on dieckol on cellular tyrosinase activity in B16F10 cells. Cells were exposed to 0.1 μM α-MSH in the presence of 40, 80, and 120 μM dieckol or 350 μM arbutin of tyrosinase inhibitor. Each percentage value for the treated cells is reported relative to that in the control cells.

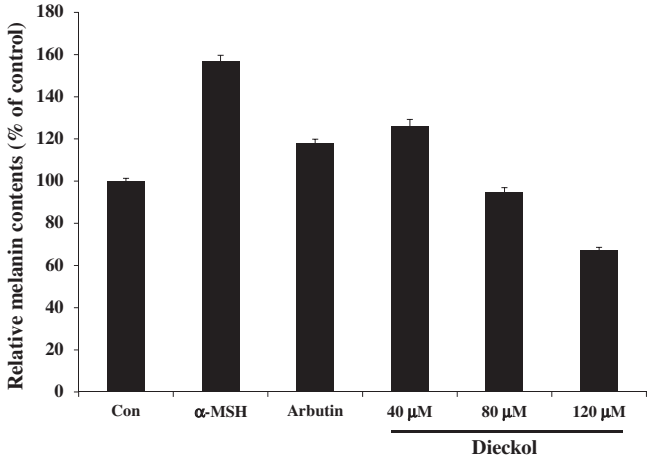

Figure 6. Effect on dieckol on cellular melanin synthesis in B16F10 cells. Cells were exposed to 0.1 μM α-MSH in the presence of 40, 80, and 120 μM dieckol or 350 μM arbutin of melanin inhibitor. Each percentage value for the treated cells is reported relative to that in the control cells.

(Fig. 7F) was formed by the residues: His208 (pi interaction bond), Met215 (hydrogen bond), and Gly46 (hydrogen bond). Moreover, the docking analysis results indicated that the following highest docking binding energy and lowest total binding energy confirmation of the most proposed complex had to be taken into account when using the CDOCKER interaction energy program of DS 3.0: arbutin: 44.01 kcal/mol and dieckol: 70.71 kcal/mol, and in the calculate program of DS 3.0: arbutin: −111.69 kcal/mol and dieckol: −126.12 kcal/mol (Table 1.). As shown in Table 1, the tyrosinase binding energy value of dieckol was more stable and strong than that of the positive control, arbutin.

4. Discussion

Melanin pigmentation protects tissues from absorption and dissipation of UV light.[25] And epidermal melanin that is synthesized by melanocytes, resulting from a cascade of enzymatic reactions,[26] is responsible for skin darkening. However, overproduction or dysregulation of melanin pigments can cause skin hyperpigmentation disorders such as freckles, melasma, senile lentigo, and age spots.[27]

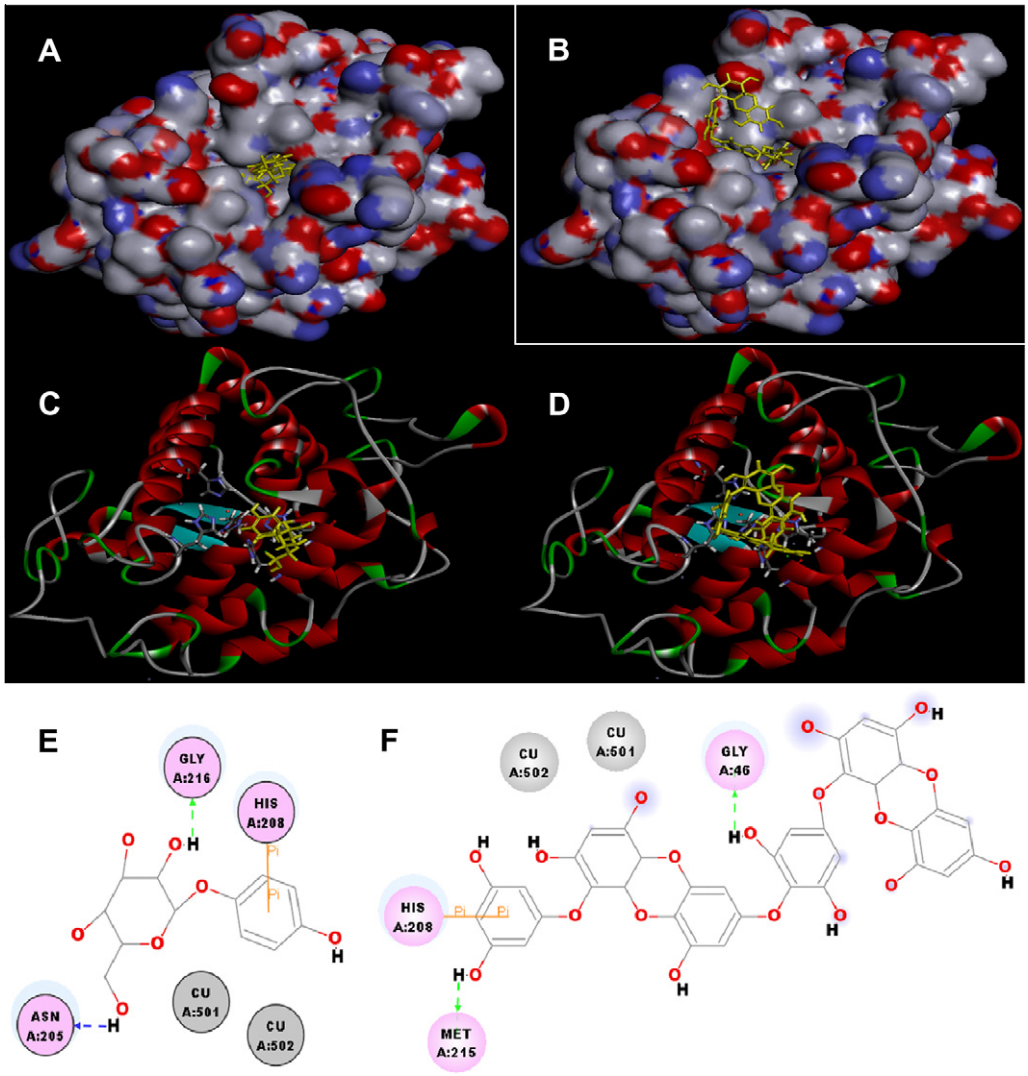

Figure 7. Computational prediction of the structure for tyrosinase and docking simulation with arbutin and dieckol. Predicted 3D structure of mushroom tyrosinase 3NM8. Tyrosinase-arbutin complex (A, C, and E); tyrosinase-dieckol complex (B, D, and F).

Table 1
Results of docking experiments of arbutin and dieckol with the tyrosinase (PDB ID: 3NM8)

Ligand	Binding energy (kcal/mol)	CDOCK interaction energy (kcal/mol)
Arbutin	−111.69	44.01
Dieckol	−126.12	70.71

Tyrosinase has long been known to be essential for melanization. In vertebrates, tyrosinase, the enzyme responsible for the initial steps of melanin synthesis, is closely associated with specialized organelles called melanosomes that are found in melanocytes.[28] Although melanin levels vary in the human population, the expression of tyrosinase does not vary significantly among human skin colors, and levels of tyrosinase mRNA were found to be similar in cultured melanocyte systems collected from black and white skin.[29]

The inhibition of tyrosinase has been the subject of numerous studies,[30,31] and several inhibitors are used as cosmetic additives and medicinal products in the treatment of hyperpigmentation. A recent ever-growing expansion of the global market demands more new products for depigmentation, cosmeceutical, and skin lighting purposes.[32] The most popular whitening agent is hydroquinone ever since its introduction in 1961, but its use has been curtailed as a cosmetic ingredient because of adverse cutaneous toxicity.[30] Additionally, several other phenolic compounds have been studied as depigmenting agents since their chemical structures are related to tyrosinase inhibitory activity. It has been suggested that the presence of a hydroxylic group and of an electron donator group in the phenol ring is a primary requirement for effective action as an alternative substrate of tyrosinase.[31] In our search for potent tyrosinase inhibitors, we found that dieckol has highly effective inhibitory activity against melanin synthesis, which is more potent than the commercial agent, arbutin.

In the present study, we investigated the effect of dieckol on tyrosinase inhibition in the anticipation of finding a new effective substance for skin whitening purposes and the prevention of hyperpigmentation, and found that dieckol clearly reduced tyrosinase activity in a dose-dependent manner. A kinetic study of the inhibition of mushroom tyrosinase by dieckol showed that this substance behaves as a non-competitive inhibitor, as evaluated by a Lineweaver–Burk kinetic analysis.

To understand the mechanism underlying the interaction between tyrosinase and dieckol and to explore their binding mode, a docking study was performed using the CDOCKER function available in Discovery Studio 3.0. These docking studies yielded crucial

information concerning the operation of the inhibitor in the binding pocket of tyrosinase. Ligand–enzyme interaction analysis found that the interaction binding residues of dieckol (His208, Met215, and Gly46) were more important than those of arbutin (His208, Gly216, and Asn205) in the active site, and were the main contributors to the receptor–ligand interaction. It was observed that for better tyrosinase inhibitory activity, the three amino acid residues mentioned above are in close vicinity to the molecule. The docking simulation supported the non-competitive inhibition observed, as this type of inhibition is generally encountered when there are multiple possible binding sites for an inhibitor.

In conclusion, dieckol was identified as a non-competitive inhibitor of mushroom tyrosinase in a kinetic study. The lack of dieckol cytotoxicity indicates that the suppression of melanogenesis by dieckol can be attributed to the inhibition of murine tyrosinase. These results suggest that dieckol has strong de-pigmenting activity without discernible cytotoxicity in B16F10 melanoma cells. Dieckol is therefore a promising candidate for the development of safe pharmacological or cosmetic agents that have potent inhibitory effects against tyrosinase activity and melanin synthesis.

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