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RESEARCH PAPER

Methyl Lucidenate F Isolated from the Ethanol-soluble-acidic Components of *Ganoderma lucidum* is a Novel Tyrosinase Inhibitor

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Abstract Tyrosinase is a key enzyme in the biosynthesis of melanin, and the use of inhibitors against tyrosinase can prevent hyperpigmentation by inhibiting enzymatic oxidation. However, the current use of tyrosine inhibitors is limited by their low activities and high toxicities. The aim of the present research was to develop novel whitening agents, or tyrosinase-targeted medicine, from a submerged culture of the fungus *Ganoderma lucidum*. Methyl lucidenate F was isolated from the ethanol-soluble-acidic components (ESACs) of *G. lucidum*, with the structure of ESACs elucidated *via* UV, LC-MS, and ^{13}C-NMR spectral analysis. The tyrosinase inhibitory activity was measured using catechol as a substrate. Methyl lucidenate F displayed uncompetitive inhibition of the potato tyrosinase activity, for which Lineweaver-Burk plots revealed a maximum reaction rate (V_{max}) of 0.4367/min, Michaelis constant (K_m) of 6.765 mM and uncompetitive inhibition constant (K_i) of 19.22 μM. Meanwhile, methyl lucidenate F (tetra cyclic triterpenoid) exhibited high tyrosinase inhibitory activity, with an IC_{50} of 32.23 μM. These results suggest that methyl lucidenate F may serve as a potential candidate for skin-whitening agents.

Keywords: inhibitor, methyl lucidenate F, tyrosinase, *Ganoderma lucidum*, submerged culture

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1. Introduction

Tyrosinase (EC 1.14.18.1) is an important polyphenoloxidase, which serves as the key enzyme in the biosynthesis of melanin. Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (monophenoloxidase activity) and the oxidation of L-dopa to dopaquinone (diphenoloxidase activity) [1]. Dopaquinones are highly reactive and can spontaneously polymerize to form melanins, the high molecular-weight brown pigments that determine the color of mammalian skin and hair (Fig. 1).

Many diseases may be induced during melanogenesis, such as malignant melanoma [2], albinism [3] and Parkinson's disease [4]. Tyrosinase inhibitors have attracted increasing interest in the fields of medicine and cosmetics due to their prevention of hyperpigmentation by inhibiting enzymatic oxidation. Many tyrosinase inhibitors have been reported, such as hydroquinone, kojic acid, vitamin C and arbutin. However, these inhibitors are associated with disadvantages, including high toxicity, low activity and insufficient penetrative ability [5]. Therefore, substantial research has focused on identifying novel tyrosinase inhibitors.

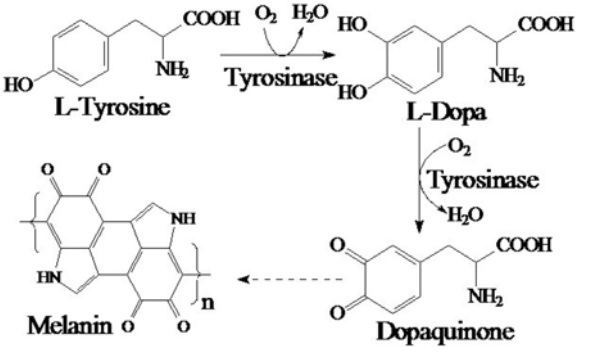

Fig. 1. Biosynthetic pathway of melanin.

G. lucidum (Fr.) Karst (Chinese: *Lingzhi*, Japanese: *Reishi*), a mushroom used in traditional Chinese medicine (TCM), is an abundant source of many bioactive substances, including polysaccharides, triterpenoids and sterols. *G. lucidum* also displays anti-hepatitis [6], anti-HIV [7], anti-tumor [8] and antihypercholesterolemic activities [9], as well as hypoglycemic effects [10] and inhibitory activity towards proteinase A [11]. However, the role of *G. lucidum* as a tyrosinase inhibitor has not been reported. The aim of the present research was to develop novel whitening agents, or tyrosinase-targeted medicine, from a submerged culture of *G. lucidum*. Methyl lucidenate F was isolated from the ethanol-soluble-acidic components (ESACs) of *G. lucidum*, with the structure of ESACs revealed via UV, LC-MS, and ^{13}C-NMR spectral analyses. The tyrosinase inhibitory activity was measured using catechol as a substrate.

2. Materials and Methods

2.1. Fungal strain and cultivation

G. lucidum SB1997 was screened and collected by the Bioresource and Biotransformation Laboratory of Jiangnan University, Wuxi, P. R. China. Cultures were maintained on potato-agar-dextrose slopes, which were inoculated, incubated at 30°C for 7 day, and stored at 4°C. To prepare an inoculum, *G. lucidum* mycelium was transferred to a 250 mL Erlenmeyer flask, containing 80 mL of medium (40 g/L glucose, 4 g/L peptone, and 1.5 g/L KH_2PO_4), at 30°C for 7 day, with shaking at 150 rpm. Subsequently, the seed culture was inoculated at 10% (v/v) into a 500-mL Erlenmeyer flask, with 150 mL of medium (20 g/L glucose, 20 g/L corn flour, 10 g/L bran meal, 10 g/L soy meal, and 1.5 g/L KH_2PO_4), at 30°C for 5 day, with shaking at 150 rpm. All the media were sterilized at 121°C for 25 min.

2.2. Preparation of crude extracts

The culture broth of *G. lucidum* was centrifuged at 4°C and 4,500 rpm for 20 min. The supernatant was precipitated by a triple volume of 95% (v/v) ethanol. The precipitate was stored at 4°C for 24 h, and then re-centrifuged at 4,500 rpm for 20 min, with the supernatant concentrated under reduced pressure using a BCHI Rotavapor R-200 (Switzerland). The ethanol extracts were dissolved in deionized water and extracted with ethyl acetate (EtOAc). The organic phase was extracted with an aqueous solution of 4% (w/v) sodium hydrogen carbonate ($NaHCO_3$). The aqueous phase was acidified on ice to pH 3.0, using 6 M hydrochloric acid. The aqueous phase was then re-extracted with EtOAc. The obtained ESACs in the EtOAc phase were vacuum-evaporated and dissolved in chloroform for further purification.

2.3. Purification of ESACs

The ESACs were fractioned on a normal-phase cilica-gal column (1.5 × 35 cm) with chloroform washing the column first, and then chloroform/methanol (98:2 v:v) eluting the aimed compound. Subsequently, the fraction was concentrated under reduced pressure and dissolved in 40 vol% methanol. The obtained sample was further purified on a reverse phase C-18 preparative column, with 40, 70, or 90 vol% methanol as the eluted solution. The 90 vol% methanol fraction was vacuum-evaporated and lyophilized to yield a primrose yellow powder.

2.4. Identification

Ultraviolet (UV) spectra of the chromatographic fraction were recorded using a UV 3000 spectrophotometer. LC-MS spectra were obtained via liquid chromatography (Waters ZMD 4000) linked to an electrospray ionization (ESI) mass spectrometry. The mobile phase was 80 vol% methanol, with a flow rate of 1 mL/min. The temperature and voltage of the ESI source were 120°C and 60 V, respectively. ^{13}C-NMR spectra were obtained using a NMR spectrometer (VXR-300) at 75 MHz in $CDCl_3$ solution, with TMS as the internal standard.

2.5. Preparation of potato tyrosinase

The preparation procedure for the potato tyrosinase was based on the method of Schmidt [12]. 150 g of peeled-potatoes were prepared by cutting to the size of soybeans. The potato pieces were then added to a pre-cooling refiner, with 2 g of polyethylene pyrrolidone (PVPP) (the impurities in the PVPP solution had been filtered out), followed by the addition of 200 mL of phosphoric acid buffer solution (0.1 mol/L, pH = 6.5). This mixture was mashed and immediately filtered through 6 levels of gauze. The filtrate was transferred into a beaker in an ice bath, and then deposited with ammonium sulphate to a 70% degree of saturation. After being allowed to settle for 30 min, the filtrate was centrifuged at 8,000 rpm and 4°C for 15 min, with the supernatant transferred into another beaker. The depositing program was then repeated. After being allowed to settle for 30 min, the filtrate was centrifuged at 8,000 rpm at 4°C for 20 min. The precipitate obtained was crude tyrosine enzyme. The prepared enzyme was lyophilized and stored in 0.1 M phosphate buffer solution (PBS) (pH 6.5) at −10°C until used [12,13].

2.6. Determination of tyrosinase inhibitory activity

By measuring the accumulation of *o*-diquinone at 420 nm, the tyrosinase inhibitory activity was determined using a $SPECTRA_{max}$ spectrophotometer, with catechol (*o*-diphenol) as the substrate. Briefly, 100 μL of 0.1 M phosphate buffer (pH 6.5), 50 μL of the test solution, with or without the test

sample, and 50 μL of a 50 mM catechol solution were mixed and incubated in a 96-well microplate at 30°C for 5 min. The reaction was initiated by the addition of 50 μL of the potato tyrosinase. The absorbance was measured every 10 sec. The slope of the initial 2 min linear reaction was used to calculate the enzyme activity. A stock solution of catechol was prepared fresh daily in 0.1 M PBS (pH 6.5). The inhibitory rate of tyrosinase (IRT) was calculated by [14]:

$$\text{IRT}\ (\%) = \frac{V_0 - V_i}{V_0} \times 100\%$$

where V_0 and V_i represent the enzymatic reaction rates ($\Delta A/\Delta t$) at 420 nm, without and with the test sample, respectively.

3. Results and Discussion

3.1. Identification of methyl lucidenate F

The UV spectra of the chromatographic fraction displayed a maximum absorption at 258.6 nm (Fig. 2A), consistent with the reported observation that ganoderic acids exhibit maximal absorption at 250 ~ 260 nm [15]. The net content of the chromatographic fraction was 95.6%, as calculated from the HPLC results via peak area regression (Fig. 2B). The molecular weight of the chromatographic fraction was 470 Da, according to the negative ion fraction (m/z) 469.5 [M-1], 505.5 [M+Cl], and positive ion fractions (m/z) 471.4 [M+1], 493.3 [M+Na] and 509.4 [M+K] in the mass spectra (Fig. 2C). The chemical shifts (δ values) of the chromatographic fraction, according to the ^{13}C-NMR spectra (Table 1), were consistent with reported ^{13}C-NMR data for triterpenoids [15,16].

The assumed structure of the chromatographic fraction is shown in Fig. 3. ^{13}C-NMR revealed the presence of an ester acyl (C_{24}, δ 173.1), a double bond between C_8 (δ 151.6) and C_9 (δ 151.9), two unconjugated carbonyl groups

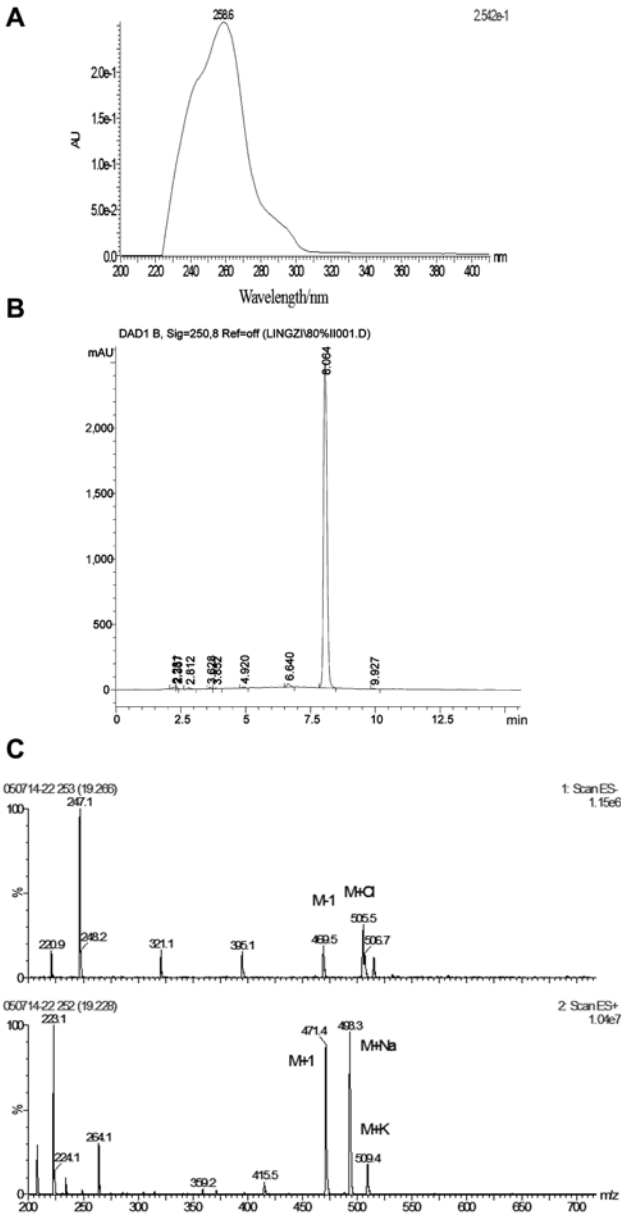

Fig. 2. UV (A), HPLC (B), and MS (C) spectra of the chromatographic fraction.

Table 1. ^{13}C-NMR chemical shifts (in $CDCl_3$ solution) of the chromatographic fraction

Atom no.	Reported δ value	Investigated δ value	Atom no.	Reported δ value	Investigated δ value	Atom no.	Reported δ value	Investigated δ value
C_1	34.0 ~ 36.2	35.0	C_{11}	197.1 ~ 201.2	198.9	C_{21}	18.1 ~ 19.9	18.5
C_2	33.8 ~ 34.5	33.9	C_{12}	44.1 ~ 52.7	44.4	C_{22}	28.7 ~ 32.5	30.6
C_3	215.2 ~ 217.9	215.4	C_{13}	45.3 ~ 49.0	44.7	C_{23}	32.2 ~ 34.7	31.4
C_4	45.9 ~ 47.5	47.0	C_{14}	58.6 ~ 59.7	61.2	C_{24}	170 ~ 178	173.1
C_5	41.1 ~ 50.7	40.1	C_{15}	206.8 ~ 217.8	215.8	C_{25}	51.8 ~ 52.3	51.9
C_6	35.0 ~ 38.2	33.5	C_{16}	36.8 ~ 39.4	36.7	C_{26}	17.0 ~ 20.8	16.0
C_7	197.1 ~ 200.3	198.9	C_{17}	45.3 ~ 49.6	45.8	C_{27}	19.0 ~ 21.1	21.6
C_8	149.5 ~ 150.3	151.6	C_{18}	16.8 ~ 19.5	20.1	C_{28}	19.0 ~ 21.1	21.6
C_9	148.2 ~ 153.8	151.9	C_{19}	17.1 ~ 19.9	22.7			
C_{10}	37.8 ~ 39.4	35.9	C_{20}	31.7 ~ 35.5	37.6			

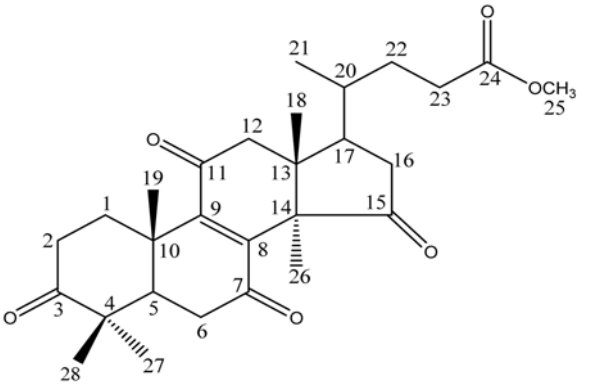

Fig. 3. Assumed structure of the chromatographic fraction.

(C_3, δ 215.4; C_{15}, δ 215.8), and two conjugated carbonyl groups (C_7, δ 198.9; C_{11}, δ 198.9), which were characteristic of the methyl lucidenate F structure.

3.2. Inhibitory effect of methyl lucidenate F on tyrosinase

The inhibitory effect of methyl lucidenate F on potato tyrosinase was investigated spectrophotometrically, using catechol as the substrate, by examining the linear relationship between the absorbance at 420 nm and the reaction time. The Michaelis-Menten and Lineweaver-Burk plots of the catechol catalyzed by potato tyrosinase at different methyl lucidenate F concentrations are shown in Fig. 4. The catalysis of catechol by tyrosinase was sharply reduced by the addition of increasing amounts of methyl lucidenate F (Fig. 4A). The Lineweaver-Burk profiles of the three reactions containing different inhibitor concentrations (0, 5.745, and 11.49 μM, Fig. 4C) were parallel to each other, suggesting that methyl lucidenate F interacts with potato tyrosinase via uncompetitive inhibition. The kinetic parameters, according to the Lineweaver-Burk plots, revealed a maximum reaction rate (V_{max}) of 0.4367/min for potato tyrosinase, a Michaelis constant (K_m) of 6.765 mM and uncompetitive inhibition constant (K_i) of 19.22 μM.

The methyl lucidenate F isolated from the submerged culture of *G. lucidum* showed a dose-dependent tyrosinase inhibition curve (Fig. 4C), with an observed tyrosinase inhibitory activity extremely close to the theoretical value. Methyl lucidenate F (tetra cyclic triterpenoid) exhibited high tyrosinase inhibitory activity, with an IC_{50} of 32.23 ìM. Tyrosinase inhibitors have been used as one of the major strategies in developing new whitening agents, and many tyrosinase inhibitors could be used as cosmetics additive [17-19]. In our research, methyl lucidenate F was proven to exhibit tyrosinase inhibition effects. In this sense, methyl lucidenate may be a potential candidate for skin-whitening agents.

To the best of our knowledge, this is the first study to show that methyl lucidenate F could be a potent tyrosinase

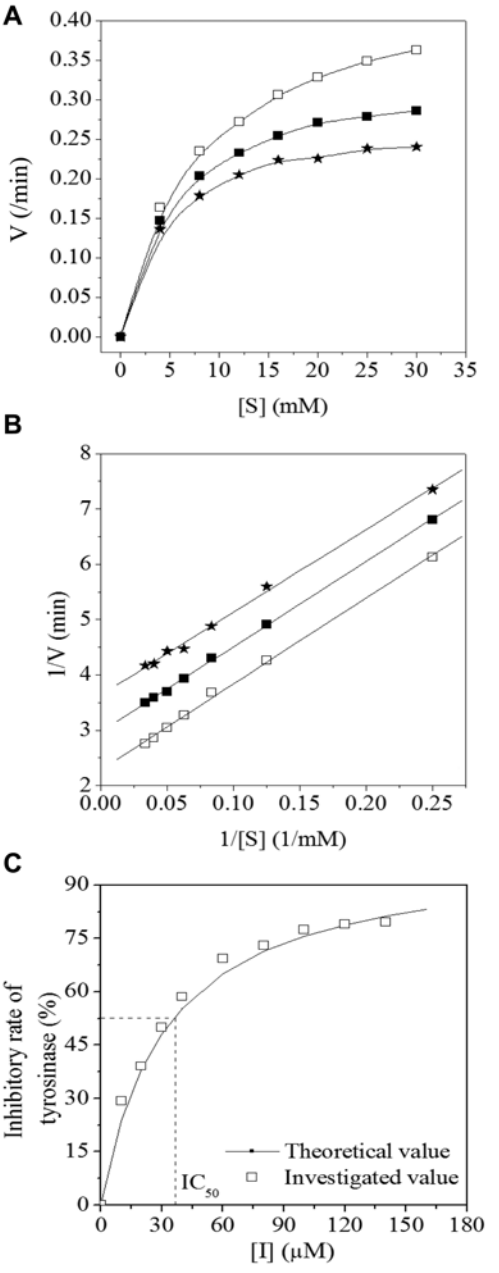

Fig. 4. Michaelis-Menten (A) and double reciprocal Lineweaver-Burk (B) plots of catechol catalyzed by potato tyrosinase with different concentrations of methyl lucidenate F: 0 μM (□), 5.745 μM (■), and 11.49 μM (★). (C) Dose-dependent relationship between methyl lucidenate F and tyrosinase inhibition. The data points were the mean of triplicate experiments.

inhibitor, suggesting that tetra cyclic triterpenoids may serve as candidates for skin-whitening agents. In addition to the potent tyrosinase inhibitory activity found in this paper, triterpenoids have also been reported to exhibit antibacterial [20,21] and antioxidant [22,23] activities. Thus, methyl lucidenate F, or other tetra cyclic triterpenoids, could be preferentially used as skin-whitening agents rather than commonly used tyrosinase inhibitors. The cellular-

level effects of tyrosinase inhibition and the potential cytotoxicity of methyl lucidenate F should be studied in greater detail.

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