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Tyrosinase Inhibitory Activities of *Meso*-dihydroguaiaretic Acid from *Machilus thunbergii*

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Machilus thunbergii (Lauraceae) is an evergreen tree cultivated in Korea and Japan. *M. thunbergii* has long been used as a traditional medicine in Korea, China, and Japan to treat various diseases, including edema, abdominal pain, and abdominal distension. In this study, dried stem bark of *M. thunbergii* extracted in methanol and extract was partitioned into *n*-hexane, $CHCl_3$, and BuOH. The $CHCl_3$-soluble extracts chromatographed on silica gel column using a $CHCl_3$/acetone and *n*-hexane/EtOAc mixture to afford Compound 1 and 2. Two dibenzylbutane lignans, macelignan (1) and *meso*-dihydroguaiaretic acid (2), were isolated from the $CHCl_3$-soluble extract of *M. thunbergii* stem bark. The structures of 1 and 2 were determined by ^{1}H- and ^{13}C-NMR spectroscopic data analyses and a comparison with literature data. The tyrosinase inhibitory activity of the isolated compounds was evaluated. Among these compounds, Compound 2 strongly inhibited the monophenolase (IC_{50}=10.2 μM) activity of tyrosinase. A kinetic analysis showed that Compound 2 was a competitive inhibitor. The apparent inhibition constant (K_i) for Compound 2 binding to free enzyme was 4.8 μM. Based on these results, it can be concluded that *meso*-dihydroguaiaretic acid (2) is a potential candidate for the treatment of melanin biosynthesis-related skin diseases.

Key words : Dibenzylbutane lignans, macelignan, *Machilus thunbergii*, *meso*-dihydroguaiaretic acid

Introduction

Machilus thunbergii (Lauraceae) is the evergreen tree grown in areas of Korea and Japan. The bark of this plant has been used as a folk medicine for the treatment of leg oederma, abdominal pain and abdominal distension in Korea [15]. Lignans, alkaloids [20], flavonoids [9], butanolids [10] and essential oils [11] have been reported as components from *M. thunbergii*, some of which have hepatoprotective activity as antioxidants [27], antibacterial activity [7], and inhibitory activity on nitric oxide synthesis in activated macrophages [9]. Macelignan and *meso*-dihydroguaiaretic acid (MDGA), an active lignan compounds were isolated from the stem bark of *M. thunbergii* [20]. Macelignan has been reported to possess antioxidant [14], anti-inflammatory [5], anticarinogenic, and hepatoprotective effects [24] and cause alteration in hepatic enzyme activities [21]. MDGA has been reported to have antioxidant [27], antifungal [1], antimicrobial [8], antiallergic [18], neuroprotective [16], and hepatoprotective activities [19].

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase, is a copper-containing mixed-function oxidase widely distributed in microorganism, animals, and plants. This oxidase catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an *o*-diphenol to the corresponding *o*-quinone. Tyrosinase is responsible for browning in plants and is considered to be deleterious to the color quality of plant-derived foods and beverages [4]. This unfavorable darkening from enzymatic oxidation generally results in a loss of nutritional and economic values and has been of great concern. However, Tyrosinase is not only the key enzyme in the browning of fruits and vegetables, but also the key enzyme of the darkening of skin, hair and eyes in animals. Hence, the discovery of new and safe tyrosinase inhibitors should have broad applications. In recent years, tyrosinase inhibitors have attracted concern owing to the hyperpigmentation [3], result-

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ing from the increased use of tyrosinase enzyme in medicinal and cosmetic products [17], and their identification and isolation from natural sources have been also increased [25]. Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low costs. Therefore, the development and utilization of more effective tyrosinase inhibitors of natural origin are desired.

In our continuous search for new tyrosinase inhibitors from *M. thunbergii*, the MeOH extracts were subsequently partitioned and isolated. As a result, two dibenzylbutane lignans, macelignan (1) and MDGA (2) were isolated from *M. thunbergii*. In this study, the isolation and structural determinations of these two compounds are described. All the isolated compounds were evaluated for their tyrosinase inhibitory activities.

Materials and Methods

Plant material

The stem bark of *M. thunbergii* was purchased from an oriental drug store in Pohang, Gyeongbuk, Korea, in July 2009. A voucher specimen (MT2009-01) has been deposited at the Laboratory of Molecular Neurophysiology, POSTECH, Pohang, Korea.

Instruments

NMR experiments were conducted on a Bruker AM 300 or 500 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. EIMS was collected on Jeol JMS-700 spectrometer. Optical rotations were measured on Perkin-Elmer 343 polarimeter. Silica gel (230-400 mesh, Merck), RP-18 (ODS-A, 12 nm, S-150 m, YMC) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (silica gel, 0.25 mm layer thickness, Merck) and RP-18 F_{254S} (Merck) plates.

Extraction and isolation

The dried stem bark (500 g) of *M. thunbergii* were chopped and extracted three times with 100% methanol (1 l ×3) for 7 days at room temperature. The combined methanol extract was concentrated *in vacuo* to yield a brown gum (11.3 g). The methanol extract was suspended in H_2O (500 ml), then partitioned in turn with *n*-Hexane, $CHCl_3$ and BuOH (each 3×500 ml) to afford *n*-Hexane extract (1.8 g), $CHCl_3$ extract (3.5 g), and BuOH extract (1.5 g). The $CHCl_3$-soluble extract was silica gel column chromatography (230-400 mesh) using $CHCl_3$/Acetone (100:1→1:1) mixtures to yield seven subfraction (D1-D7). Fraction D1 (900 mg) was applied to silica gel column (230-400 mesh) chromatography with *n*-Hexane/EtOAc (49:1→1:1) to yield fourteen subfractions (E1-E14). Subfractions E6 was subjected to silica gel column (230-400 mesh) chromatography with *n*-Hexane/EtOAc (49:1→9:1) to afford compounds 1 (21 mg) and 2 (20 mg).

Compound 1: colorless prisms; [α] +3.0^{o} (*c* 1.8 in $CHCl_3$); EIMS *m/z* 328; IR (KBr) ν_{max} 3466, 1514, 1489, 931 cm^{-1}, UV λ_{max} nm 213, 230, 285 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.84 (6H, t, *J*=6.0 Hz, 9-CH_3 and 9′-CH_3), 1.75 (2H, m, H-8 and H-8′), 2.31 (2H, m, H-7b and H-7′b), 2.74 (2H, dd, *J*=4.5, 13.6 Hz, H-7a and H-7′a), 3.86 (3H, s, OCH_3-3′), 5.45 (1H, s, OH-4′), 5.91 (2H, s, OCH_2O), 6.60 (1H, s, H-6′), 6.62 (1H, d, *J*=1.9 Hz, H-2′), 6.63 (1H, s, H-6), 6.65 (1H, s, H-2), 6.73 (1H, d, *J*=7.8 Hz, H-5), 6.83 (1H, d, *J*=7.9 Hz, H-5′); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 16.1 (C-9), 16.2 (C-9′), 38.9 (C-7′), 39.1 (C-7), 39.3 (C-8), 39.4 (C-8′), 55.9 (OCH_3-3′), 100.7 (OCH_2O), 107.9 (C-5), 109.4 (C-2), 111.5 (C-2′), 114.0 (C-5′), 121.7 (C-6), 121.8 (C-6′), 133.8 (C-1′), 135.7 (C-1), 143.6 (C-4′), 145.5 (C-4), 146.3 (C-3′), 147.5 (C-3).

Compound 2: colorless crystals, [α] +0° (*c* 0.1 in $CHCl_3$); EIMS *m/z* 330; IR (KBr) ν_{max} 3559, 3371, 1648, 1592 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.85 (6H, d, *J*=6.6 Hz, 9-CH_3 and 9′-CH_3), 1.75 (2H, m, H-8 and H-8′), 2.30 (2H, dd, *J*=9.2, 13.5 Hz, H-7b and H-7′b), 2.74 (2H, dd, *J*=5.0, 13.5 Hz, H-7a and H-7′a), 3.85 (6H, s, OCH_3-3 and OCH_3-3′), 5.40 (2H, s, OH-4 and OH-4′), 6.61 (2H, s, H-2 and H-2′), 6.66 (2H, d, *J*=8.0 Hz, H-6 and H-6′), 6.83 (2H, d, *J*=8.0 Hz, H-5 and H-5′); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 16.2 (C-9, 9′), 38.9 (C-7, 7′), 39.2 (C-8, C-8′), 55.9 (OCH_3 ×2), 111.5 (C-2, 2′), 114.0 (C-5, 5′), 121.7 (C-6, 6′), 133.8 (C-1, 1′), 143.6 (C-4, 4′), 146.3 (C-3, 3′).

High-performance liquid chromatography (HPLC) analysis of compounds

Samples were filtered through a 0.45 μm syringe filter (Millipore, Billerica, MA, USA) and analyzed by HPLC (Agilent 1260, Agilent Technologies, Waldbronn, Germany). The analytical column was a Kinetex C18 (4.6 × 150 mm, Phenomenex, CA, USA). The detection was set at ELSD (Evaporative Light Scattering Detector) and the solvent flow rate was held constant at 0.5 ml/min. The mobile phase used

for the separation consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile). A gradient elution procedure was used as 0 min 98% A, 3 min 95% A, 20 min 100% B, 23 min 98% A, 30 min 98% A. The injection volume was 3 µl for analysis. All samples were analyzed in triplicate.

Tyrosinase inhibitory activity

Mushroom tyrosinase (EC 1.14.18.1) was assayed as described previously with slight modifications [4], using L-tyrosinase or L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. In a spectrophotometric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a Spectra MAX plus spectrophotometer (Molecular device, Sunnyvale, CA) at 30℃. All test samples were dissolved in dimethyl sulfoxide (DMSO) and used for the experiment with dilution. The final concentration of DMSO in the test solution was 1.5%. First, 200 µl of a 4.5 mM L-tyrosine or 12 mM L-DOPA aqueous solution was mixed with 2785 µl of 0.25 M phosphate buffer (pH 6.8) and 10 µl of the test sample, incubated at 30℃ for 10 min. Then, 5 µl of tyrosinase solution (130 units) was added to the phosphate buffer and incubated for additional 20 min. DMSO without test samples was used as the control, and kojic acid was used as a positive control. The assay was conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc., Chicago, IL). The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by the equation as follows:

$$\text{Activity (\%)} = 100[1/(1+([I]/IC_{50}))]$$

Inhibition mode was analyzed by Enzyme Kinetics Module 1.0 (SPSS Inc.) equipped with Sigma Plot 2000.

Results and Discussion

The MeOH extract of the *M. thunbergii* was partitioned successively with *n*-Hexane, $CHCl_3$, and BuOH soluble fractions, respectively. Then, we were evaluated for the inhibitory activity of the partitioned fraction on tyrosinase. Among the partitioned fractions, the $CHCl_3$ soluble fraction showed significant tyrosinase inhibitory activity (IC_{50}=97.9± 5.2 µg/ml), whereas n-Hexane and BuOH fractions showed weaker activity. So, $CHCl_3$ soluble fraction was subject to chromatographed on a normal-phase column and a reverse-phase column, to give two dibenzylbutane lignans, macelignan (1) and *meso*-dihydroguaiaretic acid (2). Subsequently, HPLC was performed as it is more sensitive, selective, and speedy. The use of ELSD detector is convenient method to identify. The chromatogram of the result of purity analysis by HPLC is shown in Fig. 1. The purity of compounds 1 and 2 were more than 95%.

Compound 1 was obtained as colorless prisms. The EIMS of 1 had a molecular ion peak at *m/z* 328 [M^+], consistent with the molecular formula of $C_{20}H_{24}O_4$. The ^{1}H-NMR spectrum of 1 showed one hydroxyl group δ_H 5.45 (1H, s), one methoxy group δ_H 3.86 (3H, s), two methyl group δ_H 0.84 (6H, t, *J*=6.0 Hz, H-9, 9′), two benzylic methylene protons δ_H 2.31 (2H, m, H-7b, 7′b) and 2.74 (2H, dd, *J*=4.5, 13.6 Hz,

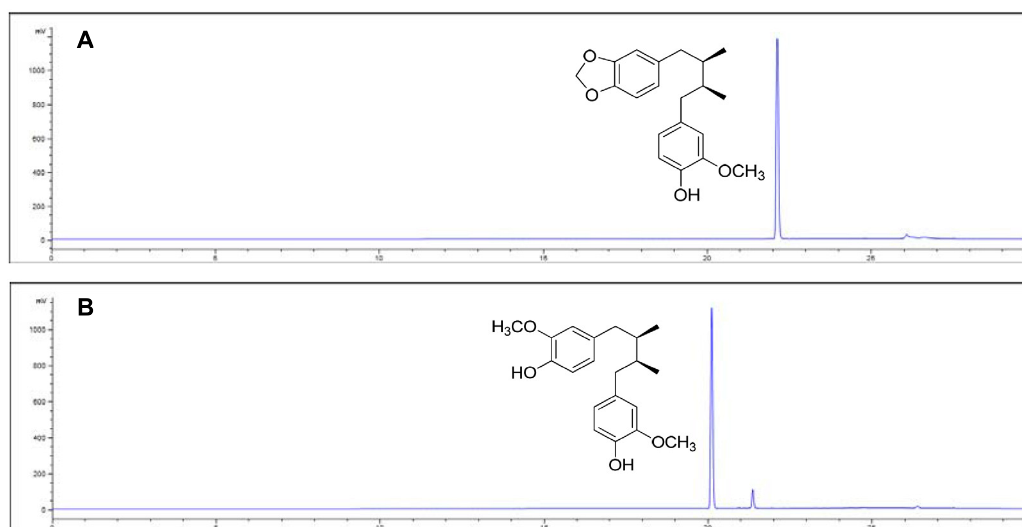

Fig. 1. HPLC chromatograms of compounds 1(A) and 2(B) from *M. thunbergii.*

H-7a, 7′a)], one methylenedioxyl group δ_H 5.91 (2H, s), two methine group δ_H 1.75 (2H, m, H-8, 8′) and six aromatic protons δ_H 6.60 (1H, s, H-6′), 6.62 (1H, d, *J*=1.9 Hz, H-2′), 6.63 (1H, s, H-6), 6.65 (1H, s, H-2), 6.73 (1H, d, *J*=7.8 Hz, H-5) and 6.83 (1H, d, *J*=7.9 Hz, H-5′). The ^{13}C-NMR spectrum showed one methoxy carbon δ_C 55.9 (OCH_3), two methyl carbons δ_C 16.1 (C-9) and 16.2 (C-9′), two methylenes δ_C 38.9 (C-7′), 39.1 (C-7), one methylenedioxy carbon δ_C 100.7 (OCH_2O), eight methins δ_C 39.39 (C-8), 39.4 (C-8′), 107.9 (C-5), 109.4 (C-2), 111.5 (C-2′), 114.0 (C-5′), 121.7 (C-6) and 121.8 (C-6′), and six quaternary carbons δ_C 133.8 (C-1′), 135.7 (C-1), 143.6 (C-4′), 145.5 (C-4), 146.3 (C-3′) and 147.5 (C-3). These spectral data suggested **1** to be a dibenzylbutane lignan. Based on of the spectral data, compound **1** was identified as (8R,8′S)-7-(3,4-methylene-dioxyphenyl)-7′-(4-hydroxy-3-methoxyphenyl)-8,8′-dimethylbutane (macelignan) by comparing its spectroscopic data with literature data [20, 26].

Compound 2 was obtained as colorless crystals. The EIMS of 2 had a molecular ion peak at *m/z* 330 $[M]^+$, consistent with the molecular formula $C_{20}H_{26}O_4$. The ^{1}H-NMR spectrum showed two hydroxyl group δ_H 5.40 (2H, s), two methoxy groups δ_H 3.85 (6H, s), two methyl groups δ_H 0.85 (6H, d, *J*=6.6 Hz, H-9, 9′), four benzylic methylene groups δ_H 2.30 (2H, dd, *J*=9.2, 13.5 Hz, H-7b, 7′b) and 2.74 (2H, dd, *J*=5.0, 13.5 Hz, H-7a, 7′a)], two methine groups [δ_H 1.75 (2H, m, H-8, 8′)] and six aromatic protons δ_H 6.61 (2H, s, H-2, 2′), 6.66 (2H, d, *J*=8.0 Hz, H-6, 6′) and 6.83 (2H, d, *J*=8.0 Hz, H-5, 5′)]. The ^{13}C-NMR spectrum showed two methoxy groups δ_C 55.9 (OCH_3), two methyl groups δ_C 16.2 (C-9, 9′), two methylene groups δ_C 38.9 (C-7, 7′), eight methin groups δ_C 39.2 (C-8, C-8′), 111.5 (C-2, 2′), 114.0 (C-5, 5′) and 121.7 (C-6, 6′), and six quaternary carbons δ_C 133.8 (C-1, C-1′), 143.6 (C-4, 4′) and 146.3 (C-3, 3′). These spectral data suggested that **2** was a dibenzylbutane lignan. As a result, compound **2** was identified as (8R,8′S)-7,7′-di-(4-hydroxy-3-methoxyphenyl)-8,8′-dimethylbutane (*meso*-dihydroguaiaretic acid, MDGA) by comparing its spectroscopic data with the previously reported data [2, 20].

Table 1. Tyrosinase inhibitory activity of compounds

Compounds	UV_{475}	
	L-Tyrosine IC_{50} (μM)	L -DOPA IC_{50} (μM)
1	82.81	>200
2	10.20	145.32
Kojic acid	11.36	14.05

The isolated compounds were evaluated for their inhibitory activities on tyrosinase. As shown in Table 1 and Fig. 2A, MDGA (2) exhibited a dose-dependent inhibitory effect on the monophenolase activity of mushroom tyrosinase (IC_{50}=10.2 μM). The result obtained indicates that MDGA (2) exhibited significantly higher inhibitory activity than positive control kojic acid. Also, MDGA (2) inhibited mushroom tyrosinase diphenolase activity with an IC_{50} of 145.32 μM (Table 1). In the previous reports, phenylpropanoids, stilbenes, and other derivatives, including 3,4-dihydroxycinnamic acid [13], oxyresveratrol were identified as tyrosinase inhibitors. Among them, phenylpropanoids containing a hydroxyl group possess potent tyrosinase inhibitory activity. In the case of oxyresveratrol the presence of a hydroxyl group in the ring seems to be related to their inhibitory potency [22]. In this study as well, we suggest that the inhibitory effect of 2 was determined by the guaiacyl

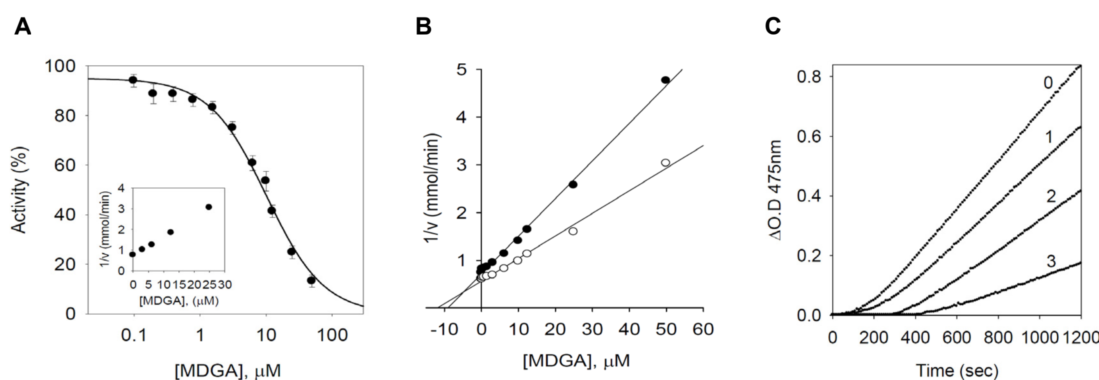

Fig. 2. Inhibitory effect of MDGA (2) on tyrosinase. (A) Effect of MDGA (2) on the tyrosinase catalyzed oxidation of L-tyrosine. Inset: Plot of 1/υ vs. concentration of MDGA (2). (B) Dixon plots for the inhibition of the monophenolase activities of tyrosinase by MDGA (2). Concentrations of substrates for curves were 300 (●) and 450 (○) μM, respectively. (C) Time dependent inhibition of tyrosinase in the presence of MDGA (2). Concentrations of MDGA (2) from top to bottom were 0 (0), 3 (1), 10 (2), 25 (3) μM.

Table 2. Kinetics and inhibition constants of mushroom tyrosinase by MDGA

Parameter	L-Tyrosine
IC_{50}	10.2 μM
K_m	388.36 μM
V_{max}	3.2 μmol/min
Inhibition Type	Competitive
K_i	4.8 μM

group.

The kinetic behavior of mushroom tyrosinase during the oxidation of L-tyrosine was studied first. Under the condition used in the present investigation, the oxidation of L-tyrosine by mushroom tyrosinase followed the Michaelis-Menten kinetics. The kinetic parameters for mushroom tyrosinase were obtained from the Dixon plot. The results illustrated in Fig. 2B show that MDGA (2) is a competitive inhibitor because increasing the MDGA (2) concentration resulted in a family of lines with a common intercept on the $1/\upsilon$ axis but with different slopes. The equilibrium constant for inhibitor binding, K_i of L-tyrosine obtained from the Dixon plot were 4.8 μM (Table 2). The time dependence of 2 on the tyrosinase catalyzed oxidation of L-tyrosine was studied. MDGA (2) showed time dependent inhibition (Fig. 2C). As shown in Fig. 2C, the lag time is known for the oxidation of monophenol substrates such as L-tyrosine and this lag can be shortened. This lag time can be extended by monophenolase inhibitors such as tropolone [6] and galangin [12]. MDGA (2) did extend this lag phase, indicating that 2 inhibit the hydroxylation of L-tyrosine.

In summary, two of dibenzylbutane lignans were isolated from $CHCl_3$-soluble fractions of *M. thunbergii*. The structures were identified as macelignan (1) and MDGA (2) by the physicochemical and spectroscopic data. The isolated compounds were evaluated for their tyrosinase inhibitory activities. Among them, MDGA (2) inhibited the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. The inhibition mechanism obtained from the Dixon plot show that MDGA (2) is a competitive inhibitor. MDGA (2) only binds the free enzyme to form an EI complex rather than bind the ES complex.

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin whitening and preventive effects. Besides being used in the treatment of some dermatological disorders associated with melanin hyperpigmentation, tyrosinase inhibitors have found an important role in the cosmetic and pharmaceutical industries for their skin-whitening effect and depigmentation after sunburn [23]. In this study, it can be concluded that MDGA (2) can be potential candidate for the treatment of melanin biosynthesis related skin diseases, likely hyper-pigmentation of human as well as animals.

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초록 : 후박나무에서 분리한 *meso*-dihydroguaiaretic acid의 tyrosinase 저해활성

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후박나무(녹나무과)는 한국과 일본 등지에 서식하는 상록 교목으로 한국, 중국, 일본에서 부종, 복통, 복부 팽만 등의 질병 치료를 위해 오랫동안 사용되어오고 있다. 본 연구에서는 후박나무 껍질을 메탄올에 추출하고 메탄올 추출물을 헥산, 클로로포름, 부탄올에 순차적으로 분획하였다. 클로로포름 분획물로부터 2종의 화합물을 분리하였으며 분리된 화합물1과 2의 구조는 ^{1}H-, ^{13}C-NMR과 참고 문헌 데이터에 의해 dibenzylbutane lignin 화합물인 macelignan (1)과 *meso*-dihydroguaiaretic acid (2)로 동정되었다. 분리된 화합물들의 tyrosinase 저해 활성을 측정한 결과, 화합물 2는 tyrosinase 저해 활성 중 monophenolase (IC_{50} = 10.2 μM)에 대해 높은 저해활성을 나타내는 경쟁적 저해제였으며 효소에 결합하는 화합물 2의 저해 상수(K_i 값)는 4.8 μM였다. 따라서 *meso*-dihydroguaiaretic acid (2)는 멜라닌 생합성과 관련된 피부 질환 치료를 위한 잠재적 후보가 될 수 있을 것으로 판단된다.