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Tyrosinase Inhibitory Constituents of *Morus bombycis* Cortex†

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Abstract – Tyrosinase is one of the important enzymes in the mammalian melanin synthesis. In the process of melanin synthesis, tyrosine is oxidized to DOPA (3,4-dihydroxyphenylalanine), and DOPA is further oxidized to dopaquinone. Tyrosinase is an enzyme catalyzing this oxidation of tyrosine, so chemicals that inhibit the activity of tyrosinase can be used as skin whitening agents. In this study, we isolated five constituents from the 80% MeOH extract of *Morus bombycis* cortex by bioactivity-guided fractionation. We performed mushroom tyrosinase inhibition assay. As a result, 7,2',4'-trihydroxyflavanone (**1**), 2',4',2,4,-tetrahydroxychalcone (**2**), and oxyresveratrol (**3**) showed the more potent inhibitory effect compared to kojic acid, a well-known skin whitening agent with anti-tyrosinase effect. Moracinoside M (**4**) and moracin M-3'-O-β-D-glucopyranoside (**5**) also showed the moderate tyrosinase inhibitory activities.
Keywords – *Morus bombycis*, skin whitening, tyrosine, tyrosinase inhibitory effect

Introduction

Tyrosinase (monophenol, dihydroxy-L-phenylalanin: oxygen oxidoreductase, EC 1.14.18.1) is a copper-dependent protein widely distributed in nature (van Gelder *et al*, 1997). It is the key enzyme responsible for enzymatic browning of many plant-derived food products (Mayer, 1987). It also contributes to the formation of melanin pigments in mammals (Slominski *et al,* 2004). Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of odiphenols to o-quinones (diphenolase activity), both utilizing molecular oxygen (Siegbahn, 2003).

These two reactions take place in the formation of mammalian melanin biosynthesis. The biosynthetic steps for the formation of melanin include the hydroxylation of *l*-tyrosine to 3-4-dihydroxyphenylalanine (*l*-dopa) and the oxidation of *l*-dopa to *o*-dopaquinone (Solano *et al.,* 2006; Martnez-Esparza *et al.*, 1998). Tyrosinase catalyzes these two hydroxylation reactions, therefore, the inhibitors of tyrosinase are thought to have potential as skin-whitening agents.

Recently, the cortex of *Morus* species has been shown to exhibit skin-whitening effect (Lee *et al.*, 2002). Cosmetics with skin-whitening effect which contain the extract of *Morus alba* or *Morus bombycis* root bark are released and received favorable responses on market. Even though oxyresveratrol isolated from *Morus* has been suggested to perform the major role in inhibiting tyrosinase followed by generating skin-whitening effect, the research on the bio-active constituents of this plants is still limited (Shin *et al*., 1998; Lee *et al.*, 2002). In this study, we isolated a number of constituents from *Morus bombycis* cortex by bioactivity-guided fractionation, and evaluated the inhibitory effects of the isolated compounds on mushroom tyrosinase.

Experimental

General Experimental Procedures – All solvents were purchased from Daejung Chemicals & Metals Co. Ltd., Korea, unless stated otherwise. All solvents for HPLC were purchased from Fisher Scientific Korea Ltd., Korea. Kiesgel 60 (40 - 60 μm, 230 - 400 mesh, Art. 9385, Merck, Germany) was used for the silica gel column chromatography. TLC was performed using Merck precoated silica gel F_{254} plates and RP-18 F_{254} plates. Ultrasonication was performed using a Branson

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5200 sonicator. MPLC was performed using Teledyne CombiFLASH RETRIEVE using Teledyne Redisep Rf C18 reverse phase columns. Preparative HPLC was performed using HITACHI L-7100 pump with HITACHI L-7420 UV detector and Phenomenex Hemini C18 5 μm column (150 × 10 mm). ESI MS data were obtained on Agilent 1100 series LC/MSD trap LC/MS. NMR spectra were recorded on JEOL GSX 400 Spectrometer (400 MHz), JEOL LA 300 Spectrometer (300 MHz), and Bruker AMX 500 Spectrometer (500 MHz). All chemicals for tyrosinase inhibition assay, including mushroom tyrosinase, L-tyrosine, and kojic acid, were purchased from Sigma-Aldrich Chimical Co..

Plant Material – The cortex of *Morus bombycis* was collected in Nambu forest of Seoul National University, Baegwoon Mountain, Gwangyang city, Jeollanamdo, Korea, in September 2008. A voucher specimen (SNU-0785) has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and Isolation – The air-dried cortex of *Morus bombycis* (4.5 kg) was extracted with 80% MeOH in an ultrasonic apparatus at room temperature and 329.3 g of total extract was obtained. This extract was suspended in distilled water and successively partitioned with *n*-Hexane, EtOAc, and *n*-BuOH. Silica gel column chromatography (CC) of EtOAc fraction (78.2 g) was carried out using $CHCl_3$ followed by a mixture of $CHCl_3$-MeOH in a gradient system, and yielded nine subfractions (E1 ~ E9). Fraction E4 was subjected to silica gel CC ($CHCl_3$-MeOH, 50 : 1 → 1 : 1) to give 10 subfrations (E4-1 ~ E4-10). Fraction E4-7 was also separated by MPLC using MeOH-H_2O gradient (30% - 90% MeOH) elution to afford compound **1** (20.0 mg), and other subfrations. Fraction E4-7-8 was purified over HPLC (55% MeOH) to yield 27.6mg of compound **2**. Fraction E5 was separated by MPLC using MeOH-H_2O gradient (20% - 80% MeOH) elution to give 20 subfractions (E5-1 ~ E5-20). Fraction E5-4 was purified over HPLC (70% MeOH) to yield 258.8 mg of compound **3**. Fraction E6 was separated by MPLC using MeOH-H_2O gradient (30% - 90% MeOH) elution to afford 19 subfractions (E6-1 ~ E6-19). Fraction E6-14 was purified using HPLC (60% MeOH) to yield 30.2 mg of compound **4**. Fraction E7 was separated by MPLC using MeOH-H_2O gradient (30% - 80% MeOH) elution and yielded 17.7 mg of compound **5**.

7,2',4'-Trihydroxyflavanone (1): $C_{15}H_{12}O_4$, colorless amorphous powder. ESI MS (*m/z*) : 271 $[M-H]^-$. ^{1}H-NMR (500 MHz, acetone-d_6): δ 7.70 (1H, d, *J* = 8.6 Hz, H-5), 7.29 (1H, d, *J* = 8.4 Hz, H-6'), 6.53 (1H, dd, *J* = 8.6, 2.3 Hz, H-6), 6.42 (1H, d, *J* = 1.9 Hz, H-3'), 6.39 (1H, dd, *J* = 2.3, 8.3 Hz, H-5'), 6.38 (1H, d, *J* = 2.3 Hz, H-8), 5.69 (1H, dd, *J* = 13.3, 2.7 Hz, H-2), 3.02 (1H, dd, *J* = 16.7, 15.4 Hz, H-3eq.), 2.65 (1H, dd, *J* = 16.7, 2.8 Hz, H-3ax.). ^{13}C-NMR (125 MHz, acetone-d_6): δ 191.7 (C-4), 165.6 (C-9), 165.4 (C-7), 159.8 (C-4'), 156.6 (C-2'), 130.0 (C-5), 129.4 (C-6'), 118.6 (C-1'), 115.6 (C-10), 111.5 (C-6), 108.3 (C-5'), 104.1 (C-8), 103.9 (C-3'), 76.3 (C-2), 44.2 (C-3).

2',4',2,4,-Tetrahydroxychalcone (2): $C_{15}H_{12}O_6$, colorless amorphous powder. ESI MS (*m/z*) : 271 $[M-H]^-$. ^{1}H-NMR (500 MHz, CD_3OD): δ 8.09 (1H, d, *J* = 15.4 Hz, H-β), 7.88 (1H, d, *J* = 8.9 Hz, H-6), 7.70 (1H, d, *J* = 15.4 Hz, H-α), 7.51 (1H, d, *J* = 8.4 Hz, H-6'), 6.39 (1H, dd, *J* = 2.3, 8.9 Hz, H-5), 6.36 (1H, dd, *J* = 2.2, 8,4 Hz, H-5'), 6.34 (1H, d, *J* = 2.2 Hz, H-3'), 6.27 (1H, d, *J* = 2.3 Hz, H-3). ^{13}C-NMR (125 MHz, CD_3OD): δ 194.9 (C = O), 168.1 (C-2), 166.9 (C-4), 163.6 (C-4'), 161.6 (C-2'), 142.9 (C-β), 133.8 (C-6), 133.1 (C-6'), 118.4 (C-α), 116.4 (C-1'), 115.6 (C-1), 109.9 (C-5'), 109.8 (C-5), 104.6 (C-3), 104.3 (C-3').

Oxyresveratrol (3): $C_{15}H_{12}O_6$, colorless amorphous powder. ESI MS (*m/z*) : 243 $[M-H]^-$. ^{1}H-NMR (300 MHz, CD_3OD): δ 7.37 (1H, d, *J* = 9.1 Hz, H-6), 7.20 (1H, d, *J* = 16.4 Hz, H-7), 6.74 (1H, d, *J* = 16.4 Hz, H-7'), 6.40 (2H, d, *J* = 2.1 Hz, H-2', 6'), 6.23 (1H, d, *J* = 2.3 Hz, H-3), 6.23 (1H, dd, *J* = 7.6, 2.3 Hz, H-5), 6.04 (1H, t, *J* = 2.0 Hz, H-4'). ^{13}C-NMR (75 MHz, CD_3OD): δ 160.2 (C-3'), 159.9 (C-4), 158 (C-2), 142.9 (C-1'), 129.1 (C-6), 127.2 (C-7'), 125.5 (C-7), 118.6 (C-1), 109.9 (C-5), 106.4 (C-2', 6'), 104.3 (C-3), 103.0 (C-4').

Moracinoside M (4): $C_{24}H_{26}O_9$, whitish amorphous powder. ESI MS (*m/z*) : 453 $[M-H]^-$, 325 [M - Xyl - H]$^-$. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.22 (1H, s, H-4), 6.99 (1H, s, H-4'), 6.86 (1H, s, H-7), 6.49 (1H, s, H-2'), 3.79 (1H, dd, *J* = 7.4, 5.4 Hz, H-2''), 3.13 (1H, d, *J* = 16.4, 5.2 Hz, H-1''a), 2.84 (1H, dd, *J* = 16.4, 7.5 Hz, H-1''b), 1.35 (3H, s, H-4''), 1.27 (3H, s, H-5''), xylopyranoside δ 4.89 (1H, d, *J* = 7.1 Hz, xyl-H1), 3.97 (1H, dd, *J* = 11.3, 5.2 Hz, xyl-H5β), 3.59 (1H, br m, xyl-H4), 3.3 - 3.46 (2H, m, xyl-H2 and xyl-H3), 3.36 (1H, br s, xyl-H5α). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 161.2 (C-5'), 160.7 (C-3'), 156.9 (C-2), 156.8 (C-7a), 153.5 (C-6), 134.5 (C-1'), 124.9 (C-3a), 122.7 (C-4), 118.6 (C-5), 107.4 (C-6'), 106.2 (C-4'), 105.9 (C-2'), 103.1 (C-3), 100.5 (C-7), 79 (C-3''), 71.3 (C-2''), 33.2 (C-1''), 26.8 (C-5''), 21.8 (C-4''), xylopyranoside δ 103.7 (xyl-C1), 78.5 (xyl-C2), 75.5 (xyl-C3), 71.8 (xyl-C4), 67.7 (xyl-C5).

Moracin M-3'-O-β-D-glucopyranoside (5): $C_{20}H_{20}O_9$, brown amorphous powder. ESI MS (*m/z*) : 405 $[M + H]^+$.

^{1}H-NMR (400 MHz, DMSO-d_6) : δ 7.17 (1H, s, H-3), 7.40 (1H, d, J = 8.4 Hz, H-4), 6.75 (1H, dd, J = 8.4, 2.0 Hz, H-5), 6.94 (1H, br s, H-7), 6.97 (1H, br s, H-2'), 6.44 (1H, br s, H-4'), 6.89 (1H, br s, H-6'), glucopyranisode δ 4.63 (1H, d, J = 7.4 Hz, glc-H1), 3.20 - 3.24 (1H, m, glc-H2), 3.27 - 3.34 (1H, m, glc-H3), 3.20 - 3.24 (1H, m, glc-H4), 3.27 - 3.34 (1H, m, glc-H5), 3.53 (1H, dd, J = 11.7, 5.8 Hz, glc-H6), 3.75 (1H, dd, J = 11.1, 3.7 Hz, glc-H6). ^{13}C-NMR (100 MHz, DMSO-d_6) : δ 159.0 (C-3'), 158.7 (C-5'), 155.8 (C-6), 155.3 (C-7a), 153.5 (C-2), 131.7 (C-1'), 121.2 (C-4), 120.7 (C-3a), 112.5 (C-5), 104.7 (C-6'), 103.6 (C-2'), 103.3 (C-4'), 102.1 (C-3), 97.5 (C-7), glucopyranisode δ 101.0 (glc-C1), 73.3 (glc-C2), 76.6 (glc-C3), 69.7 (glc-C4), 77.1 (glc-C5), 60.7 (glc-C6).

Tyrosinase Inhibitory Assay – The total extract, fractions, and isolated compounds were dissolved in DMSO, and then diluted to several concentration in 0.1 M pH 6.8 sodium phosphate buffer. DMSO was added to make the DMSO concentration of each sample equally (final conc. 0.1%). The sample solutions (70 μL) were set on the 96-well plate, followed by the addition of 30 μL of mushroom tyrosinase solution (1000 units/mL) and 40 μL of 3 mM L-tyrosine solution. 70 μL of 0.1% DMSO-buffer solution and 70 μL of kojic acid solution were used as the blank reference and positive control, respectively. The absorbance at 490 nm of the reaction mixture (140 μL) was measured using the ELISA reader, and the mixture was incubated for 10 min at 37 °C. After the incubation, the absorbance of mixture was measured again. The inhibition percentage of tyrosinase activity was calculated as follows:

$$\%\text{inhibition} = (A2 - A1) - (B2 - B1) / (A2 - A1) \times 100$$

A1 is the absorbance at 490 nm of the blank reference at 0 min, and A2 is the absorbance at 490 nm of the blank at 10 min. B1 is the absorbance at 490 nm of the test sample at 0 min, and B2 is the absorbance at 490 nm of the test sample at 10 min.

Results and Discussion

For the isolation of bio-active compounds from *Morus bombycis* cortex, the inhibitory effects of total extract and the fractions (including *n*-Hexane, EtOAc, BuOH and water fractions) of this plant on tyrosinase were evaluated. As shown in Fig. 1, the total extract showed a potent tyrosinase inhibitory activity, and the EtOAc fraction was the most potent among the four solvent fractions. EtOAc fraction was further subjected to repeated column chromatography yielded five pure compounds. The isolated

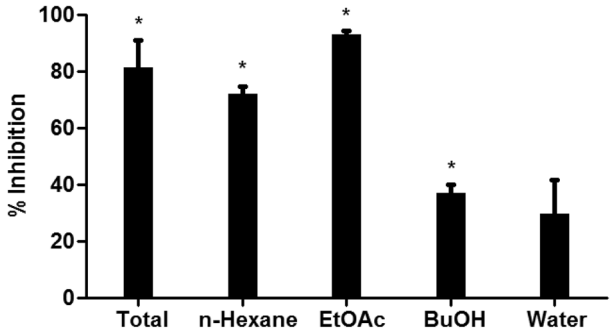

Fig. 1. Tyrosinase inhibitory effects of total extract and its fractions of *Morus bombycis*. The concentration of the treated samples was 100 μg/ml. *P < 0.05, significantly different from the blank reference.

Table 1. Mushroom tyrosinase inhibitory activity of compounds **1 - 5**

Compounds	IC_{50} (μM)	SD[a]
1	5.228	1.079
2	0.9724	1.073
3	3.66	1.122
4	61.3	1.087
5	127.5	1.285
Kojic acid[b]	112.7	1.178

[a]SD : Standard deviation. IC_{50} value is calculated by logC-%inhibition plot, so SD value is the antilog of standard error of logIC_{50}. These values mean geometric SD, or a number that you can multiply the mean by or divide the mean into.
[b]Kojic acid : Positive control.

compounds were identified as 7,2',4'-trihydroxyflavanone (**1**) (Lim *et al., 2001*), 2',4',2,4,-tetrahydroxychalcone (**2**) (Lim *et al.*, 2001), oxyreveratrol (2',4',3,5-tetrahydroxystilbene) (**3**) (Kanchanapoom *et al.*, 2002), moracinoside M (**4**) (Jeong *et al.*, 2009), and moracin M-3'-O-β-D-glucopyranoside (**5**) (Kanchanapoom *et al.*, 2002) by comparing the ^{1}H-, ^{13}C-NMR and MS spectral data with the literature values.

Compounds **1 - 5** were tested for their mushroom tyrosinase inhibitory effect (Table 1). Compounds **1**, **2**, and **3** exhibited strong inhibitory activities on tyrosinase with IC_{50} values of 5.23 ± 1.08, 0.97 ± 1.07, and 3.66 ± 1.12, respectively, which were more potent than that of positive control, kojic acid. In addition, The inhibitory effect of compound **2** was shown to be more potent than that of compound **3** (oxyresveratrol) reported previously as a major ingredient that contributes to skin whitening effect of *Morus* plants (Shin *et al.*, 1998). To exhibit their mechanism of inhibition, we performed kinetic study for compounds **1** and **2**. As a result, both compounds **1** and **2** were shown to play as competitive inhibitors on

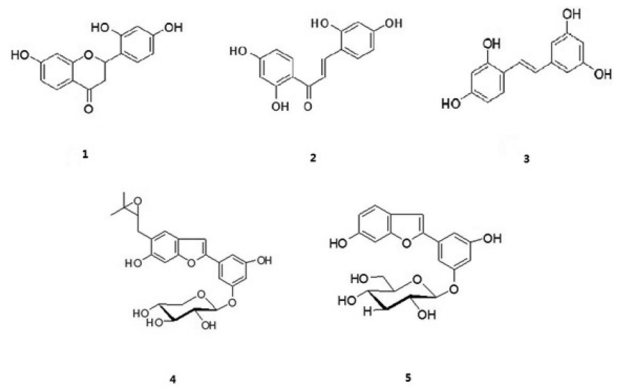

Fig. 2. The structures of **1 - 5** isolated from *M. bombycis*.

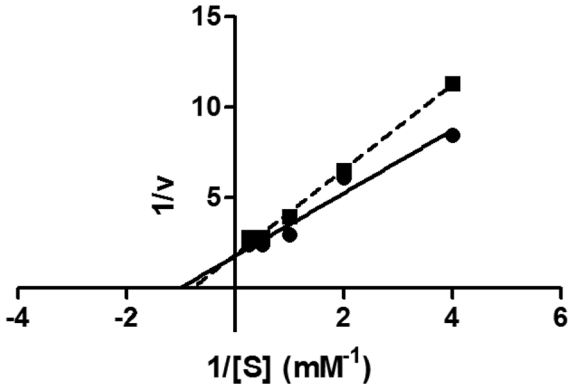

Fig. 3. Lineweaver-Burk plots of compound **1** in mushroom tyrosinase assay. Values of absorbance were measured at 490 nm in the presence of compound **1** (1 μM) (■), or buffer solution with 0.1% DMSO (●), as control.

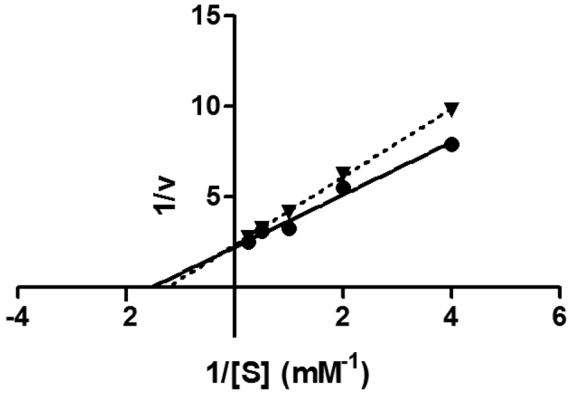

Fig. 4. Lineweaver-Burk plots of compound **2** in mushroom tyrosinase assay. Values of absorbance were measured at 490 nm in the presence of compound **2** (125 nM) (▼), or buffer solution with 0.1% DMSO (●), as control.

tyrosinase in accordance with the previous result for compound **3** (Jeong *et al.,* 2009) (Fig. 3 and Fig. 4). In addition, compound **4** and **5** also effectively attenuated tyrosinase activity which was comparable to positive control, kojic acid. Nerya *et al.* (2004) demonstrated that inhibitory activities of phenolic compounds might be due to the structural similarity to tyrosine. They also demonstrated that 2,4-resorcinol functional group perform a critical role in the tyrosinase inhibitory activity of chalcones (Katib *et al.* 2005). Compound **2** has these two functional resorcinol units, so it showed powerful inhibitory potency. The isolated compounds herein are thought to contribute at least in part to skin-whitening effect of *Morus bombycis*.

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