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ARTICLE

Tyrosinase Inhibitory Constituents from the Roots of *Morus nigra*: A Structure−Activity Relationship Study

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The phytochemical profiles of *Morus nigra* roots and twigs were compared by HPLC with those of the old and young twigs of *Morus alba* which are known to contain oxyresveratrol and mulberroside A as major components. It was found that *M. nigra* root extract contains some unknown natural products with potential tyrosinase inhibitory activity. The extract (95% ethanol) of the roots of *M. nigra* was further investigated in this study. One new compound, 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone, and twenty-eight known phenolic compounds were isolated. Their structures were identified by mass spectrometry and NMR spectroscopy. Nine compounds, 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone, steppogenin-7-*O*-β-D-glucoside, 2,4,2′,4′-tetrahydroxychalcone, moracin N, kuwanon H, mulberrofuran G, morachalcone A, oxyresveratrol-3′-*O*-β-D-glucopyranoside and oxyresveratrol-2-*O*-β-D-glucopyranoside, showed better tyrosinase inhibitory activities than kojic acid. It was noteworthy that the IC_{50} values of 2,4,2′,4′-tetrahydroxychalcone and morachalcone A were 757-fold and 328-fold lower than that of kojic acid, respectively, suggesting a great potential for their development as effective natural tyrosinase inhibitors.

KEYWORDS: *Morus nigra*; phenolic compounds; tyrosinase inhibition

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is the key enzyme responsible for enzymatic browning of many plant-derived food products (*1*). It also contributes to the formation of melanin pigments in mammals (*2*). Enzymatic browning in most fresh fruits and vegetables usually leads to deterioration in sensory and, in some cases, nutritional quality (*3*). The use of tyrosinase inhibitors appears to be a promising approach to suppress undesirable browning reactions to thus help maintain the quality attributes of the food products concerned. One of the most important requirements for an effective tyrosinase inhibitor is that the compound is safe for application in food products without causing significant negative influence on the appearance and nutritional quality. In this regard, natural agents, especially those from plants, are considered more desirable candidates than synthetic compounds (*4*−*6*). In addition, the concept of suppressing tyrosinase activity to achieve skin whitening effects with natural agents has been highly recognized by the cosmetic industry and has also been an important marketing edge for cosmetics (*5*). Well-known examples include arbutin, kojic acid and glabridin. Arbutin is a β-glycoside of 1,4-dihydroquinone from bearberry and is commonly used in the cosmetic industry as a skin-whitening agent via inhibition of tyrosinase. Arbutin exists in two forms, α- and β-arbutin. β-Arbutin inhibits the activity of tyrosinase from both mushroom and mouse melanoma noncompetitively while α-arbutin only inhibits tyrosinase from melanoma (*7*). Despite its potent tyrosinase inhibitory effect, there is still concern about the cancer risk associated with its aglycon hydroquinone, which might be formed by biotransformation in the human body (*8*, *9*). Kojic acid and glabridin are more potent tyrosinase inhibitors than arbutin (*10*). However, kojic acid is unstable when exposed to air and might have adverse effects such as skin irritation and contact allergy (*11*). On the other hand, glabridin has poor skin penetrability and is not stable in cosmetic formulations (*12*). Its low content even in the principal source (*13*), licorice root, also increases the cost of application in cosmetics. Apart from purified compounds, natural extracts such as extracts of licorice root, *Morus alba*, and *Aloe vera* have also been used in cosmetic formulations as whitening agents (*10*). To accommodate the increasing demand for natural tyrosinase inhibitors by the food and cosmetics industry, the search for potent inhibitors from natural sources has been and will likely remain a central focus of many studies in the area of enzymatic browning.

The genus *Morus* comprises more than ten species, and some of them have been used as Traditional Chinese Medicine for a long time. It has also been shown that some species of *Morus* might be rich sources of phenolic compounds which possess beneficial activities such as hypoglycemic effect and lipid homeostasis modulating activity (*14*−*18*). The roots of *Morus australis* and *Morus nigra* and the twigs of *M. alba* have been applied in skin-whitening cosmetics. However, the principal constituents of the roots of *M. nigra* responsible for the whitening effect have not yet been clearly identified. *M. nigra* mainly grows in warm

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subtropical regions of West Asia and Europe (*19*). It has been applied in traditional Chinese Medicine for a long time, and its fruits are consumed in many places. Phenolic compounds of *M. nigra* fruits were explored in previous studies (*19*, *20*). Some flavonoids and stilbenes were isolated from the bark of *M. nigra* (*21*). In this study, the HPLC profiles of several *M. nigra* extracts were compared with that of *M. alba* twigs and the extract of *M. nigra* roots was subjected to detailed phytochemical analysis because of its potent inhibitory activity against mushroom tyrosinase (IC_{50} = 21.6 μg/mL) and because it exhibited a phytochemical profile quite different from that of *M. alba*. In addition, there has been no detailed phytochemical study on the roots of *M. nigra*. As a result, twenty-nine compounds were isolated and their structures characterized. Several of them demonstrated potent activities in the tyrosinase inhibition assay, and thus represent promising candidates for further study on the potential for application in the food or cosmetics industry.

MATERIALS AND METHODS

Chemicals and Instruments. Mushroom tyrosinase (5370 units/mg), L-tyrosine, formic acid, methanol-d_6 (CD_3OD) and kojic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol (MeOH), 95% ethanol (EtOH), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$), dimethyl sulfoxide (DMSO), and HPLC grade solvents were purchased from BDH (Poole, U.K.). Sephadex LH-20 was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). MCI gel CHP 20P (75−150 μm) was purchased from Mitsubishi Chemical Corporation (Tokyo, Japan). Silica gel (200−300 mesh) for column chromatography was from Qingdao Marine Chemical Company (Qingdao, P. R. China). Anhydrous disodium hydrogen phosphate (NaH_2PO_4) and TLC plates (25 DC-platten Kieselgel 60 F_{254}) were from Merck (Darmstadt, Germany). Analytical HPLC and semipreparative HPLC were carried out on a Waters 600 system equipped with a 2487 dual-wavelength detector and an Empower Pro software. An Ultimate XB-C18 column (250 × 4.6 mm, 5 μm) and a Phenomenex Luna C18 (2) column (250 × 21.2 mm, 5 μm) were used for analytical and semipreparative HPLC, respectively. ^{1}H NMR, ^{13}C NMR, HSQC and HMBC data were acquired on a Bruker 400, 500 DRX NMR spectrometer. Mass spectrometry was carried out on an Applied Biosystems Q-trap LC−MS mass spectrometer. Spectrophotometric measurements for the tyrosinase inhibition assay were taken on a UV-1206 spectrophotometer (Shimadzu Corporation, Japan). Minigrinder (model: DF-15) was purchased from Shenzhen Laitong Company, Shenzhen, P. R. China.

Plant Material. The fresh roots and twigs of *M. nigra* were collected at Urumqi, Xinjiang Province, P. R. China, in October 2008. Voucher specimens (accession number 20081001 and 20081002) were deposited at the School of Biological Sciences, The University of Hong Kong. The young and old twigs of *M. alba* were purchased from a traditional Chinese medicine store in Hong Kong.

HPLC Analysis of Different Morus Plant Parts. Different plant parts were ground into powders, which were extracted with methanol by sonication for 60 min. After cooling, the extract solutions were filtrated and analyzed by HPLC. An Ultimate XB-C18 column (250 × 4.6 mm, 5 μm) was used for the analysis. The mobile phase consisted of solvent A (0.2% formic acid in Milli-Q water) and solvent B (acetonitrile). The following gradient grogram was used for the elution: 95% A, 5% B; 0−10 min; 5%−15% B; 10−20 min; 15%−25% B; 20−30 min; 25%−40% B; 30−40 min; 40%−55% B; 40−50 min; 55%−70% B; 50−60 min; 70%−100% B; 60−75 min, 5% B. The sample injection volume was 10 μL, and the flow rate was set at 1.0 mL/min. The UV detector was set at 254 nm.

Extraction and Isolation of *M. nigra* Root Phytochemicals. *M. nigra* roots (474 g) were ground using a minigrinder and transferred into glass flasks. 95% ethanol (1.5 L) was added, and the extraction was performed by sonication. The extraction was repeated three times, and the extracts were combined and concentrated on a rotor evaporator at 40 °C under vacuum. The dried extract (28.8 g) was dissolved in methanol and subjected to MCI gel column chromatography by successive elution with different proportions of MeOH−H_2O (20:80 → 100:0) to obtain 12 fractions (Frs. 1−12). Fr. 1 was fractionated by Sephadex LH-20 column chromatography [MeOH−H_2O (1:1)], which led to 3 subfractions (Sub-Fr. 1−Sub-Fr. 3). Sub-Fr. 1 contained a single component characterized as compound **4** (4.1 g). Sub-Fr. 2 was purified by semipreparative HPLC [ACN−H_2O (12:88), UV 254 nm] which gave compound **3** (50.0 mg); and semipreparative HPLC [ACN−H_2O (12:88), UV 254 nm] of Sub-Fr. 3 gave **5** (5.0 mg) and **9** (1.5 mg). Fr. 2 was processed on a Sephadex LH-20 column [MeOH−H_2O (1:1)] into 3 subfractions (Fr. A1−C1). Semipreparative HPLC [ACN−H_2O (11:89), UV 254 nm] of Fr. A1 gave compound **6** (6.3 mg) and **7** (2.3 mg), and that of Fr. B1 [ACN−H_2O (14:86), UV 254 nm] gave compound **2** (6.5 mg). Fr. C1 was separated on a silica gel column eluted with ethyl acetate, followed by semipreparative HPLC [ACN−H_2O (12:88), UV 254 nm] to give compound **27** (29.2 mg) and **28** (74.5 mg). Fr. 3 was separated on a Sephadex LH-20 column [MeOH−H_2O (1:1)] to offer 3 subfractions (Fr. A2−C2). Fr. A2 was fractionated by silica gel chromatography eluted with ethyl acetate to obtain compound **10** (18.5 mg). Fr. C2 was purified by semipreparative HPLC [ACN−H_2O (14:86), UV 254 nm] to obtain compound **8** (4.6 mg). Fr. 4 was separated by Sephadex LH-20 [MeOH−H_2O (1:1)], followed by silica gel column chromatography [$CHCl_3$−MeOH (10:1)] to give compound **11** (5.8 mg). Sephadex LH-20 [MeOH−H_2O (1:1)] and then silica gel chromatography (ethyl acetate) of Fr. 5 led to compound **12** (25.7 mg). Fr. 6 was isolated by Sephadex LH-20 chromatography [MeOH−H_2O (1:1)] to offer 3 subfractions (Fr. A3−C3). Fr. A3 was separated on a silica gel column (*n*-hexane−ethyl acetate, 3:4, v/v) to give compound **13** (9.0 mg). Fr. B3 was purified by semipreparative HPLC [ACN−H_2O (38:62), UV 254 nm] to give compound **14** (10.8 mg). Fr. C3 was purified by semipreparative HPLC [ACN−H_2O (14:86), UV 254 nm] to give compound **15** (2.0 mg). Fr. 7 was fractionated by silica gel chromatography (*n*-hexane−ethyl acetate, 1:2, v/v) to give compound **16** (628.7 mg). Fr. 8 was chromatographed on a Sephadex LH-20 column [MeOH−H_2O (1:1)], followed by a silica gel column ($CHCl_3$−MeOH, 10:1, v/v) to give compound **17** (25.9 mg). Fr. 10 was separated into 5 subfractions (Fr. A5−E5) by Sephadex LH-20 chromatography [MeOH−H_2O (1:1)]. Fr. A5 was purified by silica gel chromatography ($CHCl_3$−MeOH, 20:1, v/v) to give compound **18** (20.2 mg), and purification of Fr. B5 ($CHCl_3$−MeOH, 20:1.5, v/v) gave compounds **19** (2.0 mg) and **20** (26.2 mg). Fr. D5 was purified by silica gel chromatography ($CHCl_3$−MeOH, 20:1.5, v/v) to give compound **22** (25.4 mg). Fr. 11 was chromatographed on a Sephadex LH-20 column [MeOH−H_2O (1:1)], followed by a silica gel column ($CHCl_3$−MeOH, 10:1, v/v) to give compound **21** (10.2 mg) and **23** (18.2 mg). Fr. 12 was separated into four subfractions (Fr. A6−D6) by Sephadex LH-20 chromatography (MeOH−H_2O, 1:1, v/v). Silica gel chromatography ($CHCl_3$−MeOH, 25:1, v/v) of Fr. A6 gave compound **24** (117.2 mg), and that (CH_2Cl_2−MeOH, 20:1, v/v) of Fr. B6 gave compound **25** (32.9 mg), **26** (440.4 mg) and **29** (2.7 mg). Fr. C6 was purified by silica gel chromatography (CH_2Cl_2−MeOH, 25:1, v/v) to give compound **1** (3.0 mg).

Spectral Data. 5′-Geranyl-5,7,2′,4′-tetrahydroxyflavone (**1**): yellow powders; ^{1}H NMR (acetone-d_6, 400 MHz) δ 13.16 (1H, OH-5), 7.95 (1H, s, H-6′), 7.05 (1H, s, H-3′), 6.66 (1H, s, H-3), 6.46 (1H, d, J = 2.0 Hz, H-8), 6.24 (1H, d, J = 2.0 Hz, H-6), 5.40 (1H, m, H-2″), 5.13 (1H, m, H-7″), 3.33 (2H, d, J = 7.3 Hz, H-1″), 2.13 (2H, m, H-5″), 2.08 (2H, m, H-6″), 1.77 (3H, s, H-4″), 1.61, 1.58 (each 3H, s, H-9″, 10′); ^{13}C NMR (acetone-d_6, 100 MHz) δ 183.4 (C=O, C-4), 164.6 (C, C-7), 163.4 (C, C-5), 163.1 (C, C-2), 159.9 (C, C-4′), 158.8 (C, C-9), 157.2 (C, C-2′), 136.6 (C, C-3″), 131.8 (C, C-8″), 130.3 (CH, C-6′), 125.0 (CH, C-7″), 123.6 (CH, C-2″), 121.5 (C, C-5′), 110.4 (C, C-1′), 108.6 (CH, C-3), 105.2 (C, C-10), 104.1 (CH, C-3′), 99.4 (CH, C-6), 94.4 (CH, C-8), 40.5 (CH_2, C-6″), 28.1 (CH_2, C-1″), 27.5 (CH_2, C-5″), 25.8, 17.7 (CH_3, C-9″, 10″), 16.2 (CH_3, C-4″); ESI-MS m/z 423.4 $[M + H]^+$, m/z 421.3 $[M - H]^-$.

Mulberroside A (**4**): white powders; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.85 (OH), 9.44 (OH), 7.45 (1H, d, J = 8.5 Hz, H-6), 7.22 (1H, d, J = 16.4 Hz, H-α), 6.94 (1H, d, J = 16.4 Hz, H-β), 6.64 (1H, br s, H-2′), 6.57 (1H, br s, H-6′), 6.54 (1H, br s, H-4′), 6.51 (1H, H-5), 6.33 (1H, br s, H-3), 4.79 (2H, d, J = 7.4 Hz, H-1″, 1‴); ^{13} C NMR (DMSO-d_6, 100 MHz) δ 118.1 (C, C-1), 156.1 (C, C-2), 104.0 (CH, C-3), 158.2 (C, C-4), 106.8 (CH, C-5), 123.7 (CH, C-6), 126.2 (CH, C-α), 127.6 (CH, C-β), 140.0 (C, C-1′), 102.9 (CH, C-2′), 159.2 (C, C-3′), 105.6 (CH, C-4′), 158.8 (C, C-5′), 107.7 (CH, C-6′), 101.0, 100.6 (CH, C-1″, 1‴), 73.5 (CH, C-2″, 2‴), 76.9 (CH, C-3″, 3‴), 69.9, 69.8 (CH, C-4″, 4‴), 77.3 (CH, C-5″, 5‴), 60.8 (CH_2, C-6″, 6‴); ESI-MS m/z 567.0 $[M - H]^-$.

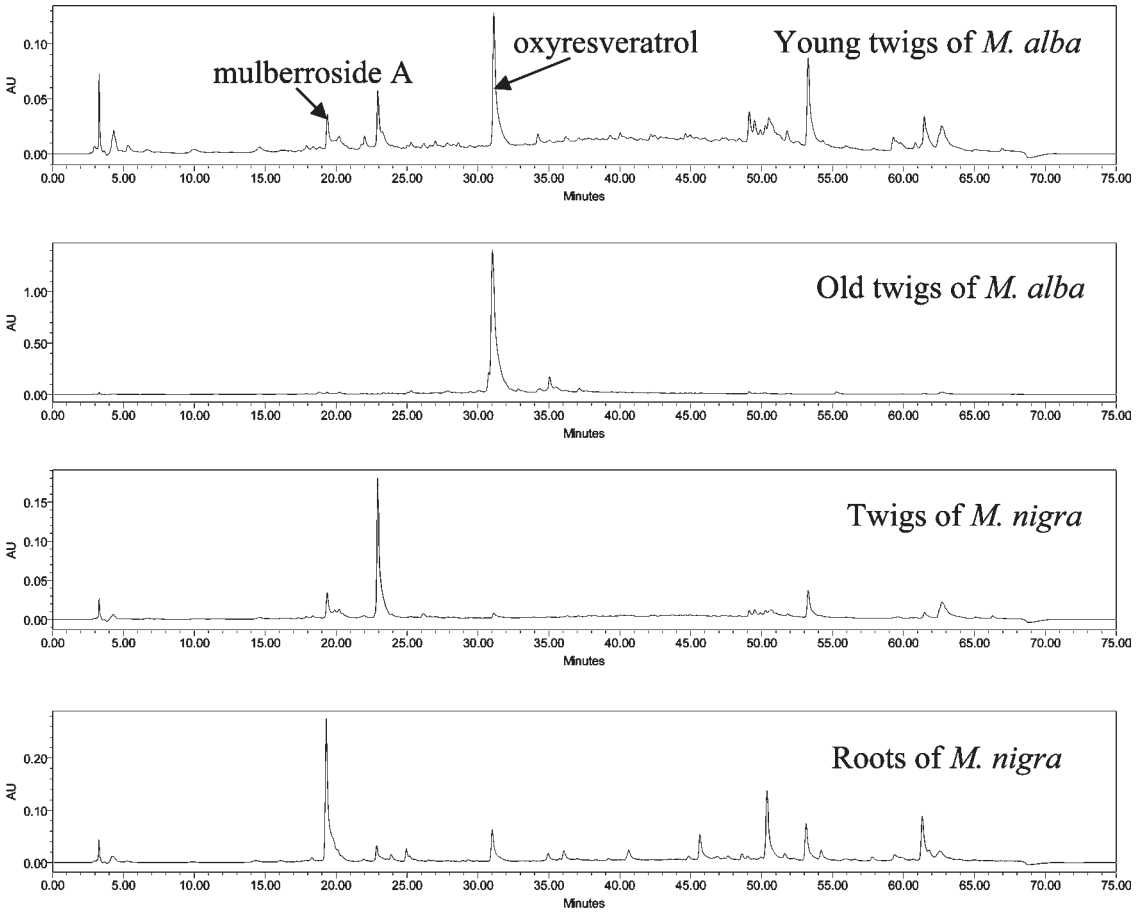

Figure 1. HPLC chromatograms of extracts from the twigs and roots of *M. nigra* and twigs of *M. alba*.

Oxyresveratrol: pale yellow powders; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.32 (1H, d, J = 9.1 Hz, H-6), 7.26 (1H, d, J = 16.4 Hz, H-α), 6.81 (1H, d, J = 16.4 Hz, H-β), 6.45 (2H, d, J = 2.0 Hz, H-2′, 6′), 6.31 (2H, overlapped, H-3, 5), 6.14 (1H, t, J = 2.0 Hz, H-4′); ^{13}C NMR (CD_3OD, 100 MHz) δ 159.8 (C, C-3′, 5′), 159.4 (C, C-4), 157.6 (C, C-2), 142.5 (C, C-1′), 128.7 (CH, C-6), 126.8 (CH, C-β), 125.1 (CH, C-α), 118.1 (C, C-1), 108.7 (CH, C-5), 106.0 (CH, C-2′, 6′), 103.9 (CH, C-3), 102.6 (CH, C-4′).

Mushroom Tyrosinase Inhibitory Assay. Tyrosinase inhibitory activities of plant extracts and isolated compounds were determined by spectrophotometric method as described in our previous study (*22*). The extracts and compounds were first dissolved in DMSO at a concentration of 1.0 mg/mL and then diluted to different concentrations with DMSO. The sample solutions (30 μL) were diluted with 970 μL of 0.05 mM sodium phosphate buffer (pH 6.8) in test tubes, followed by the addition of 1 mL of 0.1 mg/mL L-tyrosine and finally 1.0 mL of mushroom tyrosinase solution (200 units/mL). Thirty microliters of DMSO and 30 μL of kojic acid solution were used as the blank reference and positive control, respectively. The reaction mixtures (3.0 mL) were mixed by Vortex, and the initial absorbance at 490 nm was measured. After incubation for 20 min at 37 °C, the final absorbance at the same wavelength was taken. The IC_{50} values were determined which represent the concentrations of plant extracts or compounds at which 50% of the tyrosinase activity was inhibited. The percent inhibition of tyrosinase activity was calculated as follows:

$$\%\ \text{inhibition} = [(A_2 - A_1) - (B_2 - B_1)]/(A_2 - A_1) \times 100$$

A_1 is the absorbance at 490 nm of the blank at 0 min, and A_2 is the absorbance at 490 nm of the blank at 20 min; B_1 is the absorbance at 490 nm of test sample at 0 min, and B_2 is the absorbance at 490 nm of test sample at 20 min.

RESULTS AND DISCUSSION

HPLC Analysis of Several *Morus* Plants. The HPLC profiles of different extracts are shown in **Figure 1**. By comparison with that of *M. alba*, the major component of the roots of *M. nigra* was found to be mulberroside A, a compound with weak tyrosinase inhibitory activity. The twigs of *M. nigra* also contained mulberroside A as one of the main components. The young and old twigs of *M. alba* had very different HPLC profiles with the former having a much more complex phytochemical composition. Interestingly, the old twigs contained only oxyresveratrol as the major constituent, whereas in the young twigs, both oxyresveratrol and mulberroside A were present in high concentrations in addition to other minor components. Oxyresveratrol was also found in the twigs of *M. nigra*, but the content was relatively lower than that in the twigs of *M. alba*. Although *M. nigra* roots also contain oxyresveratrol, it is not a major component in it. Other components which are present in much higher concentrations might contribute to its tyrosinase inhibitory effect, and investigation was thus performed to characterize these purported compounds. As a result, 29 compounds were purified and their structures were elucidated based on their spectral data.

Structural Elucidation of the New Compound. Compound **1** gave a molecular ion peak $[M - H]^-$ at m/z 421.3 in negative ESI-MS and $[M + H]^+$ at m/z 423.4 in positive ESI-MS, together with 25 carbons in the ^{13}C NMR spectrum, suggesting its molecular formula as $C_{25}H_{26}O_6$. The ^{1}H NMR and ^{13}C NMR spectra showed compound **1** was likely a flavone. The ^{1}H NMR spectrum showed a meta-substitution on the A ring [δ 6.46 (1H, d, J = 2.0 Hz), 6.24 (1H, d, J = 2.0 Hz)]. The singlet at δ 6.66 (1H, s) was assigned to the 3 position on ring C and the two at δ 7.05 (1H, s) and δ 7.95 (1H, s) to the 3′ and 5′ position respectively on ring B. Proton signals at δ 1.58, 1.61, 1.77 (each 3H, br s), 2.08, 2.13 (each 2H, m), 3.33 (2H, d, J = 7.3 Hz), and 5.13, 5.40 (each 1H, m) support the presence of a geranyl group. The signal at δ 13.16 (1H, br s) is evident of a hydrogen

bonded hydroxyl group. In the HMBC spectrum, the H-1″ proton provided a good starting point for the assignment of the proton and carbon resonances of the geranyl group. Proton H-1″ exhibited correlations with the C-4′, C-5′, C-6′, C-2″ and C-3″ carbons, which allowed assignment of the C-5′ position of the geranyl group (**Figure 2**). Accordingly, compound **1** was identified as 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone. The structure and all assignments were confirmed by HSQC and HMBC.

Identification of Known Compounds. Structures of the known compounds (**Figure 3**) from the roots of *M. nigra* were identified by comparing ESI-MS, ^{1}H NMR and ^{13}C NMR data with the literature as steppogenin-di-7,4′-*O*-*β*-D-glucoside (**2**), 5,7-dihydroxycoumarin-7-(6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranoside) (**3**), mulberroside A (**4**), mulberroside B (**5**), 7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-*O*-*β*-D-glucopyranosyl]-oxy]-2*H*-1-benzopyran-2-one (**6**), xeroboside (**7**), morin-7-*O*-*β*-D-glucoside (**8**), 5,7-dihydroxycoumarin-7-*O*-*β*-D-glucopyranoside (**9**) (*29*), steppogenin-7-*O*-*β*-D-glucoside (**10**), moracin M (**11**), moracinoside M (**12**), 2,4,2′,4′-tetrahydroxychalcone (**13**), moracin O (**14**), norartocarpetin (**15**), kuwanon G (**16**), moracin C (**17**), cudraflavone C (**18**), moracin N (**19**), kuwanon H (**20**), mulberrofuran G (**21**), morachalcone A (**22**), mulberrofuran J (**23**), cudraflavone B (**24**), kuwanon U (**25**), kuwanon E (**26**), oxyresveratrol-2-*O*-*β*-D-glucopyranoside (**27**), oxyresveratrol-3′-*O*-*β*-D-glucopyranoside (**28**), and mulberrofuran B (**29**).

Tyrosinase Inhibition Activities of Purified Compounds and Their Structure−Activity Relationships. Twenty-nine compounds isolated from the roots of *M. nigra* were tested on mushroom tyrosinase, and some of the compounds including 5′-geranyl-5,7,2′,4′-tetrahydroxyflavone, steppogenin-7-*O*-*β*-D-glucoside, moracin O, moracin C, kuwanon H, mulberrofuran G, mulberrofuran J, oxyresveratrol-2-*O*-*β*-D-glucopyranoside, and oxyresveratrol-3′-*O*-*β*-D-glucopyranoside were tested on mushroom tyrosinase for the first time. The 25 tested compounds could be divided into four classes: (1) flavonoids; (2) stilbene glycosides; (3) 2-arylbenzofuran

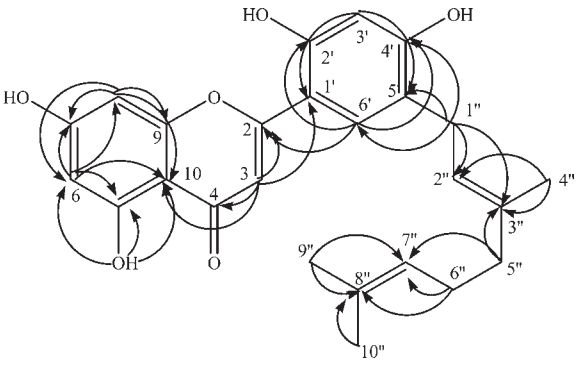

Figure 2. Key HMBC correlations of compound **1**.

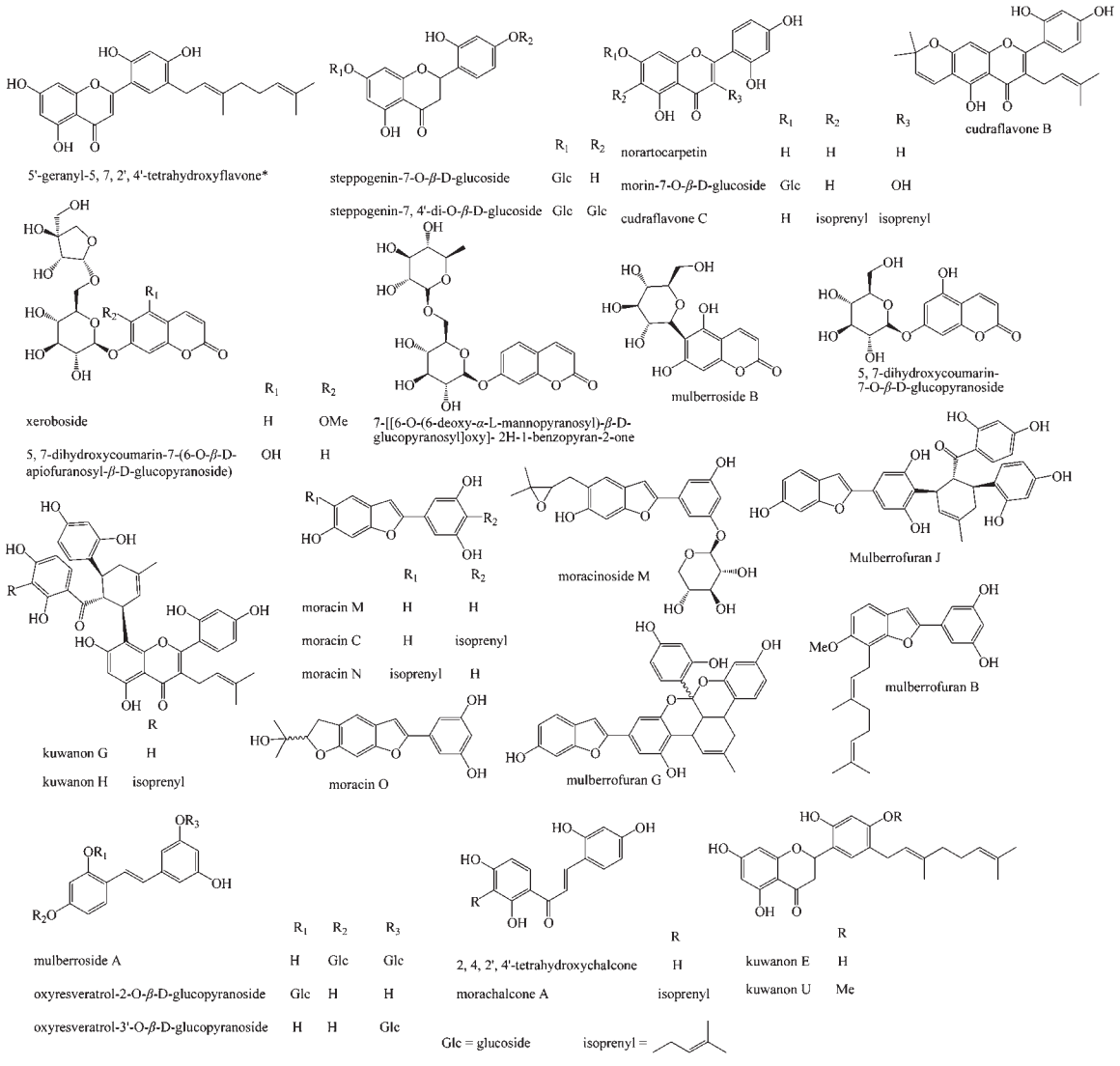

Figure 3. Compounds from the roots of *M. nigra*.

derivatives; and (4) coumarin glycosides. The isolated flavonoids included compounds **1**, **2**, **8**, **10**, **13**, **15**, **16**, **18**, **20**, **22**, **24**, **25**, and **26**. 5′-Geranyl-5,7,2′,4′-tetrahydroxyflavone is a novel flavone compound with an IC_{50} of 37.09 μM in the tyrosinase inhibition assay, which is weaker than norartocarpetin and artocarpesin (*23*), suggesting that the presence of an isoprenyl/geranyl group at different positions, especially the B-ring of the flavonoid skeleton, significantly affects tyrosinase inhibitory activity. The other three monoisoprenyl substituted flavone compounds, cudraflavone C, cudraflavone B, and kuwanon G, had very low to undetectable tyrosinase inhibitory activities. However, it was noted that the presence of an additional isoprenyl group at the 24 position on the E ring in kuwanon H led to much more potent activity relative to kuwanon G, which has only one isoprenyl group at the 24 position on the E ring. The two chalcones, 2,4,2′,4′-tetrahydroxychalcone and morachalcone A, were the two strongest inhibitors among the isolated compounds, with IC_{50} values of 0.062 and 0.14 μM, respectively. Morachalcone A isolated from the stem of *M. nigra* was reported to inhibit mushroom tyrosinase activity and melanin biosynthesis (*24*), but this study has been the first time to identify 2,4,2′,4′-tetrahydroxychalcone from *M. nigra*. Kuwanon U, an isogeranyl flavanone, showed much lower inhibitory activity than kuwanon E. This observation suggested that the substitution of a methyl group at the 4′-OH group on B-ring (kuwanon U) would greatly compromise the tyrosinase inhibitory effect. Steppogenin-7,4′-di-*O*-β-D-glucoside and steppogenin-7-*O*-β-D-glucoside differ only in the presence of one more glycoside unit in the former compound at the 4′ position of the B ring, but they demonstrated tremendously different activities in the tyrosinase inhibition assay (**Table 1**). The former was more than 50-fold weaker than the latter, and both of them were weaker than steppogenin (*23*). These data indicate that glycosidation of hydroxyl groups, especially at the 4′ position, negatively influences tyrosinase inhibitory activity of flavonones. Three stilbene glycosides, including oxyresveratrol-2-*O*-β-D-glucopyranoside, oxyresveratrol-3′-*O*-β-D-glucopyranoside, and mulberroside A, were isolated. Their tyrosinase inhibitory activities are in the following order: oxyresveratrol-3′-*O*-β-D-glucopyranoside $\gg$ oxyresveratrol-2-*O*-β-D-glucopyranoside $\gg$ mulberroside A. Like flavanones, glycosidation significantly affects the activity of the stilbenes. It appears that glycosidation at the 2 or 4 position suppresses the activity to a much larger extent than at the 3′ or 5′ position. Furthermore, a free −OH group at the 4 position of the resorcinol skeleton likely plays an important role in mediating the inhibitory activity based on the observation that glycosidation at this site (as in the case of mulberroside A, IC_{50} > 200 μM) led to substantially weakened activity. Eight 2-arylbenzofuran derivatives (moracin M, moracinoside M, moracin O, moracin C, moracin N, mulberrofuran G, mulberrofuran J, and mulberrofuran B) were isolated. Moracin O, moracin C, and moracin N belong to isoprenyl-substituted 2-arylbenzofuran derivatives. In moracin O, the isoprenyl group forms a five-membered ring with the −OH group at the 6 position, whereas in moracin N, the isoprenyl group remains intact, which might contribute to its higher tyrosinase inhibitory activity relative to moracin O. Different positions of isoprenyl substitution also result in different tyrosinase inhibitory activities as in the case of moracin C (IC_{50} = 111.47 μM) and moracin N (IC_{50} = 30.52 μM). Mulberrofuran B (IC_{50} > 200 μM) has a geranyl substituent at the 7 position and its 6 position is methoxylated. However, data obtained for this part of the study did not lead to conclusive evidence about the relative importance of these two substituents in lowering the tyrosinase inhibitory activity of the 2-arylbenzofuran derivatives. Moracinoside M, which has a glycoside at the 3′ position, also exhibited very weak activity with IC_{50} > 200 μM. Two Diels−Alder-type 2-arylbenofuran derivatives (mulberrofuran G and mulberrofuran J) were isolated, and their activities differed by nearly 10-fold. It was observed that the 2-arylbenzofuran derivatives generally had lower tyrosinase inhibitory activities than the corresponding stilbene derivatives, suggesting that part of the tyrosinase-inhibitory functionality is lost upon formation of the five-membered ring in the 2-arylbenzofuran derivatives. All the five purified coumarin glycosides (compounds **3**, **5**, **6**, **7**, **9**) exhibited weak tyrosinase inhibitory activities.

Table 1. Tyrosinase Inhibition Activity of Compounds from the Roots of *M. nigra*

compounds	IC_{50} (μM) ± SD
5′-geranyl-5,7,2′,4′-tetrahydroxyflavone (**1**)	37.09 ± 1.74
steppogenin-7,4′-di-*O*-β-D-glucoside (**2**)	>300
5,7-dihydroxycoumarin-7-(6-*O*-β-D-apiofuranosyl-β-D-glucopyranoside) (**3**)	>400
mulberroside A (**4**)	>200
mulberroside B (**5**)	>500
7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2*H*-1-benzopyran-2-one (**6**)	>400
xeroboside (**7**)	>400
morin-7-*O*-β-D-glucoside (**8**)	nt[a]
5,7-dihydroxycoumarin-7-*O*-β-D-glucopyranoside (**9**)	>400
steppogenin-7-*O*-β-D-glucoside (**10**)	5.99 ± 0.03
moracin M (**11**)	nt
moracinoside M (**12**)	>200
2,4,2′,4′-tetrahydroxychalcone (**13**)	0.062 ± 0.002
moracin O (**14**)	93.58 ± 0.65
norartocarpetin (**15**)	nt
kuwanon G (**16**)	>200
moracin C (**17**)	111.47 ± 6.96
cudraflavone C (**18**)	>200
moracin N (**19**)	30.52 ± 1.46
kuwanon H (**20**)	10.34 ± 0.19
mulberrofuran G (**21**)	17.53 ± 0.26
morachalcone A (**22**)	0.14 ± 0.01
mulberrofuran J (**23**)	191.28 ± 4.48
cudraflavone B (**24**)	nt
kuwanon U (**25**)	>200
kuwanon E (**26**)	77.99 ± 2.35
oxyresveratrol-2-*O*-β-D-glucopyranoside (**27**)	29.75 ± 2.07
oxyresveratrol-3′-*O*-β-D-glucopyranoside (**28**)	1.64 ± 0.10
mulberrofuran B (**29**)	>200
kojic acid	46.95 ± 1.72

[a] Not tested.

In conclusion, HPLC analyses showed that extracts of *M. alba* and *M. nigra* all contain oxyresveratrol, but the contents vary greatly. In the *M. nigra* root extract, oxyresveratrol only represents a very minor component, and other phytochemicals may thus contribute to the strong tyrosinase inhibitory activity of the extract. Examination of the root extract of *M. nigra* and subsequent analysis of the tyrosinase inhibitory activities of the twenty-nine purified components established preliminary structure−activity relationship data, which might be useful for future studies in this area. Moreover, several of the compounds were found to potently inhibit tyrosinase activity, indicating that *M. nigra* root extract or some of its constituents could be promising natural agents to counteract tyrosinase activity in food products or cosmetics, which warrants further investigation.

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