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Bioassay-guided screening and isolation of α-glucosidase and tyrosinase inhibitors from leaves of *Morus alba*

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ABSTRACT

In this study, bioassay-guided fractionation of extracts from the leaves of *Morus alba* L. led to the isolation of 15 bioactive constituents with α-glucosidase and tyrosinase inhibitory activities, among which prenylated stilbenes were proved to be a new group of α-glucosidase inhibitors apart from iminosugars derived from *Morus alba*. Their structures were identified on the basis of extensive spectroscopic analysis and chemical evidence, as well as comparing with data from the literature. Among them, compounds (2R)/(2S)-Euchrenone a_7 (**6a/6b**), Chalcomoracin (**7**), Moracin C (**8**), Moracin D (**9**) and Moracin N (**10**) exhibited a significant degree of α-glucosidase inhibitory activity with IC_{50} of 6.28, 2.59, 4.04, 2.54 and 2.76 μM, respectively, while (2R)/(2S)-Euchrenone a_7 (**6a/6b**), Moracin N (**10**), Quercetin (**13**), Norartocarpetin (**14**), the interconvertible epimeric mixture of (2R)/(2S)-7-methoxyl-8-ethyl-2′,4′-dihydroxylflavane-2″-*O*-β-D-glucopyranoside (**1a/1b**) and the interconvertible enantiomers of (2R)/(2S)-7-methoxyl-8-hydroxyethyl-2′,4′-dihydroxylflavane (**5a/5b**) displayed a potent tyrosinase inhibitory effect with IC_{50} of 0.260, 0.924, 0.523, 0.0824, 0.616 and 0.528 μM, respectively. Especially, (2R)-7-methoxyl-8-ethyl-2′,4′-dihydroxylflavane-2″-*O*-β-D-glucopyranoside (**1a**), (2S)-7-methoxyl-8-ethyl-2′,4′-dihydroxylflavane-2″-*O*-β-D-glucopyranoside (**1b**), (2S)-8-hydroxyethyl-7,4′-dimethoxylflavane-2′-*O*-β-D-glucopyranoside (**2**), (2R)-7-methoxyl-8-hydroxyethyl-2′,4′-dihydroxylflavane (**5a**) and (2S)-7-methoxyl-8-hydroxyethyl-2′,4′-dihydroxylflavane (**5b**) were identified as new compounds.

1. Introduction

Because of the trend of going back to nature, there is increasing enthusiasm to develop nutritional food, functional food, food medicine and even new drug from plants which contain a rich source of bioactive components with fewer side-effects (Cordain et al., 2005; Fallon, Zhong, Furne, & Levitt, 2008). *Morus alba* L. an deciduous tree belonging to the family of *Moraceae*, is widely cultivated in tropical, subtropical and temperate areas (Agarwal & Kanwar, 2007). Its leaves have been used in East Asia as herbal medicine for healing of various diseases (i.e., treat fever, protect the liver, improve eyesight and lower blood pressure) for thousands of years (Zhishen, Mengcheng, & Jianming, 1999). Leaves and extracts of *Morus alba* L. are commonly used as functional foods in Asian regions, including China, Korea, Japan and Thailand (Kimura et al., 2007; Sattayasai, Tiamkao, & Puapairoj, 2008). Traditionally, mulberry leaves have been consumed regionally as herbal tea. The consumption of mulberry-leaf tea has increased over the past decades because of its hypoglycemic, antidepressant, antioxidant and hepatoprotective effects (Nookabkaew, Rangkadilok, & Satayavivad, 2006; Zhong, Furne, & Levitt, 2006). Recently, various other food-grade mulberry products (i.e., drink powders, snacks and tablets) have been developed and marketed in these countries (Yoshihashi, Do, Tungtrakul, Boonbumrung, & Yamaki, 2010).

It was reported that the extracts from some species of *Morus* showed potent antihyperglycemic and antihyperpigmentation activities (Chen, Nakashima, Kimura, & Kimura, 1995; Shin et al., 1998). Iminosugars, such as 1-deoxynojirimycin (1-DNJ) and *N*-methyl-1-deoxynojirimycin (*N*-methyl-1-DNJ), isolated from species of *Morus*, have been studied continuously because of their potent antihyperglycemic effect (Asano, 2003). Polyphenols, such as flavonoids and stilbenes contained in this species, have been proven to possess excellent antihyperpigmentation property (Jeong et al., 2009). Our research aimed to further discover effective substances from mulberry (*Morus alba* L.) leaves with antihyperglycemic and antihyperpigmentation activities. In the present work, a bioassay-guided fractionation and purification process was used to obtain 15 bioactive constituents with α-glucosidase and tyrosinase inhibitory activities. Particularly, (2S)-8-hydroxyethyl-7,4′-dimethoxylflavane-2′-*O*-β-D-glucopyranoside (**2**), the interconvertible epimers (2R)-7-methoxyl-8-ethyl-2′,4′-dihydroxylflavane-2″-*O*-β-D-glucopyranoside (**1a**) and (2S)-7-methoxyl-8-ethyl-2′,4′-dihydroxylflavane-2″-*O*-β-D-glucopyranoside (**1b**), and the

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interconvertible enantiomers (2R)-7-methoxyl-8-hydroxyethyl-2′,4′-dihydroxylflavane (**5a**) and (2S)-7-methoxyl-8-hydroxyethyl-2′,4′-dihydroxylflavane (**5b**) were identified as new compounds.

2. Materials and methods

2.1. Chemicals and instruments

Yeast α-glucosidase (EC 3.2.1.20), mushroom tyrosinase (EC 1.14.18.1), 4-nitrophenyl-α-D-glucopyranoside, pyridine, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and chlorotrimethylsilane were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Acarbose was obtained from Melone Biotechnology Co. Ltd. (Dalian, China). Kojic acid was obtained from J&K Chemical Ltd. (Shanghai, China). L-Tyrosine was purchased from Kangjie Biotechnology Development Co. Ltd. (Shanghai, China). 1-DNJ was a product of Ronghe Pharmaceutical Technology Development Co. Ltd. (Shanghai, China). *N*-methyl-1-DNJ was obtained from Toronto Research Chemicals Inc. (North York, Canada). HPLC-grade methanol and acetonitrile (Merck, Darmstadt, Germany) were utilised for the HPLC analysis. Deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical grade. Silica gel (100–200 mesh) for column chromatography was from Qingdao Marine Chemical Company (Qingdao, China). Preparative HPLC was carried out on a Shimadzu LC-8A system with a photodiode array detector and an Agilent Zorbax SB-C18 column (250 × 21.2 mm, 7 μm). GC–MS was performed on an Agilent 6890 N system hyphenated to a 5973 network mass selective detector using a ZB-5MS column (0.25 mm × 30 m × 0.25 μm). Optical rotations were recorded on a Jasco P-1030 polarimeter. IR spectra (KBr) were acquired on a Jasco FT-IR 4100 spectrometer. UV detections were measured on a Persee UV-T6 spectrophotometer. CD spectra were measured on a Jasco J-815 spectrometer. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AVANCE DMX 500 NMR spectrometer with TMS as internal standard. HR-FT-ICR-MS experiments were performed on a Bruker Apex III spectrometer. ESI mass spectra were acquired on a Thermo Finnigan LCQ Deca XP^{plus} ESI ion trap mass spectrometer. Spectrophotometric measurements for the α-glucosidase and tyrosinase inhibition assay were taken on an infinite F200 microplate reader instrument.

2.2. Plant material

Mulberry (*Morus alba* L.) leaves were collected at Huzhou, Zhejiang province in China in September 2009. The plant material was identified by Associate Professor Liurong Chen, Department of Chinese Medicine Science & Engineering, Zhejiang University. Voucher specimens (No. MA090916) were deposited at the College of Pharmaceutical Sciences, Zhejiang University, China.

2.3. Extraction and isolation

The dried leaves (10 kg) were extracted with 70% ethanol (2 × 90 L, 2 h each). The extract was concentrated under reduced pressure to syrup, which was suspended in distiled water. The suspension was then partitioned sequentially with petroleum ether, ethyl acetate and *n*-butanol. The different fractions of petroleum ether, ethyl acetate, *n*-butanol and water were separately collected. Among these, the ethyl acetate fraction showed the strongest inhibitory activity against both α-glucosidase and tyrosinase with IC_{50} of 171 and 11.9 μg/ml, respectively. The bioactive ethyl acetate fraction (160 g) was then adsorbed onto silica gel (176 g) and subjected to chromatography on silica gel (44 × 10 cm, 1500 g, 100–200 mesh), eluting with the petroleum ether–ethyl acetate system and the dichloromethane–methanol system, to afford 20 fractions (Fr. 1–20). The Fr. 15 was further separated by preparative HPLC using a elution program in a total run time of 85 min (linear gradient from 25–44% methanol over 32 min, then isocratic for 15 min, then to 55% methanol over 1 min, then to 60% methanol over 23 min, then to 100% methanol over 4 min, and finally isocratic for 10 min) at flow rate of 10 ml/min to obtain a mixture of **1a** and **1b** (26.3 mg, t_R 72.3 min) and **2** (11.8 mg, t_R 76.6 min). A mixture of **5a** and **5b** (2.2 mg, t_R 47.5 min) was obtained from the Fr. 11 using the following elution program at flow rate of 10 ml/min for separation in a total run time of 60 min: linear gradient from 30–50% methanol over 20 min, then isocratic for 7 min, then to 60% methanol over 1 min, then isocratic for 7 min, then to 70% methanol over 1 min, then isocratic for 14 min, and finally to 80% methanol over 10 min. The other known compounds were all separated using the similar method combining of the silica gel column chromatography and the preparative HPLC.

2.3.1. Compound ***1a*** (***1b***)

Yellowish oil; $[\alpha]^{22}_D$-10.0° (c 0.38, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.09) nm, 281 (3.34) nm; IR (KBr) ν_{max} 3403, 2921, 1615, 1524, 1489, 1454, 1379, 1318, 1268, 1218, 1168, 1113 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 1; HR-FT-ICR-MS *m/z* 501.1731 $[M+Na]^+$ (Calc. for $C_{24}H_{30}NaO_{10}$, 501.1737).

2.3.2. Compound ***2***

Yellowish oil; $[\alpha]^{22}_D$-34.8° (c 0.61, MeOH); UV (MeOH) λ_{max} (log ε) 219 (4.08) nm, 278 (3.55) nm; IR (KBr) ν_{max} 3412, 2926, 1612, 1508, 1491, 1269, 1108, 1074 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 1; HR-FT-ICR-MS *m/z* 515.1888 $[M+Na]^+$ (Calc. for $C_{25}H_{32}NaO_{10}$, 515.1893).

2.3.3. Compound ***3***

Yellowish oil; $[\alpha]^{22}_D$-40.7° (c 11.29, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.47 (1H, dd, *J* = 9.3, 2.2 Hz, H-2), 2.09 (1H, m, H-3a), 1.69 (1H, m, H-3b), 2.82 (1H, m, H-4a), 2.54 (1H, m, H-4b), 6.72 (1H, d, *J* = 8.3 Hz, H-5), 6.37 (1H, d, *J* = 8.3 Hz, H-6), 6.79 (1H, d, *J* = 2.4 Hz, H-3′), 6.65 (1H, dd, *J* = 8.5, 2.4 Hz, H-5′), 7.28 (1H, d, *J* = 8.5 Hz, H-6′), 2.77 (1H, m, H-1″), 3.49 (1H, m, H-2″), 3.75 (3H, s, OCH_3-4′), 4.81 (1H, d, *J* = 7.2 Hz, H-1‴), 3.27–3.34 (2H, m, H-2‴, 3‴), 3.18 (1H, m, H-4‴), 3.39 (1H, m, H-5‴), 3.76 (1H, overlap, H-6‴a), 3.52 (1H, overlap, H-6‴b); ^{13}C NMR (125 MHz, DMSO-d_6): δ 71.8 (C-2), 29.1 (C-3), 24.3 (C-4), 112.7 (C-4a), 127.2 (C-5), 107.6 (C-6), 154.6 (C-7), 112.2 (C-8), 153.9 (C-8a), 123.9 (C-1′), 154.9 (C-2′), 102.1 (C-3′), 159.7 (C-4′), 107.5 (C-5′), 126.6 (C-6′), 27.2 (C-1″), 60.5 (C-2″), 55.4 (OCH_3-4′), 101.8 (C-1‴), 73.6 (C-2‴), 76.8 (C-3‴), 70.2 (C-4‴), 77.4 (C-5‴), 61.1 (C-6‴).

2.3.4. Compound ***4***

Yellowish oil; $[\alpha]^{22}_D$-27.7° (c 6.84, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6): δ 5.47 (1H, dd, *J* = 9.3, 2.2 Hz, H-2), 2.10 (1H, m, H-3a), 1.70 (1H, m, H-3b), 2.86 (1H, m, H-4a), 2.59 (1H, m, H-4b), 6.89 (1H, d, *J* = 8.5 Hz, H-5), 6.48 (1H, overlap, H-6), 6.61 (1H, d, *J* = 2.1 Hz, H-3′), 6.48 (1H, overlap, H-5′), 7.14 (1H, d, *J* = 8.3 Hz, H-6′), 2.76 (1H, m, H-1″), 3.42 (1H, m, H-2″), 3.73 (3H, s, OCH_3-4′), 4.74 (1H, d, *J* = 7.4 Hz, H-1‴), 3.21–3.30 (4H, m, H-2‴-5‴), 3.74 (1H, overlap, H-6‴a), 3.53 (1H, *J* = 11.8, 5.3 Hz, H-6‴b); ^{13}C NMR (125 MHz, DMSO-d_6): δ 72.0 (C-2), 28.9 (C-3), 24.2 (C-4), 114.6 (C-4a), 127.2 (C-5), 109.1 (C-6), 156.5 (C-7), 113.3 (C-8), 153.7 (C-8a), 122.0 (C-1′), 154.9 (C-2′), 103.1 (C-3′), 157.8 (C-4′), 103.0 (C-5′), 126.4 (C-6′), 27.0 (C-1″), 60.1 (C-2″), 55.7 (OCH_3-7), 101.8 (C-1‴), 73.5 (C-2‴), 76.6 (C-3‴), 69.7 (C-4‴), 77.1 (C-5‴), 60.8 (C-6‴).