Phenolic Constituents from the Stems of Morus nigra and their α-Glucosidase Inhibitory Activities

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Introduction

As a metabolic chronic disease, diabetes has severely affected people’s health. Evidence suggested that α-glucosidase inhibitors, such as acarbose, miglitol as well as voglibose, can lower the glucose levels in plasma by delaying the absorbance of carbohydrates, and are used clinically to treat diabetes; however, they also bring adverse reactions such as abdominal pain, flatulence, and diarrhea.1 Thus, the discovery of natural, side-effect-free, and effective α-glucosidase inhibitors from widely sourced medicinal plants are of important value for the treatment of diabetes.

Hyperglycemia is the major symptom of diabetes. It is well known that Morus plants are famous for their antihyperglycemia effects, and has received much attention in diabetes treatment. The isolated alkaloids from Morus plants, such as 1-deoxynojirimycin, and phenolic compo-

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Abstracts

A new sanggenon-type flavanone, nigragenon F (1), together with 11 known compounds, trans-resveratrol (2), (E)-4-isopentenyl-3,5,2’,4’-tetrahydroxystilbene (3), notabilisin E (4), notabilisin A (5), morusin (6), petalopurpurinol (7), 8-geranyl-5,7-dihydroxycoumarin (8), 2,4-dihydroxybenzaldehyde (9), 4-ethoxy-2,6-dihydroxybenzoic acid (10), 3-hydroxy-4-methoxybenzaldehyde (11), and 4-hydroxybenzaldehyde (12), were isolated from the stems of Morus nigra. Compound 10 was a new natural product, compounds 3, 4, 7, and 8 were reported from the Morus genus for the first time. All of the isolated compounds were evaluated for their α-glucosidase inhibition activity. Among them, six compounds showed obvious inhibitory effects against α-glucosidase with IC50 values ranging from 1.24 to 19.00 µmol/L.

Keywords

► Moraceae
► Morus nigra
► sanggenon-type flavanone
► nigragenon F
► α-glucosidase
Materials and Methods

General Methods
Ultraviolet (UV) spectra were collected using a UV-2500 PC instrument (Shimadzu Corporation, Japan). Mass spectrometry was determined on a Waters Xevo G2-XS-Q-TOF. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV III instrument. Electronic circular dichroism spectra were obtained using a JASCO-810 spectropolarimeter. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd.), Sephadex LH-20 (GE Healthcare, Sweden), and RP-C18 (YMC Co., Ltd., Japan) were used for column chromatography (CC). Thin layer chromatography was performed on silica gel HF254 plates using 10% H2SO4 in ethanol (v/v) spray reagents followed by heating. Semipreparative high-performance liquid chromatography (HPLC) was carried out on a LC3050N HPLC using a C18 column (10 × 250 mm, 5 µm, Waters Corporation, United States) and characteristic UV absorption at 210 nm. Reagents were of analytical reagent grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) except for acetonitrile and methanol which were of chromatographic grade.

Plant Materials
The stems of M. nigra L. (Moraceae) were collected from Hetian town, in the Xinjiang province of China in September 2016. The plant was identified by Prof. Tong Wu, who comes from China State Institute of Pharmaceutical Industry, China. A voucher specimen (No. 201609001) was deposited in our department.

Extraction and Isolation
The dried and powered stems of M. nigra (15 kg) were extracted twice with 90% aqueous EtOH under hot reflux (1.5 hours each time). The concentrated extract was suspended in water and partitioned successively with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EtOAc), and n-butanol. The crude extracts of DCM portion (120 g) and EtOAc portion (80 g) were mixed and then subjected to silica gel CC, eluted with CH2Cl2–CH3OH (80:1 → 1:1) to obtain six fractions (Fr.A–Fr.F). Fr.A (42.5 g) was purified by silica gel CC (PE–acetone, 15:1 → 1:1) to obtain 18 subfractions (Fr.A-1–Fr.A-18). Fr.A-8 (13.3 g) was decolorized with HP-20 macroporous-absorbing resin eluted with EtOH to get a fraction (Fr.A-8-1). Fr.A-8-1 was further separated by CC successively over Sephadex LH-20 (CH2Cl2–CH3OH, 1:1) and RP-C18 (CH3OH–H2O, 50:50 → 100:0) to obtain five fractions (Fr.A-8-1-a–Fr.A-8-1-e). Fr.A-8-1-a (56.0 mg) was purified by semipreparative HPLC (CH3CN–H2O, 18:82) to yield compound 9 (1.7 mg) and compound 11 (3.5 mg). Fr.A-8-1-b (14.7 mg) was separated by semipreparative HPLC eluted with a gradient of CH3CN–H2O (28:72 → 35:65) to afford compound 10 (3.0 mg). Fr.A-9 (1.3 g) was chromatographed over Sephadex LH-20 (CH2Cl2–CH3OH, 1:1), followed by CC on RP-C18 (CH3OH–H2O, 60:40 → 90:10), to give two fractions (Fr.A-9-1–Fr.A-9-2). Fr.A-9-2 (30.0 mg) was further subjected to semipreparative HPLC (CH3CN–H2O, 25:75) to yield compound 12 (3.0 mg). Fr.A-11 (0.8 g) was chromatographed on Sephadex LH-20 (CH2Cl2–CH3OH, 1:1) to give a fraction (Fr.A-11-3). Fr.A-11-3 (125.7 mg) was separated by silica gel CC (CH2Cl2–CH3OH, 100:1 → 10:1) to obtain two fractions (Fr.A-11-3-1–Fr.A-11-3-2). Fr.A-11-3-1 (18.5 mg) and Fr.A-11-3-2 (35.4 mg) were further purified by semipreparative HPLC to yield compound 6 (2.0 mg) and compound 7 (11.4 mg), respectively. Fr.A-13 (3.4 g) was chromatographed over Sephadex LH-20 (CH2Cl2–CH3OH, 1:1) to produce a fraction (Fr.A-13-1), which was subjected to CC on RP-C18 (CH3OH–H2O, 50% → 100%) to produce three fractions (Fr.A-13-1-1–Fr.A-13-1-3). Fr.A-13-1 (174.5 mg) and Fr.A-13-1-2 (82.7 mg) were further purified by semipreparative HPLC (CH3CN–H2O, 70:30) to afford compounds 1 (5.6 mg) and 4 (3.6 mg), respectively. By a similar procedure to Fr.A-13, Fr.A-14 (1.4 g) was subjected successively to CC on Sephadex LH-20 (CH2Cl2–CH3OH, 1:1) and RP-C18 (CH3OH–H2O, 50:50 → 90:10), followed by semipreparative HPLC, to yield compound 5 (26.6 mg). Fr.B (6.6 g) was initially isolated by Sephadex LH-20 (CH2Cl2–CH3OH, 1:1), followed by RP-C18 eluted with CH3CN–H2O (20:80 → 70:30) to yield six fractions (Fr.B-1–Fr.B-6). Fr.B-2 (107.3 mg) was chromatographed on silica gel CC (CH2Cl2–CH3OH, 60:1, 50:1, 40:1, 20:1, 10:1) to give one fraction (Fr.B-2-1), which afforded compound 8 (1.0 mg) by semipreparative HPLC (CH3CN–H2O, 48:52). Fr.D (15.0 g) was initially divided into three fractions (Fr.D-1–Fr.D-3) by CC on silica gel (PE–acetone, 5:1, 3:1, 2:1, 1:1). Fr.D-2 (5.0 g) was separated by Sephadex LH-20 (CH2Cl2–CH3OH, 1:1) to give four subfractions (Fr.D-2-1–Fr.D-2-4). Fr.D-2-2 (1.0 g) was further separated by three fractions (Fr.D-2-2-1–Fr.D-2-2-3) by CC on RP-C18 (CH3OH–H2O, 10:90 → 90:10). Fr.D-2-2-1 (47.7 mg) was further purified by semipreparative HPLC (CH3CN–H2O, 23:77) to yield compound 2 (3.3 mg). Fr.D-3 (4.1 g) was subjected to CC on Sephadex LH-20 twice, eluted with CH2Cl2–CH3OH (1:1) to get one portion (Fr.D-3-2), which was further separated by CC on RP-C18 (CH3OH–H2O, 10:90 → 90:10) to afford four fractions (Fr.D-3-2-a–Fr.D-3-2-d). Fr.D-3-2-d (10.2 mg) was purified by semipreparative HPLC to obtain compound 3 (2.0 mg).

α-Glucosidase Inhibition Assay
The α-glucosidase inhibitory activity was assessed with a spectrophotometric method using acarbose as the positive control. Sample solution with six different concentrations was preincubated with α-glucosidase (0.2 U/mL, Sigma Chemical Co. St. Louis, Missouri, United States) in 96-well plates at 37°C for 10 minutes. Then the substrate 4-nitrophenyl-α-D-glucosidase (PNPG, 100 µL, 2 mmol/L, Sigma Chemical Co., United States) was added to each well. After incubation at 37°C for 20 minutes, the reaction was terminated with Na2CO3 solution (50 µL, 1.06 g/50 mL). The absorbance of the system was measured at 405 nm using a microplate reader. The IC50 was performed in triplicate and calculated with Graphpad Prism 7.0.

Results and Discussion

Structure Identification
Compound 1 was obtained as yellow powder. The molecular formula was established as C26H25O8 according to
Table 1 1H NMR (400 MHz) and 13C NMR (100 MHz) data of 1 (in acetone-d6)

| Position | δH (J in Hz) | δC |
|----------|--------------|----|
| 2        | 7.07 br s (OH) | 92.2 |
| 3        | 11.77 s (OH) | 188.4 |
| 4        | 5.80 s | 100.8 |
| 5        | 3.13 dd (14.8, 9.2) | 32.1 |
| 6        | 5.80 s | 100.8 |
| 7        | 5.24 br t (7.6) | 91.9 |
| 8        | 1.51 s | 136.6 |
| 8a       | 1.61 s | 18.1 |
| 9a       | 3.04 d (8.4) | 26.5 |
| 9b       | 4.77 br t (8.4) | 93.2 |
| 10       | 1.23 s | 25.9 |
| 11       | 1.22 s | 25.2 |
| 12       | 1.51 s | 25.9 |
| 13       | 1.61 s | 18.1 |
| 14       | 3.04 d (8.4) | 26.5 |
| 15       | 4.77 br t (8.4) | 93.2 |
| 16       | 1.23 s | 25.9 |
| 17       | 1.22 s | 25.2 |
| 18       | 1.51 s | 25.9 |
| 19       | 1.61 s | 18.1 |
| 20       | 3.13 dd (14.8, 9.2) | 32.1 |
| 21       | 5.80 s | 100.8 |
| 22       | 6.39 d (2.0) | 99.5 |
| 23       | 8.72 br s (OH) | 161.3 |
| 24       | 6.52 dd (8.4, 2.0) | 109.8 |
| 25       | 7.36 d (8.4) | 125.8 |

to the [M – H]− ion peak at m/z 453.1555 (calcd. for C25H25O8, 453.1549) in its HRESIMS (high-resolution electrospray ionization mass spectrometry) spectrum. UV absorption maxima of compound 1 were recorded at 235 (sh), 285 (sh), and 310 nm, indicating the presence of a sanggenon-type flavanone framework (3-hydroxy-2-prenylflavanones with a furan moiety between the B and C rings) in this compound. Besides, IR spectrum of compound 1 showed the existence of OH (3,395 cm⁻¹), C = O (1,657 cm⁻¹), and benzene ring (1,608 and 1,463 cm⁻¹). Furthermore, the 1H NMR spectrum of compound 1 (Table 1) showed (1) the signals of a hydrogen-bonded hydroxy group at δH 11.77 (1H, br s, OH-5); (2) an aromatic ABX spin system at δH 7.36 (1H, d, J = 8.4 Hz, H-6′), 6.52 (1H, dd, J = 8.4, 2.0 Hz, H-5′), and 6.39 (1H, d, J = 2.0 Hz, H-3′); (3) an aromatic proton at δH 5.80 (1H, s, H-6); and (4) a characteristic isoprenyl of sanggenon-type flavanone at δH 5.24 (1H, br t, J = 7.6 Hz, H-10), 3.13 (1H, br dd, J = 14.8, 9.2 Hz, H-9a), 2.82 (1H, br dd, J = 14.8, 6.0 Hz, H-9b), 1.61 (3H, br s, H-13), and 1.51 (3H, br s, H-12). In addition, signals of another cyclized isoprenyl group were observed at δH 4.77 (1H, br t, J = 8.4 Hz, H-15), 3.04 (2H, br d, J = 8.4 Hz, H-14), 1.23 (3H, s, H-17), and 1.22 (3H, s, H-18). A total of 25 carbon signals appeared in the 13C NMR spectrum (Table 1), including 20 carbon signals from the sanggenon skeleton and 5 carbon signals from the substituent. The key HMBC correlations of H-2 to C-3 and C-1′ assigned the isoprenyl group at C-2, confirming the sanggenon skeleton of 1. The HMBC correlations from H-2′ to C-8a and C-16, and from H-15 to C-7, C-17, and C-18 indicated that the cyclized isoprenyl group was fused at C-7 and C-8. Thus, its planar structure was established as shown in Fig. 1. Furthermore, the absolute configurations of C-2 and C-3 in 1 were assigned as 2R and 3S respectively, according to the positive Cotton effects at 219, 251, 296, and 317 nm, and negative Cotton effects at 239 and 276 nm in its circular dichroism (CD) spectrum (see Supplementary Figs. S1, S2, S3, S4, S5, S6 [online only]). The absolute configuration of C-15 remained to be determined. Therefore, compound 1 was elucidated as (6α,11β)-6α,11β-dihydro-5,6a,9-trihydroxy-2-(1-hydroxy-1-methylethyl)-11β-(3-methyl-2-buten-1-yl)-1H,2H,6H-benzofuro[3,2-b]pyrano[2,3-e]-[1]benzofuro-6-one, and was named nigragenon F.

The structures of the remaining 11 compounds (Fig. 1) were elucidated as trans-resveratrol (2), 4-isopentenyl-3,5,2′,4′-tetrahydroxystilbene (3), 5,6a,9-trihydroxy-2-(1-hydroxy-1-methylethyl)-11β-(3-methyl-2-buten-1-yl)-1H,2H,6H-benzofuro[3,2-b]pyrano[2,3-e]-[1]benzofuro-6-one, and was named nigragenon F.

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ranging from 1.24 to 19.00 µmol/L (Table 2). Of these, compound 5 showed the highest α-glucosidase inhibitory effect with IC50 value of 1.24 µmol/L, approximately 800 times stronger than the positive control drug acarbose.

**Conclusion**

In the present study, phytochemistry investigation on the stems of *M. nigra* afforded 12 compounds (1–12), including a new sanggenon-type flavanone, a new natural product, and four compounds firstly reported from the *Morus* genus. The α-glucosidase inhibitory effect test provided different structure-type α-glucosidase inhibitors from *M. nigra*, not only enriching the library of natural α-glucosidase inhibitors, but also laying experimental basis for the development and utilization of *M. nigra* as hypoglycemic medicinal plant resources.

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**Conflict of Interest**

The authors declare no conflict of interest.

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