SUPPLEMENTARY MATERIAL

Quantification and bio-assay of α -glucosidase inhibitors from the roots of *Glycyrrhiza uralensis* Fisch.

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Abstract: This work aimed to investigate the α -glucosidase inhibitor from the roots of *Glycyrrhiza uralensis* Fisch.. Seven flavonoids were isolated, and the total content of compounds **1-7** were more than 50% in *Glycyrrhiza* total flavones (GTF), and the content of compound **1** and **2** was abundant in GTF. The results of the α -glucosidase inhibitory activities indicated that compound **1-6** and GTF respectively with the IC₅₀ values of 31.303, 30.263, 23.363, 19.528, 10.854, 26.454 and 21.641 μ g/mL exhibited the more potent activity than acarbose with the IC₅₀ values of 38.995 μ g/mL. These result suggested that *Glycyrrhiza* flavonoids may become a valid alternative of potential basis for new hypoglycaemic and antidiabetic agents.

Keywords: *Glycyrrhiza uralensis*; Licorice; α -glucosidase; flavonoid; hypoglycaemic; antidiabetic

Experimental

Equipment and materials

ESI-MS were obtained on a Navigator Mass Thermoquest spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The melting point was measured using a Kofler Bank (Coesfeld GmbH & Co., Dortmund, Germany). NMR spectra were recorded on a 300 MHz Bruker Avance DPX instrument (Bruker, Karlsruhe, Germany). Open column chromatography was performed in glass columns filled with polyamide gel (30-60 mesh), D101 macroporous resin, silica gel (200–300 mesh), octadecyl silane (ODS) silica gel (AAG12S50, YMC, Tokyo, Japan) and Sephadex LH-20. Various fractions were analyzed on glass slide pre-coated with silica gel GF₂₅₄ (2.5×7.6cm, 0.2 mm thick) and TLC silica gel 60 RP-18 F₂₅₄S (5×7.5cm; Merck, Darmstadt, Germany) and were detected under UV lamp. Liquid chromatography was performed on a Waters 2998 HPLC system (Waters Corporation, Massachusetts, USA). The p-nitrophenyl glucopyranoside (pNPG) (Marque: N1377, Lot number: BCBN2675V) and α -glucosidase (Marque: G5003, Lot number: SLBG6702V) were purchased from Sigma-Aldrich. Analytes of the enzyme activity were determined on 96-well plates (Costar, USA).

Plant material

The dried root and stem of *Glycyrrhiza uralensis* Fisch. (Leguminosae) were obtained from Guazhou county of Jiuquan, China and purchased in Lanzhou Yellow River herbal market, China, during March 2013. The herb medicine was authenticated by Prof. Zhigang Ma at the School of Pharmacy, Lanzhou University, Lanzhou, Republic of China. The whole plant specimen of *Glycyrrhiza uralensis* was collected in Jiuquan, China and preserved in the museum of Chinese herbal medicine specimen of Lanzhou University, during summer 2012. The voucher specimen number is 620922120825003LY.

Extraction and Isolation

The dried and crushed decoction pieces of *G. uralensis* Fisch.(5kg) was extracted by solvothermal reflux extraction with 95% ethanol two times (each for 1.5 h) and the licorice ethanolic extract was subjected to polyamide gel column chromatography (2.5kg, 30-60 mesh, 12×150cm) with distill water and 95% ethanol as the mobile

phase. The fractions demonstrated positive by Mg-HCl reaction was GTF (306g), which was eluted with a gradient using ethanol-distill water (3:7, 5:5, 7:3, 9:1, 1:0; v/v) were further submitted to D101 macroporous resin (7L, 12×150cm) and divided into five fractions was A, B, C, D and E, respectively.

A and C did not get any pure compound. Some small-polar compounds were obtained from fraction E. B (87g) was submitted to a Sephadex LH-20 column (250 g, 4×100 cm) and eluted with MeOH-CHCl₃ (1:1, v/v) and divided into 4 fractions was B-Fraction1 (B-Fr1), B-Fr2, B-Fr3 and B-Fr4.

Subfractions B-Fr1 (16g) was submitted to a Sephadex LH-20 column and eluted with MeOH-CHCl₃ (1:1, v/v). The target fractions, B-Fr1-2 (13g) was submitted to silica gel column chromatography (100 g, 200–300 mesh, 2.5 × 40 cm) using a MeOH-CHCl₃ (50:1-1:1, v/v) eluent to gain compound **3** (61 mg).

Subfractions B-Fr2 (23g) was submitted to a Sephadex LH-20 column and eluted with MeOH-CHCl₃ (1:1, v/v). The target fractions, B-Fr2-2 (9.6g) was subjected to ODS silica gel column chromatography (20g, 50μm, 1×20cm) with H₂O-MeOH (5:1-1:1, v/v) as the mobile phase to obtain compound **1** (82 mg). At this moment, B-Fr2-3 (3.4g) was subjected to ODS silica gel column chromatography (40g, 50μm, 2×30cm) with H₂O-MeOH (5:1-1:1, v/v) as the mobile phase to get compound **2** (45 mg).

Subfractions B-Fr3 (17g) was submitted to a Sephadex LH-20 column and eluted with MeOH-CHCl₃ (1:1, v/v). The target fractions, B-Fr3-2 (6.6g) was subjected to a Sephadex LH-20 column. The quaternary fractions B-Fr3-2-5 (2.4g) was submitted to silica gel column chromatography (50 g, 200–300 mesh, 2×40cm) using a MeOH-CHCl₃ (100:1-0:1, v/v). Its 202–214 fractions (0.5g) was subjected to ODS silica gel column chromatography (25g, 50μm, 1×20cm) with H₂O-MeOH (1:1, v/v) as the mobile phase to get compound **4** (32 mg).

Subfractions B-Fr4 (19g) was submitted to a Sephadex LH-20 column and eluted with MeOH. Trifractions B-Fr4-3 (1g) was subjected to ODS silica gel column chromatography (30g, 50μm, 1.5×25cm) with H₂O-MeOH (6:1-1:1, v/v) as the mobile phase to obtain compound **5** (44 mg) and compound **6** (35mg) .

D (45g) was submitted to a Sephadex LH-20 column and eluted with MeOH, then the subfractions D-Fr5 (7.6g) was submitted to silica gel column chromatography (200 g, 200–300 mesh, 3×60cm) with CHCl₃-MeOH (500:1-1:1, v/v) as the mobile phase. The target fractions, D-Fr5-2 (2.0g) was subjected to ODS silica gel column chromatography (25g, 50μm, 1×20cm) with H₂O-MeOH (5:1-1:1, v/v) as the mobile phase. The obtained crude product was submitted to a Sephadex LH-20 column (20g, 1×50cm) and eluted with MeOH to yield compound **7** (16 mg).

The spectral data of six flavonoids

Compound **1** was obtained as white amorphous powder (82 mg) : mp: 164 °C ~166 °C; ¹H NMR (methanol-*d*₄, 300 MHz) δ: 7.74 (1H, d, *J*=8.6 Hz, H-5), 6.52 (1H, dd, *J*=2.1, 8.6 Hz, H-6), 6.36 (1H, d, *J*=2.2 Hz, H-8), 7.45, 7.47 (2H, d, *J*=8.6 Hz, H-2', 6'), 7.12, 7.21 (2H, d, *J*=8.6 Hz, H-3', 5'), 5.46 (1H, dd, *J*=2.3, 13.1 Hz, H-2), 3.06 (1H, dd, *J*=12.9, 16.9 Hz, H-3), 2.76 (1H, dd, *J*=2.7, 16.9 Hz, H-3), 4.92 (1H, d, *J*=7.5 Hz, H-1''), 3.47 (1H, m, H-2''), 3.45-3.48 (2H, m, H-3'', 5''), 3.41 (1H, m, H-4''), 3.93 (1H, dd, *J*=2.2, 12.2 Hz, H-6a), 3.67 (1H, dd, *J*=5.5, 12.2 Hz, H-6b). ¹³C NMR (methanol-*d*₄, 100 MHz) δ: 80.6 (C-2), 44.9 (C-3), 193.1 (C-4), 129.1 (C-5), 111.0 (C-6), 144.1 (C-7), 104.1 (C-8), 165.2 (C-9), 112.5 (C-10), 130.2 (C-1'), 122.1 (C-2', C-6'), 118.1 (C-3', C-5'), 160.2 (C-4'), 102.4 (C-1''), 75.2 (C-2''), 78.4 (C-3'', C-5''), 71.7 (C-4''), 60.9 (C-6a, C-6b); MS *m/z* 419.4132 [M+H]⁺. Compared with the literature (Nakanishi et al. 1985), compound **1** was identified as neoliquiritin.

Compound **2** was obtained as white needle-shape crystals from MeOH (45mg): mp: 212 °C ~213 °C; ¹H NMR (DMSO-*d*₆, 300 MHz) δ: 5.52 (1H, d, *J*=12.4 Hz, H-2), 2.67 (1H, d, *J*=14 Hz, H-3), 3.13 (1H, dd, *J*=16.4, 12.4 Hz, H-3), 7.68 (1H, d, *J*=8.2 Hz, H-5), 6.49 (1H, d, *J*=8.7 Hz, H-6), 6.42 (1H, d, *J*=2.3 Hz, H-8), 7.46 (1H, d, *J*=8.2 Hz, H-2', 6'), 7.11 (1H, d, *J*=8.4 Hz, H-3', 5'), 4.86 (1H, d, *J*=7.4 Hz, H-1''), 3.16 (1H, m, H-2''), 3.38-3.38 (2H, m, H-3'', 5''), 3.09 (1H, m, H-4''), 3.70 (1H, dd, *J*=2.0, 12.1 Hz, H-6a), 3.70 (1H, dd, *J*=2.0, 12.1 Hz, H-6b); ¹³C NMR (DMSO-*d*₆, 100 MHz) δ: 78.6 (C-2), 43.1 (C-3), 189.8 (C-4), 128.5 (C-5), 110.6 (C-6), 165.2 (C-7), 101.9 (C-8), 163.1 (C-9), 113.4 (C-10), 130.2 (C-1'), 125.9 (C-2', 6'), 115.9 (C-3', 5'), 157.3 (C-4'), 100.3 (C-1''), 76.7 (C-2''), 77.1 (C-3''), 70.2 (C-4''), 76.2 (C-5''), 60.1 (C-6''); MS *m/z* 419.3911 [M+H]⁺. Compound **2** was identified as liquiritin from these spectral data (Adila et al. 2009).

Compound **3** was obtained as yellowish amorphous solid (61mg): mp: 207°C; ^1H NMR (methanol- d_4 , 300 MHz) δ : 7.71 (1H, d, $J=8.8$ Hz, H-5), 6.48 (1H, dd, $J=2.1, 8.6$ Hz, H-6), 6.18 (1H, d, $J=2.1$ Hz, H-8), 7.45 (2H, d, $J=8.5$ Hz, H-2', 6'), 6.94 (2H, d, $J=8.5$ Hz, H-3', 5'), 5.05 (1H, dd, $J=3.0, 13.0$ Hz, H-2), 2.96 (1H, dd, $J=13.0, 16.8$ Hz, H-3), 2.66 (1H, dd, $J=3.0, 16.8$ Hz, H-3). ^{13}C NMR (methanol- d_4 , 100 MHz) δ : 80.6 (C-2), 44.5 (C-3), 193.1 (C-4), 128.9 (C-5), 111.1 (C-6), 164.9 (C-7), 103.2 (C-8), 166.8 (C-9), 114.2 (C-10), 131.2 (C-1'), 128.8 (C-2'), 116.1 (C-3'), 158.2 (C-4'), 116.2 (C-5'), 128.9 (C-6'); MS m/z 257.2512 $[\text{M}+\text{H}]^+$. Compound **3** was identified as liquiritigenin from the spectral data, and the literature (Ma et al. 2005).

Compound **4** was obtained as yellow needle-shape crystals from MeOH (32mg): mp: 231°C~238°C; ^1H NMR (DMSO- d_6 , 300 MHz) δ : 1.73 (3H, s, CH_3), 1.64 (3H, s, CH_3), 3.26 (2H, d, $J=6.8$ Hz, H-1''), 3.77 (3H, s, OCH_3), 5.15 (1H, s, $J=6.8$ Hz, H-2''), 6.28 (1H, dd, $J=2.1, 8.3$ Hz, H-5'), 6.35 (1H, d, $J=2.1$ Hz, H-3'), 7.14 (1H, d, $J=8.3$ Hz, H-6'), 7.81 (1H, s, H-4), 9.36 (2H, s, 2',3'-OH), 10.54 (1H, s, 7-OH). ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.9 (C-2), 120.3 (C-3), 136.3 (C-4), 106.1 (C-4a), 158.3 (C-5), 62.7 (5- OCH_3), 113.4 (C-6), 159.2 (C-7), 97.8 (C-8), 152.9 (C-8a), 118.3 (C-1'), 155.9 (C-2'), 102.6 (C-3'), 155.2 (C-4'), 105.8 (C-5'), 131.5 (C-6'), 22.2 (C-1''), 122.6 (C-2''), 130.6 (C-3''), 17.7 (3''- CH_3), 25.4 (3''- CH_3); MS m/z 369.1247 $[\text{M}+\text{H}]^+$. Thin layer chromatography (TLC) of compound **4** visualized under UV lamp at 254 nm had blue fluorescent. Compared with the literature (Fu et al. 2013), and compound **4** was identified as glycycomarin.

Compound **5** was obtained as yellow needle-shape crystals from MeOH (44mg): mp: 117°C~119°C; ^1H NMR (DMSO- d_6 , 300MHz) δ 7.90: (1H, d, $J=3.6$ Hz, H-2'), 7.86 (1H, dd, $J=8.4, 6.6$ Hz, H-6'), 6.95 (1H, d, $J=8.7$ Hz, H-5'), 6.41 (1H, d, $J=1.2$ Hz, H-8), 6.18 (1H, d, $J=1.2$ Hz, H-6), 5.44 (1H, m, H-2''), 3.41 (2H, m, H-1''), 1.69 (3H, s, CH_3 -4''), 1.68 (3H, s, CH_3 -5''). ^{13}C NMR (DMSO- d_6 , 100MHz) δ 175.8 (C-4, s, C=O), 164.1 (C-7, s), 160.6 (C-5, s), 156.9 (C-4', s), 156.1 (C-9, s), 146.9 (C-2, s), 135.6 (C-3, s), 131.8 (C-3', s), 129.1 (C-2', d), 127.6 (C-3', s), 126.9 (C-6', d), 122.4 (C-1', s, C-2', d), 114.8 (C-5', d), 102.9 (C-1', s), 98.2 (C-6, d), 93.4 (C-8, d), 28.1 (C-1'', t), 25.5 (C-4', q), 17.7 (C-5', q); MS m/z 355.1109 $[\text{M}+\text{H}]^+$. Compound **5** was identified as isolicoflavonol from the spectral data, and the literature (Zheng et al. 2008).

Compound **6** was obtained as yellow needle-shape crystals from MeOH (35mg): molecular formula ($C_{21}H_{22}O_4$); 1H NMR (DMSO- d_6 , 300MHz) δ 1.47 (6H, Me-4'', 5'') 3.82 (3H, s, OMe) 4.78 (1H, dd, $J=1.5, 10.7$ Hz, H-3''a) 4.97 (1H, dd, $J=3.6, 15.6$ Hz, H-3''b) 6.29 (1H, dd, $J=2.4, 5.1$ Hz, H-2'') 6.57 (1H, s, H-3) 6.91 (2H, d, $J=8.4$ Hz, H-3', 5') 7.53 (1H, s, H-6) 7.62 (1H, d, $J=15.6$ Hz, H- α) 7.94 (2H, d, $J=15.3$ Hz, H-2', 6') 8.00 (1H, d, $J=8.7$ Hz, H- β) ^{13}C NMR (DMSO- d_6 , 100MHz) δ 187.9 (C=O, s), 162.2 (C-4', s), 160.23 (C-4, s), 158.3 (C-2, d), 147.5 (C-2'', s), 138.7 (C- β , s), 130.7 (C-2', 6', s), 129.3 (C-1', s), 127.7 (C-6, d), 117.4 (C- α , s), 115.3 (C-3', 5', s), 113.2 (C-1, s), 112.5 (C-3'', s), 99.9 (C-3, s), 55.3 (OCH₃, d), 38.8 (C-1'', t), 26.9 (C-4'', 5'', d); MS m/z 339.4029 $[M+H]^+$. Compound **6** was identified as licochalcone A from the spectral data (Wang et al. 2004).

Compound **7** was obtained as amorphous powder (16mg), mp: 131 °C ~ 132 °C; 1H NMR (acetone- d_6 , 300 MHz) δ : 7.28 (1H, d, $J=8.2$ Hz, H-1), 6.5 (1H, dd, $J=2.2$ Hz, H-2), 6.4 (1H, d, $J=2.2$ Hz, H-4), 3.6 (1H, d, $J=7.5$ Hz, H-6), 3.59 (1H, d, $J=6.2$ Hz, H-6a), 7.24 (1H, d, $J=8.2$ Hz, H-7), 6.46 (1H, dd, $J=8.2$ Hz, H-8), 6.41 (1H, d, $J=2.3$ Hz, H-10), 5.53 (1H, d, $J=6.0$ Hz, H-11a), 3.69 (3H, s, 3-O Me); ^{13}C NMR (acetone- d_6 , 100 MHz) δ : 132.3 (C-1), 111.0 (C-2), 156.8 (C-3), 105.0 (C-4), 156.8 (C-4a), 66.6 (C-6), 39.7 (C-6a), 119.6 (C-6b), 125.3 (C-7), 106.5 (C-8), 161.9 (C-9), 97.0 (C-10), 160.9 (C-10a), 78.6 (C-11a), 111.0 (C-11b), 55.3 (-OMe); MS m/z 270.2541 $[M+H]^+$. Compound **7** was identified as medicarpin from these spectral data (Hasan et al. 2012).

Detection of six purified flavonoids in GTF by HPLC

Liquid chromatography was performed on a Waters 2998 HPLC system equipped with a photo diode array (PDA) system and a thermostatically controlled column compartment. The sample was separated on a Waters Symmertry SB-C18 column (250 $\times$ 4.6 mm, 5 μ m) and the temperature was maintained at 25 °C. The mobile phase consisted of phase A (H₂O) and phase B (ACN). A gradient program was used according to the following profile: 0–4 min, 19% B; 4–8 min, 19%–45% B; 8–10 min, 45%–55% B; 10–18 min, 55%–58% B; 18–20 min, 58%–65% B; 20–30 min, 65%–100% B. The flow rate was 1.0 mL/min and the injection volume was 20 μ L. All the operations, acquisition and analysis of data were controlled by Empower 3 software (Waters Corporation, Massachusetts, USA).

For the construction of calibration curves, the standard stock solution of the six isolated flavonoids were prepared and diluted with MeOH to six appropriate concentrations. The analyte stock solution of GTF samples were prepared and diluted with methanol, the resultant solution was filtered through a 0.22 μm filter for HPLC analysis. The peak sequence of compound **1-7** was consistent with the order of isolated from GTF and shown in Figure 2.

Figure 1. The corresponding peak positions of seven isolates in chromatogram of GTF.

Determination of the activity of α -glucosidase inhibitory

The reaction mixture contained 20 μL of 1.6Unit/mL enzyme (diluted with PBS, pH=6.8), 120 μL (2.5, 5, 10, 30, 50, 75, 100, 125 and 250 $\mu\text{g/mL}$) of test samples (compound **1-6** and GTF) diluted with DMSO, after three contents were mixed in 96-well plates, pre-incubated for 15 min at 37°C, the reaction was initiated by the addition of 20 μL of 10 mM substrate (pNPG) diluted with PBS (pH=6.8), for 30 min of incubation at 37°C and addition of 80 μL of sodium carbonate solution to terminate the reaction. Acarbose was used as positive control. Absorbance was measured at 405 nm using Tecan Infinite M200 Pro Multimode reader (Tecan, Männedorf, Switzerland). The inhibitory percentage rate was calculated by the following equation:

$$\text{Inhibition \%} = 1 - (A_{\text{Test}} - A_{\text{Background}}) / A_{\text{Blank}} \times 100\%$$

A_{Test} : The absorbance of test [enzyme + Sample (acarbose or compound **1-7** or GTF) + pNPG]

$A_{\text{Background}}$: The absorbance of background (PBS + Sample + pNPG)

A_{Blank} : The absorbance of blank (enzyme + DMSO + pNPG)

IC₅₀ values of the samples were calculated by SPSS 16.0 software bundle (IBM, Chicago, USA).

The 7 compounds and GTF were evaluated preliminarily for α -glucosidase (yeast) inhibitory activities with p-nitrophenyl glucopyranoside (pNPG) as the substrate and acarbose as the positive control. The results are presented in Figure 2.

Figure 2. The effects of compound **1-7**, BTFL and acarbose concentration on the rate of α -glucosidase inhibition.

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