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Bioassay-guided purification of α-amylase, α-glucosidase inhibitors and DPPH radical scavengers from roots of *Rheum turkestanicum*

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ABSTRACT

Rheum turkestanicum has been used in Iranian folk medicine to treat diabetes mellitus. The present study was designed to isolate bioactive phytochemicals from the roots of *R. turkestanicum* using "bioassay-guided fractionation and purification" method. The α-amylase, α-glucosidase and DPPH inhibitory activities of various extracts (*n*-hexane, dichloromethane, ethyl acetate, methanol and water) were evaluated. Also, the total phenolic contents of the extracts were evaluated and compared to their activities. Among all the extracts, ethyl acetate extract, as the most effective agent, was selected for isolation and identification of its active phytochemicals. The ethyl acetate extract was fractionated by column chromatography and all the fractions were assessed by α-amylase, α-glucosidase, DPPH, and total phenolic assays. As a result, chrysophanol, physcion, emodin, daucosterol and rhododendrin (betuloside) were isolated and identified from the most bioactive fractions by ^{1}H-, ^{13}C-, 2D-NMR, EI-MS and single-crystal X-ray diffraction. Also, the highest inhibitory activities were exerted by daucosterol with IC_{50} values of 46.4 and 17.0 μM (similar to that of acarbose with IC_{50} values of 47.4 and 16.4 μM) against α-amylase and α-glucosidase, respectively. Moreover, emodin and rhododendrin showed significant inhibitory activities against α-glucosidase with IC_{50} values of 42.5 μM and 77.9 μM, respectively. Also, rhododendrin exhibited high antioxidant activity against DPPH radicals (IC_{50} = 80.4 μM), more potent than that of BHT (IC_{50} = 95.7 μM), as a commercial antioxidant. The overall results suggested that *R. turkestanicum* roots can be considered as a source of bioactive phytochemicals for developing novel lead compounds in drug design.

1. Introduction

Blood glucose is mainly obtained by the hydrolysis of dietary polysaccharides such as starch. Pancreatic α-amylase and intestinal α-glucosidase are key enzymes in the digestive system that catalyze digestion of starch by hydrolyzing the α-1,4-glucoside linkages. The inhibition of these enzymes significantly delays carbohydrate digestion, prolongs the overall carbohydrate digestion time, and thus reduces the rate of glucose absorption in the type 2 diabetes mellitus patients (Bischoff, 1995). Current α-amylase and α-glucosidase inhibitors such as acarbose, miglitol and voglibose are widely used for the treatment of patients with type 2 diabetes, but it was also reported that they cause various side-effects such as abdominal distention, meteorism, bloating and diarrhea. Therefore, safer natural α-glucosidase inhibitors are desired (Van de Laar et al., 2005).

Several studies have demonstrated that oxidative stress play an accelerated role in development of complications in diabetic mellitus (Rahimi et al., 2005). Moreover, high blood glucose concentration in diabetes patients might have cause to increase oxidative stress by auto-oxidation of glucose (Pazdro and Burgess, 2010). Therefore, antioxidants can decrease diabetes complications by reducing oxidative stress and be helpful for prevention of diabetes mellitus.

Medicinal plants and their active constituents have been shown to exert antioxidant and anti-diabetic activities in various studies (Lin et al., 2016; Krishnaiah et al., 2011). Many secondary metabolites, such as flavonoids, alkaloids, terpenoids, anthocyanins, glycosides, phenolic compounds, and other types of phytochemicals, have been reported as antioxidant and glucosidase inhibitors (Kumar et al., 2011; Carocho and Ferreira, 2013).

Rheum (rhubarb) species, from Polygonaceae family, are significant medicinal plants in Iranian, Chinese and Indian traditional medicine (Ahvazi et al., 2012; Wink and van Wyk, 2004). These species are usually used as laxative, antibacterial and anti-gastrointestinal agents (Das Prajapati, 2003). *Rheum turkestanicum* is one of the three native *Rheum* species in Iran (*R. ribes*, *R. persicum* and *R. turkestanicum*) that grows in central Asia and also in north-eastern regions of Iran (Ahvazi

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et al., 2012). The roots of the plant have traditionally been used as a folk medicine with strong anti-diabetic and anticancer activities and have also been used as agents to reduce blood pressure (Shiezadeh et al., 2013).

As a part of ongoing project on screening of medicinal plants used as anti-diabetic agents in Iranian folk medicine (Salehi et al., 2013), the inhibitory effect of the plants on α-amylase and α-glucosidase were evaluated. It was revealed that the roots of *R. turkestanicum* possessed significant activities. This high potency prompted us to further investigation on *R. turkestanicum*. The objective of the study was the bioassay-guided isolation of α-amylase, α-glucosidase inhibitors and DPPH radical scavengers from the roots of *R. turkestanicum*. The study was designed to evaluate the inhibitory activities of *n*-hexane, dichloromethane, ethyl acetate, methanol and water extracts of roots of *R. turkestanicum* and to fractionate the most active extract using column chromatography. The inhibition of α-amylase and α-glucosidase by obtained fractions was evaluated, and additionally the active phytochemicals were purified and identified by column chromatography, nuclear magnetic resonance (NMR), mass spectrometry (MS) and single-crystal X-ray crystallography. Furthermore, the correlation of enzymes inhibitory activities, antioxidant activity and the amount of phenolic compounds of the extracts, fractions and isolated phytochemicals were examined. This study is the first report of the isolation of phytochemicals from *R. turkestanicum*.

2. Materials and methods

2.1. General

Dimethyl sulfoxide (DMSO), *n*-hexane, dichloromethane, chloroform, ethyl acetate, methanol, $CDCl_3$, DMSO-d_6, CD_3OD, silica gel (for column chromatography) and Phosphomolybdic acid were purchased from Merck (Germany). α-Amylase, α-glucosidase, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 3,5-dinitrosalicylic acid (DNS), *p*-nitrophenyl α-D-glucopyranoside (PNPG) were purchased from Sigma-Aldrich (Germany). ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Avance III (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C) NMR spectrometer, using TMS as an internal standard. 2D-NMR (HMQC) spectra were obtained on a Bruker Avance III (500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C) NMR spectrometer, using TMS as an internal standard. The X-ray diffraction measurements were made on a STOEI-PDS-II diffractometer with graphite-monochromated Cu/Kα radiation. EI-MS spectra were recorded on an Agilent 5975C system with a quadrupole analyzer and with scan range between *m/z* 50–700. Silica gel (230–400 mesh, Merck, Germany) was used for column chromatography. TLC analysis was conducted on silica gel 60 F_{254} pre-coated plates (Merck, Germany) and the spots were detected by the use of UV light (254 and 366 nm) and also by 3% phosphomolybdic acid (in ethanol) followed by heating (100 °C). The mobile phase varies according to the polarity and nature of the samples, using different solvents (*n*-hexane, dichloromethane, chloroform, ethyl acetate and methanol). Absorbance of the samples in assays were determined by using Biotek power wave XS2 spectrophotometer.

2.2. Plant material

The roots of *R. turkestanicum* were collected in June 2012 from Chenar, Kalat Country, Razavi Khorasan Province, Iran. The plant was identified by M. R. Joharchi as voucher specimen of 42082 in Ferdowsi University of Mashhad Herbarium.

2.3. Extraction and isolation

The dried roots of *R. turkestanicum* were grounded prior for extraction. Sequential extraction was designed by using different solvents with different polarities in 5 steps, starting with the most nonpolar solvent (*n*-hexane, dichloromethane, ethyl acetate, methanol and water, respectively). Step 1: The dried and fine roots (3.8 kg) was extracted with 8 L *n*-hexane (24 h × 2) in a maceration tank. Step2: The residue obtained in step 1 was extracted by 8 L of dichloromethane (24 h × 2). Step 3: The residue obtained from step 2 was extracted by 8 L of ethyl acetate (24 h × 2). Step 4: By addition of 8 L methanol to the dried plant's residue from step 3 (24 h × 2), methanol extract was obtained. Step 5: The residue obtained in step 4 was extracted by 8 L of water (24 h × 2). The extracts were filtered and subsequently evaporated using rotary evaporator under reduced pressure at 40 ° C. The extraction yield was calculated as the ratio of the dry extract weight to the dry starting material weight multiplied by 100.

The ethyl acetate extract (90 g) was subjected to column chromatography over silica gel (800 g, mesh: 230–400) using a gradient of chloroform: methanol (1:0 to 0:1). The isolated fractions were screened by TLC and the ones with similar compositions were pooled to obtain 17 combined fractions (F1-F17).

Fraction F1 (800 mg) was subjected to a silica gel column chromatography (130 g, mesh: 70–230) eluted with a gradient of *n*-hexane: chloroform (50:50 to 25:75) to give 5 fractions (F1-1 to F1-5). Fraction F1-1 was recrystallized in *n*-hexane-chloroform (1:1) to give compound **1** (85 mg) in a pure form. From F1-3, crude crystals were obtained, which were recrystallized from *n*-hexane: chloroform (1:3) to obtain compound **2** (57 mg). Fraction F5 (0.80 g) was washed with methanol to separate impurities, which was recrystallized from chloroform to obtain compound **3** (21 mg). Fraction F9 (0.51 g) was washed with methanol and acetone to separate impurities, which was recrystallized from chloroform: methanol (3:1) to obtain compound **4** (45 mg). From fraction F13 (15.3 g), a crude solid was obtained, which was washed with *n*-hexane and acetone, and was recrystallized from methanol: water (1:1) to give compound **5** (11.8 g).

2.4. Characteristic data of compounds

2.4.1. Chrysophanol (1)

Orange crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 12.11 (1H, s, OH-12), 12.00 (1H, s, OH-8), 7.80 (1H, d, J = 7.2 Hz, H-5), 7.67 (1H, m, H-6), 7.64 (1H, s, H-4), 7.29 (1H, d, J = 8 Hz, H-7), 7.09 (1H, s, H-2), 2.47 (3H, s, CH_3).^{13}C NMR (100 MHz, $CDCl_3$): δ_c192.5 (C-9), 181.9 (C10), 162.7 (C-8), 162.4 (C-1), 149.4 (C-3), 137.0 (C-6), 133.6 (C-11), 133.3 (C-14), 124.6 (C-13), 124.4 (C-7), 121.4 (C-5), 119.9 (C-4), 115.9 (C-12), 113.7 (C-2), 22.3 (CH_3-3); ESI–MS *m/z*: 254 $[M]^+$ (Guo et al., 2011).

2.4.2. Physcion (2)

Orange crystals; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 12.35 (1H, s, OH-1), 12.15 (1H, s, OH-8), 7.66 (1H, s, H-5), 7.39 (1H, d, J = 2.8 Hz, H-4), 7.11 (1H, s, H-7), 6.71 (1H, d, J = 2.8 Hz, H-2), 3.97 (3H, s, OCH_3), 2.48 (3H, s, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_c 190.7 (C-9), 181.3 (C-10), 167.1 (C-3), 163.1 (C-1), 161.6 (C-8), 146.5 (C-6), 134.7 (C-12), 131.5 (C-13), 129.8 (C-2), 123.0 (C-7), 121.7 (C-5), 112.3 (C-14), 110.7 (C-11), 108.2 (C-4), 55.9 (OCH_3-3), 22.3 (CH_3-6); ESI–MS *m/z*: 284 [M] (Basu et al., 2005).

2.4.3. Emodin (3)

Orange crystals; ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 12.06 (1H, s, OH-1), 11.99 (1H, s, OH-8), 7.44 (1H, s, H-5), 7.13 (1H, s, H-4), 7.09 (1H, d, J = 2.4 Hz, H-7), 6.57 (1H, d, J = 2.4 Hz, H-2), 2.40 (3H, s, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ_c 190.1 (C-9), 181.8 (C10), 166.1 (C-3), 164.9 (C-8), 161.9 (C-1), 148.7 (C-6), 135.5 (C-11), 133.2 (C-14), 124.6 (C-7), 120.9 (C-5), 113.8 (C-12), 109.3 (C-13), 109.3 (C-4), 108.3 (C-2), 22.0 (CH_3-6); ESI–MS *m/z*: 270 [M] (Zhang et al., 2007).

2.4.4. Daucosterol (4)

White powder; ^{1}H NMR (400 MHz, DMSO-d_6): δ_H 5.34 (1H, br s, H-