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Identification, quantification and antioxidant activity of acylated flavonol glycosides from sea buckthorn (*Hippophae rhamnoides* ssp. *sinensis*)

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ABSTRACT

A novel acylated flavonol glycoside: isorhamnetin (3-*O*-[(6-*O*-*E*-sinapoyl)-*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside) (**1**), together with two known acylated flavonol glycosides: quercetin (3-*O*-[(6-*O*-*E*-sinapoyl)-*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside) (**2**) and kaempferol (3-*O*-[(6-*O*-*E*-sinapoyl)-*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside) (**3**) were isolated from the *n*-butanol fraction of sea buckthorn (*Hippophae* rhamnoides ssp. *sinensis*) berries for the first time by chromatographic methods, and their structures were elucidated using UV, MS, ^{1}H and ^{13}C NMR, and 2D NMR. Compounds **1**–**3** showed good scavenging activities, with respective IC_{50} values of 8.91, 4.26 and 30.90 μM toward the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical; respective Trolox equivalent antioxidant capacities of 2.89, 4.04 and 2.44 μM μM^{-1} toward 2,2′-azino-bis-3-ethyl-benzothiazoline-6-sulphonate (ABTS) radical. The quantitative analysis of the isolated acylated flavonol glycosides was performed by HPLC–DAD method. The contents of compounds **1**–**3** were in the range of 12.2–31.4, 4.0–25.3, 7.5–59.7 mg/100 g dried berries and 9.1–34.5, 75.1–182.1, 29.2–113.4 mg/100 g dried leaves, respectively.

1. Introduction

Sea buckthorn (*Hippophae* sp.) is a berry-bearing, mainly hardy bush of the family Elaeagnaceae, naturally distributed in Asia and Europe (Rousi, 1971). Sea buckthorn berries have been used as fruits and food materials for a long history and lately as a candidate for functional food ingredient. Besides the berries, the leaves of sea buckthorn are rich in nutritional components and can be steeped to yield a nutritious herbal tea (Beveridge, Li, Oomah, & Smith, 1999). In addition to its nutritional effects, sea buckthorn has been used medicinally, especially in the traditional Tibetan and Mongolian medicines. Since 1950s, many medicinal products of wild and cultivated sea buckthorn have been used in Asian and European countries, particularly in China and Russia (Bal, Meda, Naik, & Satya, 2011; Guliyev, Gulb, & Yildirima, 2004). Previous phytochemical studies indicated that sea buckthorn berries and leaves contain high levels of flavonoids which are known to have many beneficial effects on health. For instance, the total flavonoids from the aqueous ethanol extract of sea buckthorn berries have been clinically used in China since 1980, for the treatment of cardiovascular disorders (Wang, Feng, Yu, Zhang, & Zhu, 1993; Wang et al., 2000). In addition, it was reported that the dietary intake of flavonoids, from fruits and vegetables, may reduce cardiovascular mortality with epidemic proof (Cheng et al., 2003; Eccleston et al., 2002; Hertog, Feskens, Hollman, Katan, & Kromhut, 1993). Flavonol glycosides are an important group of flavonoids in sea buckthorn berries. Several flavonol glycosides, from different origins of sea buckthorn berries, were quantitatively analysed by HPLC–DAD using reference compounds (Chen, Zhang, Xiao, Yong, & Bai, 2007; Yang, Halttunen, Raimo, Price, & Kallio, 2009). These flavonol glycosides were: quecertin 3-*O*-sophoroside-7-*O*-rhamnoside, kaempferol 3-*O*-sophoroside-7-*O*-rhamnoside, isorhamnetin 3-*O*-sophoroside-7-*O*-rhamnoside, isorhamnetin 3-*O*-glucoside-7-*O*-rhamnoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside, isorhamnetin 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside and kaempferol 7-*O*-rhamnoside. Four flavonol glycosides were isolated from sea buckthorn pomace by chromatographic methods, and their structures were identified as: isorhamnetin 7-*O*-*α*-L-rhamnoside, isorhamnetin 3-*O*-*β*-D-glucoside-7-*O*-*α*-L-rhamnoside, isorhamnetin 3-*O*-*β*-D-sophoroside-7-*O*-*α*-L-rhamnoside and kaempferol 3-*O*-*β*-D-sophoroside-7-*O*-*α*-L-rhamnoside. In addition to the isolated glycosides, 30 flavonol glycosides were tentatively characterised by HPLC–DAD–ESI-MS^n, including eight flavonol

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derivatives that are acylated by hydroxybenzoic or hydoxycinnamic acids (Rösch, Krumbein, Mügge, & Kroh, 2004). Unfortunately, none of acylated flavonol glycoside was isolated from sea buckthorn berries for unambiguous identification and bioactive evaluation. To further characterise the flavonoids of sea buckthorn, we have investigated acylated flavonol glycosides present in the berry extract of sea buckthorn. Although the detection of acylated flavonol glycosides during LC–MS/MS analysis of plant extracts is relatively straightforward, full characterisation requires isolation of individual constituents as a prerequisite for structure elucidation and further bioactive evaluation. In the present study, effective separation and purification of the complex matrix of acylated flavonol glycosides in sea buckthorn berries was achieved using combined chromatographic methods. On that basis, the antioxidant capacities of the purified acylated flavonol glycosides were determined by measuring the scavenging activities on 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethyl-benzothiazoline-6-sulphonate (ABTS) radicals, and their contents in sea buckthorn berry and leaf samples collected from different natural growth sites were quantitatively analysed as well.

2. Materials and methods

2.1. Instruments

NMR spectra were acquired in DMSO-d_6 at 25 °C on a Varian Unity Inova 400 NMR spectrometer (Palo Alto, CA, USA), and standard pulse sequences and parameters were used to obtain ^{1}H and ^{13}C NMR, and 2D NMR spectra. The ESI-MS^n data were acquired on a Thermo Finnigan LCQ^{DECA} system (San Jose, CA, USA), and the operation parameters were as follows: nebulizer sheath gas, N_2 (80 uit); nebulizer auxiliary gas, N_2 (20 uit); capillary temperature, 300 °C; capillary voltage, −13 V; spray voltage, 4.5 kV; lens voltage, 18 V. The HR-ESI-MS spectra were recorded using a Bruker micrOTOF-Q II mass spectrometer (Bremen, Germany), and the ESI conditions were set as follows: ion polarity, positive; capillary voltage, 4500 V; end plate offset voltage, −500 V; collision cell RF, 550 Vpp; nebulizer pressure, 0.3 bar; dry heater, 180 °C; dry gas flow, 4.0 L/min. UV–Vis absorbance was measured on a Jingke UV759S spectrophotometer (Shanghai, China). GC analysis was conducted on an Agilent 7890A GC–FID instrument (Palo Alto, CA, USA). HPLC analysis was performed on a Waters ACQUITY UHPLC system equipped with a photodiode array detector (Milford, MA, USA).

2.2. Chemicals and reagents

Materials for column chromatography were HPD-100 macroporous adsorption resin (Baoen Chemical Co., Cangzhou, China), YMC-Pack ODS-A (50 μm; YMC, Kyoto, Japan) and Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). 2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethyl-benzothiazoline-6-sulphonate (ABTS) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ascorbic acid and quercetin were obtained from National Institute of the Control of Pharmaceutical & Biological Products (Beijing, China). HPLC grade acetonitrile was purchased from TEDIA Co. (Fairfield, OH, USA). Deionized water was obtained from a Milli-Q water system (Bedford, MA, USA). All other chemicals and solvents used in this study were of analytical grade or better.

2.3. Plant materials

For the isolation and identification of the acylated flavonol glycosides, the sea buckthorn berries were harvested at Xiaojin County, Sichuan Province of China in November 2005. For quantitative analysis of the acylated flavonol glycosides, the berry and leaf samples were collected from different natural growth sites (longitudes 102°38′E–105°02′E, latitudes 31°57′N–34°22′N, altitudes 2000–3100 m) in Sichuan and Gansu Provinces of China in September 2011. Berry and leaf samples sharing the same code were collected from an identical plant. According to the morphological characterisation and microscopic analysis (Chen, Ma, Sun, & Lian, 2003; Lian & Chen, 1996), the original plants of collected samples were identified as *Hippophae rhamnoides* ssp. *sinensis*. The reference specimens were deposited at the Department of Pharmacognosy, West China School of Pharmacy, Sichuan University.

2.4. Extraction and isolation

The fresh berries of *H. rhamnoides* ssp. *sinensis* (5 kg) were extracted with 95% EtOH (3 × 10 L) at room temperature. After solvent evaporation in vacuo (<40 °C), the residue was suspended in H_2O. The suspension was successively partitioned with petroleum ether, EtOAc and *n*-BuOH. The dried *n*-BuOH extract (60 g) was subjected to an HPD-100 macroporous adsorption resin column previously equilibrated with H_2O. After the column was rinsed with H_2O (discarded), fractions were collected by increasing the EtOH content of the eluent (15%, 30%, 50%, 70%, 95%, v/v, successively). The dried residue of the 15% ethanol-eluted fraction was applied to a RP-C18 column (50 μm, 230 mm × 49 mm i.d.) and eluted with 10–50% aq. MeOH. Collected fractions were combined based on their TLC profiles. The acylated flavonol glycosides were mainly eluted with 30% aq. MeOH. Then the further separation was carried out on a Sephadex LH-20 column (600 × 25 mm i.d.) using 60% aq. MeOH as the mobile phase to yield pure compound **1** (32 mg) and a mixture of compounds **2** and **3**. The mixture was separated on a Kromasil semi-preparative HPLC C18 column (5 μm, 250 mm × 10 mm i.d.) eluting with acetonitrile–H_2O (18:82, v/v) at a flow rate of 4 ml/min. The eluates were monitored by measuring the absorbance at 260 nm. After solvent evaporation, pure compounds **2** (28 mg) and **3** (24 mg) were yielded by lyophilization, respectively.

To check the purity of the isolated acylated flavonol glycosides, thin layer chromatography (TLC) analysis were carried out on polyamide films using two developing agents, respectively: H_2O–EtOH–2-butanone–acetylacetone (30:3:3:1, v/v/v/v) and $CHCl_3$–Me_2CO–MeOH–HAc (10:4:3:1, v/v/v/v). After development, the films were visualised under UV light at 365 nm using 1% $AlCl_3$–EtOH solution as the spray reagent for flavonoids.

2.5. Acid hydrolysis

A solution of each compound (4 mg) dissolved in 1 N HCl (2 ml) was heated at 90 °C for 2 h and then extracted with EtOAc to remove the flavonol aglycone. The resulting monosaccharides were treated with 1-(trimethylsilyl)imidazole and analysed by GC. Peaks of the hydrolysate were detected by comparing the retention times with those of the authentic trimethylsilylated sugars.

2.6. Spectrometric identification

2.6.1. Isorhamnetin 3-O-[(6-O-E-sinapoyl)-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranosyl-7-O-α-L-rhamnopyranoside (*1*)

UV: 245, 269 sh, 334 nm; The ESI-MS spectrum gave a quasi-molecular ion at *m*/*z* 1015 $[M+Na]^+$, fragment ions at *m*/*z* 869 $[(M+Na)\text{-Rha}]^+$, *m*/*z* 699 $[(M+Na)\text{-isorhamnetin}]^+$, *m*/*z* 553 $[(M+Na)\text{-Rha-isorhamnetin}]^+$, *m*/*z* 535 $[(M+Na)\text{-Rha-isorhamnetin-}H_2O]^+$, *m*/*z* 501 $[(M+Na)\text{-Rha-sinapoylGlc}]^+$; The HR-ESI-MS gave, *m*/*z*: 1015.2708 $[M+Na]^+$, calcd. for $C_{45}H_{52}O_{25}Na$ *m*/*z*