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Flavonoid glycosides from seeds of *Hippophae rhamnoides* subsp. *Sinensis* with α-glucosidase inhibition activity

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ABSTRACT

Hippophae rhamnoides subsp. *Sinensis* is a famous traditional medicinal plant in Tibet and Mongolia of China. Three novel flavonoid glycosides and ten known analogues were obtained from the seeds of *H. rhamnoides*. The structures of new compounds were elucidated by spectroscopics, chemical methods as well as literature data. In vitro assay, compounds **5–9**, kaempferol and 70% ethanolic elution fraction showed prominent α-glucosidase inhibitory activities with IC_{50} values ranging from 8.30 to 112.11 μM, better than that of the positive control, acarbose, whose IC_{50} value was 1727.07 μM.

1. Introduction

α-Glucosidase inhibitors are a class of oral hypoglycemic agents that delay the absorption of intestinal carbohydrates (such as starch and table sugar) to treat diabetes. They can competitively inhibit various α-glucosidases, and then retard the decomposition of starch into glucose. Thereby they can slow down the absorption of glucose in intestine to reduce postprandial hyperglycemia. α-Glucosidase inhibitors are relatively mature drug for the treatment of diabetes, but synthetic α-glucosidase inhibitors (e.g., acarbose, miglitol and voglibose), exhibit adverse side effects, including headaches, gastrointestinal disorders, fatigue, insomnia, and vertigo. On the other hand, a number of herbal medicines have been reported to possess potential α-glucosidase inhibitory activity along with low toxicity and high activity to treat diabetes. Clinical studies have demonstrated that polyphenol-rich (such as phenolic acids, flavonoids, anthocyanins, tannins and quinones) diets exhibit α-glucosidase inhibitory activity [1,2].

Seabuckthorn (*Hippophae rhamnoides* L.), belongs to the Elaeagnaceae family, which is naturally distributed across the Eurasian continent. *Hippophae rhamnoides* subsp. *Sinensis* is famous for being used as a medicine material in Tibet and Mongolia of China. Earlier pharmacological investigations have shown that the seeds of *H. rhamnoides* had a variety of bioactivities, including treating complications in diabetes, protecting cardiovascular system, anti-oxidation, antibacterial and immunomodulatory [3]. Flavonoids, as one of the bioactive constituents in *H. rhamnoides*, can treat diabetes by inhibiting of α-glucosidase [4,5]. As a part of our ongoing investigation on bioactive components from *H. rhamnoides* [6–10], three novel flavonoids, named hippophin N−P (**1–3**) along with ten known compounds were identified (Fig. 1). This paper reports the isolation and structural elucidation of these flavonoid glycosides and the results of their inhibitory activities against α-glucosidase.

2. Experimental

2.1. General experimental procedures

Optical rotation was determined in MeOH on a Rudolph Autopol IV-T polarimeter. NMR spectra (^{1}H NMR, ^{13}C NMR, DEPT, HSQC, HMBC, H−H COSY, TOCSY and NOESY experiments) were obtained with a Bruker-400 instrument (at 400 MHz for ^{1}H and 100 MHz for ^{13}C) or a Varian INOVA-500 instrument (at 500 MHz for ^{1}H and 125 MHz for ^{13}C) in DMSO-d_6 relative to TMS as internal standard. HR-MS were performed on a Waters Xevo G2-XS Q-TOF mass spectrometer. Semipreparative HPLC was carried out on an Agilent 1100 HPLC equipped with a C_{18} column (Waters XBridge BEH C_{18}, 5 μm, 10 × 250 mm). HPLC was conducted on an Agilent 1260 HPLC with C_{18} column (Intersil ODS-3, 5 μm, 4.6 × 250 mm or Agilent Extend-C_{18}, 5 μm, 4.6 × 250 mm). Flash column chromatography was performed on a Biotage Isolera one with a Flash-ODS column (Biotage SNAP Cartridge KP-C_{18}-HS 30 g). TLC was carried out with silica gel $HSGF_{254}$ (Yantai Jiangyou Guijiao Kaifa Co., Ltd., Yantai, China). Column

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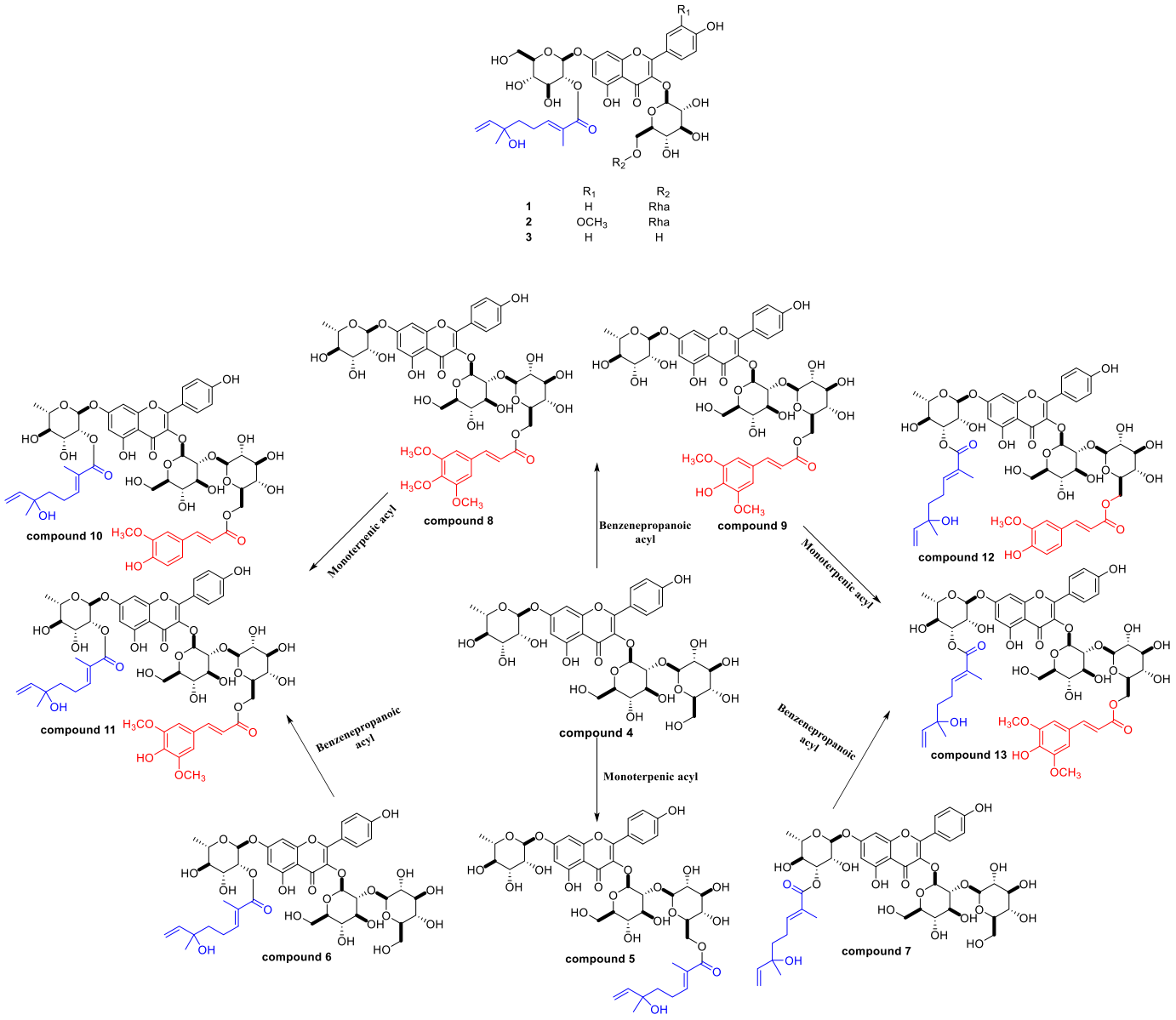

Fig. 1. The structures of compounds **1–13**.

chromatography (C.C.) was performed on macroporous resin (D101; Cangzhou Bon Adsorber Technology Co., Ltd., Cangzhou, China), silica gel (100–200 mesh; Shanghai Sanpont Co., Ltd., P. R. China) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Spectrophotometric measurements for the tyrosinase inhibition assay were taken on a Synergy 2 multi-mode microplate reader (Bio-Tek Instruments Inc.). All solvents were used with HPLC grade (Adamas-beta Co., Ltd., China).

2.2. Plant material

The seeds of *H. rhamnoides* were collected from Datong County of Qinghai Province, China, in July 2017, and identified by Dr. Min-Sheng Cao of Qinghai General Health Bio-science Co., Ltd. A voucher specimen (SIPITCM-1707001) has been deposited at the Shanghai Institute of Pharmaceutical Industry.

2.3. Sample preparation and isolation

The seed residues of *H. rhamnoides,* which had been extracted through supercritical fluid extraction, were obtained from Qinghai General Health Bio-Science Co., Ltd. (Xining, Qinghai, China). The air-dried seed residues (40 Kg) were extracted twice with 70% EtOH for 1 h and concentrated to give an extract (3.5 Kg). The extract passed through a D101 macropourous resin column (20 Kg), and eluted with EtOH-H_2O (0:100, 4 CV; 30:70, 4 CV; 70:30, 4 CV; 95:5, 4 CV) to yield four fractions (Fr.1–4). Fr.3 (500 g), the 70% ethanol elution fraction, was subjected to C.C. on Silica gel column (10 × 120 cm), and eluted gradiently with CH_2Cl_2-MeOH-H_2O (9:1:0.1, 8:2:0.1, 7:3:0.5, and 6:4:1; each 80 L) to yield 13 fractions (Fr. 3A– 3 M) according to TLC. Fr. 3E (10 g) was purified by C.C. (Sephadex LH-20; H_2O) and preparative HPLC [11% MeCN (0.1% formic acid), 5.0 mL/min, UV detection at λ 254 nm] to afford compound **4** (326 mg, t_R = 20 min). Fr. 3D (23 g) was purified by Sephadex LH-20 [CH_3OH-H_2O (0:100 → 100:0)] to give 9 fractions (Fr. 3D1-3D9). Fr. 3D1 and Fr. 3D8 were further purified by Flash-ODS [CH_3OH-H_2O (0:100 → 100:0), UV detection at λ 254 nm] to yield 6 fractions (Fr. 3D1a-3D1f) and 8 fractions (Fr. 3D8a-3D8h), responsibility. Fr. 3D1b was purified by preparative HPLC [11% MeCN (0.1% formic acid), 2.0 mL/min, UV detection at λ 254 nm] to obtain compound **9** (18 mg, t_R = 20 min). Fr. 3D1e was purified by preparative

Table 1
^{1}H NMR spectral data of compounds **1–2**, **6** (400 MHz) and **3** (500 MHz) [in DMSO-d_6, TMS, δ (ppm) (J = Hz)].

Position	1	2	3	6
6	6.35 (1H, s)	6.41 (1H, d, J = 2.0 Hz)	6.35 (1H, s)	6.48 (1H, s)
8	6.74 (1H, s)	6.81 (1H, d, J = 2.0 Hz)	6.75 (1H, s)	6.90 (1H, s)
2′	8.03 (1H, d, J = 8.4 Hz)	7.92 (1H, d, J = 2.0 Hz)	8.06 (1H, d, J = 8.5 Hz)	8.09 (1H, d, J = 8.8 Hz)
3′	6.91 (1H, d, J = 8.4 Hz)		6.90 (1H, d, J = 8.5 Hz)	6.91 (1H, d, J = 9.2 Hz)
5′	6.91 (1H, d, J = 8.4 Hz)	6.98 (1H, d, J = 8.4 Hz)	6.90 (1H, d, J = 8.5 Hz)	6.91 (1H, d, J = 9.2 Hz)
6′	8.03 (1H, d, J = 8.4 Hz)	7.62 (1H, dd, J = 8.4,1.6 Hz)	8.06 (1H, d, J = 8.5 Hz)	8.09 (1H, d, J = 8.8 Hz)
-OCH_3		3.90 (3H, s)		
Glc I				
1″	5.36 (1H, d, J = 7.2 Hz)	5.51 (1H, d, J = 7.2 Hz)	5.47 (1H, d, J = 7.5 Hz)	5.70 (1H, m)
2″	3.19 (1H, m)	3.30 (1H, m)	3.17 (1H, m)	3.51 (1H, m)
3″	3.21 (1H, m)	3.33 (1H, m)	3.20 (1H, m)	3.54 (1H, m)
4″	3.08 (1H, m)	3.11 (1H, m)	3.05 (1H, m)	3.17 (1H, m)
5″	3.24 (1H, m)	3.48 (1H, m)	3.08 (1H, m)	3.54 (1H, m)
6″	3.69 (1H, m), 3.31 (1H, m)	3.75 (1H, m), 3.41 (1H, m)	3.58 (1H, m), 3.35 (1H, m)	3.29 (1H, m), 3.49 (1H, m)
Rha				
1‴	4.40 (1H, s)	4.48 (1H, s)		5.71 (1H, br.s)
2‴	3.42 (1H, m)	3.46 (1H, m)		5.11 (1H, m)
3‴	3.11 (1H, m)	3.14 (1H, m)		4.09 (1H, m)
4‴	3.09 (1H, m)	3.12 (1H, m)		3.87 (1H, m)
5‴	3.28 (1H, m)	3.31 (1H, m)		3.58 (1H, m)
6‴	0.99 (3H, d, J = 6 Hz)	1.03 (3H, d, J = 6.4 Hz)		1.19 (3H, s)
Glc II				
1⁗	5.44 (1H, d, J = 8.0 Hz)	5.48 (1H, d, J = 8.0 Hz)	5.44 (1H, d, J = 8.0 Hz)	4.62 (1H, d, J = 7.6 Hz)
2⁗	4.87 (1H, t, J = 8.8 Hz)	4.92 (1H, t, J = 9.2 Hz)	4.86 (1H, t, J = 8.8 Hz)	3.10 (1H, m)
3⁗	3.58 (1H, m)	3.62 (1H, m)	3.56 (1H, m)	3.14 (1H, m)
4⁗	3.30 (1H, m)	3.34 (1H, m)	3.29 (1H, m)	3.17 (1H, m)
5⁗	3.42 (1H, m)	3.60 (1H, m)	3.55 (1H, m)	3.14 (1H, m)
6⁗	3.74 (1H, m), 3.53 (1H, m)	3.80 (1H, m), 3.55 (1H, m)	3.73 (1H, m), 3.49 (1H, m)	3.33 (1H, m), 3.58 (1H, m)
MT				
3⁗′	6.69 (1H, t, J = 7.2 Hz)	6.74 (1H, t, J = 7.6 Hz)	6.68 (1H, t, J = 7.2 Hz)	6.79 (1H, t, J = 7.4 Hz)
4⁗′	2.11 (2H, m)	2.15 (2H, m)	2.09 (2H, m)	2.20 (2H, m)
5⁗′	1.44 (2H, t, J = 8.0 Hz)	1.49 (2H, m)	1.43 (2H, t, J = 8.0 Hz)	1.53 (2H, t, J = 8.2 Hz)
7⁗′	5.81 (1H, dd, J = 17.6, 10.8 Hz)	5.86 (1H, dd, J = 17.2, 10.4 Hz)	5.80 (1H, dd, J = 17.5,11.0 Hz)	5.89 (1H, dd, J = 17.2, 10.4 Hz)
8⁗′	5.12 (1H, d, J = 17.2 Hz) , 4.93 (1H, d, J = 10.4 Hz)	5.17 (1H, dd, J = 17.2, 2.0 Hz) , 4.98 (1H, dd, J = 10.4, 2.0 Hz)	5.13 (1H, d, J = 17.5 Hz) , 4.92 (1H, d, J = 10.5 Hz)	5.18 (1H, dd, J = 17.6, 2.0 Hz), 4.99 (1H, dd, J = 10.8, 2.0 Hz)
9⁗′	1.75 (3H, s)	1.80 (3H, s)	1.74 (3H, s)	1.80 (3H, s)
10⁗′	1.12 (3H, s)	1.17 (3H, s)	1.10 (3H, s)	1.19 (3H, s)

HPLC [25% MeCN (0.1% formic acid), 5.0 mL/min, UV detection at λ 270 nm] to obtain compounds **1** (35 mg, t_R = 32 min), **2** (20 mg, t_R = 33 min), **3** (11 mg, t_R = 36 min), **5** (48 mg, t_R = 17 min), **7** (24 mg, t_R = 48 min) and **8** (10 mg, t_R = 19 min). Fr. 3D1f was purified by preparative HPLC [29% MeCN (0.1% formic acid), 5.0 mL/min, UV detection at λ 270 nm] to obtain compounds **6** (20 mg, t_R = 28 min). Fr. 3D8e was purified by preparative HPLC [31% MeCN (0.1% formic acid), 2.0 mL/min, UV detection at λ 254 nm] to obtain compounds **10** (26 mg, t_R = 24 min), **11** (12 mg, t_R = 26 min), **12** (4 mg, t_R = 18 min) and **13** (4 mg, t_R = 16 min).

2.3.1. Hippophin N (=kaempferol-3-O-β-D-rutinosyl-7-O-{2-O-[(2E)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]}-β-D-glucoside; 1)

Yellow amorphous powder. [α_D^{25}] - 9.600 (c 0.125, MeOH); UV λ_{MeOH} nm: 265.1, 330.7; negative ESI-Q-TOF-MS (m/z): 921.3072 [M - H]$^-$ (calcd. For $C_{43}H_{53}O_{22}^-$, 921.3028, error 4.8 ppm); ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) spectroscopic data see Tables 1 and 2.

2.3.2. Hippophin O (=isorhamnetin-3-O-β-D-rutinosyl-7-O-{2-O-[(2E))-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]}-β-D-glucoside; 2)

Yellow amorphous powder. [α_D^{25}] - 9.709 (c 0.103, MeOH); UV λ_{MeOH} nm: 254.4, 355.9; negative ESI-Q-TOF-MS (m/z): 951.3160 [M - H]$^-$ (calcd. For $C_{44}H_{55}O_{23}^-$, 951.3134, error 2.7 ppm); ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) spectroscopic data see Tables 1 and 2.

2.3.3. Hippophin P (=kaempferol-3-O-β-D-glucosyl-7-O-{2-O-[(2E)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]}-β-D-glucoside; 3)

Yellow amorphous powder. [α_D^{25}] - 3.704 (c 0.054, MeOH); UV λ_{MeOH} nm: 265.1, 349.9; negative ESI-Q-TOF-MS (m/z): 775.2446 [M - H]$^-$ (calcd. For $C_{37}H_{43}O_{18}^-$, 775.2449, error 6.8 ppm); ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) spectroscopic data see Tables 1 and 2.

*2.4. Hydrolysis of **1–3***

Compounds **1–3** (10 mg each) were separately dissolved in 1% aqueous $NaHCO_3$ (10 mL) and then heated at 90 °C for 6 h. To get the flavonoid glycosides which lose the monoterpenyl moiety, the reaction mixture was purified by Flash-ODS after cooling. Then 2 mol/L HCl solution (2 mL) was added to the sample solution and heated at 90 °C for 5 h (Fig. 2). When cooling, the mixture was neutralized with $NaHCO_3$, and then dried by vacuum concentration to get the sample.

The identification of sugars was used PMP (1-phenyl-3-methyl-5-pyrazolone) derivatization in comparison with glucose and rhamnose standards (Institute for the Control of Pharmaceutical and Biological Products of China, China) [11]. The sample (5 mg) was dissolved in PMP (0.5 mL of 0.5 mol/L) and NaOH solution (0.5 mL of 0.3 mol/L) and then heated at 70 °C for 1 h (Fig. 3). After the reaction was completed, the mixture was neutralized with 0.3 mol/L HCl solution. For clearance of PMP residue, the mixture was partitioned between CH_2Cl_2 and water, and the sugar derivation was isolated from the water layer. Finally, the sugar derivation was analyzed by HPLC under the following conditions: column, Agilent Extend-C_{18} (5 μm, 4.6 × 250 mm); solvent,

Table 2
^{13}C NMR spectral data of compounds **1–2**, **6** (100 MHz) and **3** (125 MHz) [in DMSO-d_6, TMS, δ (ppm) (J = Hz)].

Position	1	2	3	6
2	157.8	157.4	156.9	156.6
3	134.0	133.8	133.4	133.7
4	178.0	178.0	177.6	178.1
5	161.4	161.4	160.9	160.6
6	99.8	99.8	99.1	99.9
7	162.7	162.7	162.1	161.4
8	95.2	95.3	94.5	94.9
9	156.5	156.4	155.9	156.3
10	106.4	106.4	105.9	106.3
1′	121.1	121.2	120.6	121.2
2′	131.5	113.7	130.9	131.5
3′	115.6	147.4	115.1	115.8
4′	160.6	150.2	160.3	160.6
5′	115.6	115.7	115.1	115.8
6′	131.5	122.9	130.9	131.5
-OCH_3		56.1		
Glc I				
1″	101.6	101.5	100.6	98.4
2″	74.6	71.0	74.2	82.8
3″	76.8	76.9	76.4	77.0
4″	70.3	70.5	69.8	69.9
5″	76.8	76.5	77.5	77.0
6″	67.2	67.2	60.8	61.3
Rha				
1‴	101.2	101.3		95.6
2‴	70.8	70.7		72.1
3‴	72.3	72.2		68.7
4‴	70.3	70.5		72.5
5‴	68.7	68.7		70.6
6‴	18.2	18.2		18.4
Glc II				
1⁗	98.1	98.2	97.5	104.5
2⁗	74.0	74.0	73.5	74.9
3⁗	74.3	74.3	73.8	77.5
4⁗	70.2	70.2	69.7	70.2
5⁗	77.8	77.9	77.3	78.0
6⁗	60.9	60.7	60.4	61.3
MT				
1⁗′	166.8	166.8	166.2	167.0
2⁗′	127.1	127.1	126.6	127.1
3⁗′	143.6	143.6	143.0	144.2
4⁗′	23.6	23.6	23.1	23.7
5⁗′	40.9	40.9	40.4	41.0
6⁗′	71.7	71.7	71.2	71.8
7⁗′	146.0	146.1	145.6	146.2
8⁗′	111.8	111.8	111.3	111.8
9⁗′	12.7	12.7	12.2	12.6
10⁗′	28.2	28.2	27.7	28.2

CH_3CN-0.05% phosphate buffer (PH 6.8) =18:82; flow rate, 0.8 mL/min; UV detection, λ 245 nm.

2.5. α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was tested by using a previously described method [2,12]. The 70% ethanol elution fraction and kaempferol (Institute for the Control of Pharmaceutical and Biological Products of China, China) of different concentrations were dissolved in 5% DMSO and others were dissolved in PBS buffer (PH 6.8). Sample solutions were preincubated with α-glucosidase (0.2 U/mL, Sigma Chemical Co., USA) in 96-well plates at 37 °C for 10 min. Then PNPG (4-nitrophenyl-α-D-glucopyranoside, 1 mM/L, Macklin biochemical Co. China) was added to incubate at 37 °C for 20 min. The absorbance at 405 nm was measured immediately using a microplate reader. The percentage inhibition (%) for each sample was calculated using the following equation.

$$\text{Inhibition (\%)} = (1 - (Aa - Ab)/(Ac - Ad)) \times 100$$

where Aa was the absorbance of the sample group (with sample, with enzyme), Ab was the absorbance of the sample control group (with sample, without enzyme), Ac was the absorbance of the control group (with enzyme, without sample), and Ad was the absorbance of blank control group (without sample and enzyme).

The IC_{50} was performed in triplicate and calculated via nonlinear analysis of the dose-response curves using Prism 7.0 software. Acarbose (purity ≥98%, Aladdin Industrial Corporation, China) was used as the positive control for α-glucosidase.

3. Results and discussion

3.1. Compounds identification

Hippophin N (**1**) was obtained as yellow amorphous powder. The molecular formula of **1** was confirmed as $C_{43}H_{54}O_{22}$ on the basis of its negative-ion peak at m/z 921.3072 $[M-H]^-$ (calcd. for $C_{43}H_{53}O_{22}^-$, 921.3028) in the UPLC-ESI-Q-TOF-MS/MS, implying 17 degrees of unsaturation.

^{1}H NMR spectrum displayed signals at low field for a kaempferol skeleton, which was characterized by two aromatic protons [δ_H 6.35 (1H, s) and 6.74 (1H, s)] and AA'BB'-type aromatic protons [δ_H 8.03 (2H, d, J = 8.4 Hz) and 6.91 (2H, d, J = 8.4 Hz)]. In addition, signals of glucose and rhamnose in ^{13}C NMR spectra were showed at δ_C 98.1, 101.2 and 101.6. The ^{1}H NMR further confirmed the presence of one rhamnose at δ_H 0.99 (3H, d, J = 6.0 Hz). Beside the above pieces of evidence, ^{1}H NMR and ^{13}C NMR spectroscopies also displayed the characterization of one monoterpenyl moiety (MT) (Tables 1 and 2) [6,13]. The ^{1}H NMR data showed the presence of an ABX-type aromatic protons [δ_H 5.81 (1H, dd, J = 17.6, 10.8 Hz), 5.12 (1H, d, J = 17.2 Hz) and 4.93 (1H, d, J = 10.4 Hz)], an olefinic proton signal [δ_H 6.69 (1H, t, J = 7.2 Hz)], two methyl signals [δ_H 1.75 (3H, s) and 1.12 (3H, s)] and two methylene signals [δ_H 2.11 (2H, m) and 1.44 (2H, t, J = 8.0 Hz)]. Furthermore, in HMBC spectrum, the anomeric proton signals of sugars at δ_H 5.44 (1H, d, J = 8.0 Hz, H-1‴), 5.36 (1H, d, J = 7.2 Hz, H-1″) and 3.69 (1H, m, H-6″) accordingly displayed the long-rang correlation with the carbon signals at δ_C 162.7 (C-7), 134.0 (C-3) and 101.2 (C-1‴), which suggested that glucose II was located at C-7 of kaempferol, glucose I was connected to C-3 of kaempferol and rhamnose was linked to C-6″ of glucose I (Fig. 4). And also, the anomeric proton signal at δ_H 4.87 (1H, t, J = 8.8 Hz, H-2⁗) accordingly displayed the long-rang correlation with the carbon signal at δ_C 166.8 (C-1⁗′), which suggested that the location of the monoterpenyl moiety was at C-2⁗ of glucose II (Fig. 4). From the above, although **1** had the similar fragments with **6**,

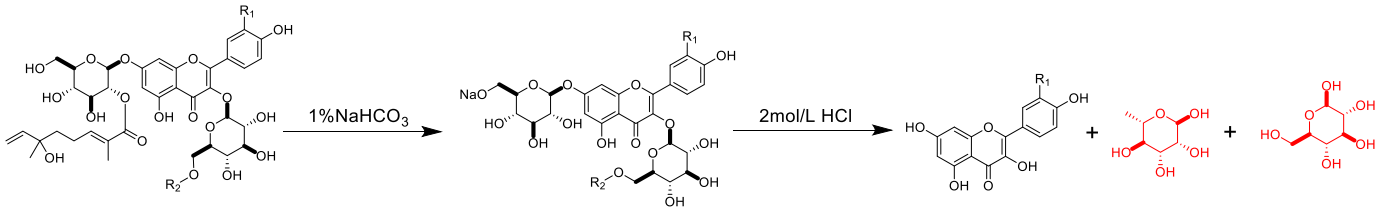

Fig. 2. Hydrolysis of compounds **1–3**.

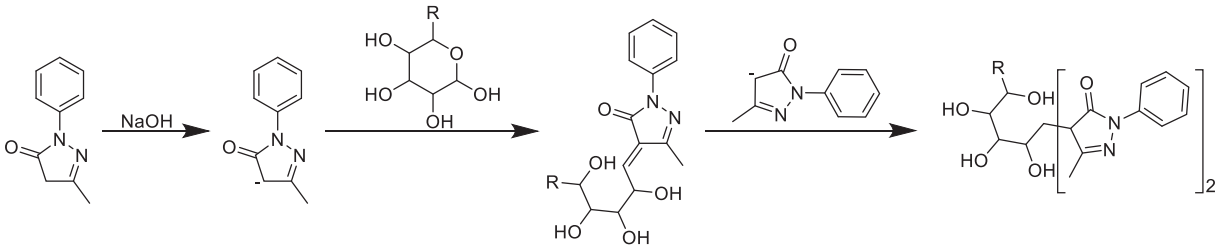

Fig. 3. Derivation process of sugar by PMP derivatization.

there are a little bit different shifts between them at combing of sugars (Tables 1 and 2). Moreover, the signal at δ_H 6.69 (1H, t, J = 7.2 Hz, H-3‴″) indicated the presence of the (*E*)–substituted double bond. Therefore, the monoterpenoic acid moiety of **1** were determined to be (2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoic acid. In the ^{1}H-NMR spectrum, the chemical shifts indicated *β*-configuration [δ_H 5.44 (1H, d, J = 8.0 Hz, H-1‴), 5.36 (1H, d, J = 7.2 Hz, H-1″)] for the two glucopyranosyl group [6,13]. On the acid hydrolysis of **1** with hydrochloric acid, *β*-D-glucose and *α*-L-rhamnose were detected by HPLC in comparison with the authentic samples (See Experimental). Thus, compound **1** was characterized as kaempferol-3-O-*β*-D-rutinosyl-7-O-{2-O-[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]}-*β*-D-glucoside.

Hippophin O (**2**) was obtained as yellow amorphous powder. Its molecular formula was confirmed as $C_{43}H_{54}O_{22}$ on the basis of its negative-ion peak $[M-H]^-$ at m/z 951.3160 (calcd. for $C_{44}H_{55}O_{23}^-$, 951.3134) in the UPLC-ESI-Q-TOF-MS/MS, implying 17 degrees of unsaturation. The ^{1}H NMR data exhibited signals at low field for a isorhamnetin skeleton, which was characterized by two meta-coupled aromatic protons [δ_H 6.41 (1H, d, J = 2.0 Hz) and 6.81 (1H, d, J = 2.0 Hz)], ABX-type aromatic protons [δ_H 6.98 (1H, d, J = 8.4 Hz), 7.62 (1H, dd, J = 8.4, 1.6 Hz) and 7.92 (1H, d, J = 2 Hz)] and methoxy protons [δ_H 3.90 (3H, s)]. The ^{1}H NMR and ^{13}C NMR spectral data of **2** were resembled to those of **1**, except for the isorhamnetin skeleton. Three sugars were identified by HPLC analysis after hydrolysis (see Experimental). The HMBC spectrum displayed glucose II [δ_H 5.48 (1H, d, J = 8.0 Hz, H-1‴)] was connected to C-7 of isorhamnetin (δ_C 162.7, C-7), monoterpenyl moiety (δ_C 166.8, C-1⁗) was linked to C-2‴ of glucose II [δ_H 4.92 (1H, t, J = 9.2 Hz, H-2⁗)], and rhamnose (δ_C 101.3, C-1⁗) was located at C-6″ of glucose I δ_H 3.41 (1H, m, H-6″) (Fig. 2). However, 2D-NMR spectra failed to display the long-rang correlation of the glucose I and C-3 of isorhamnetin. Comparing the NMR data of **2** with **1**, there was no obviously different shift between the H-1″ of glucose I and C-3 of isorhamnetin (Fig. 4) and 1D-NMR spectra of H-1″ of glucose I existed glycosylation shift, which suggested that the location of the glucose I was at C-3 of isorhamnetin. Thus, the structure of compound **2** was assigned as isorhamnetin-3-O-*β*-D-rutinosyl-7-O-{2-O-[(2*E*))-2,6-dimethyl-6-hydroxy-2,7-octadienoyl]}-*β*-D-glucoside.

Hippophin P (**3**) was obtained as yellow amorphous powder. Its molecular formula was confirmed as $C_{37}H_{44}O_{18}$ on the basis of its negative-ion peak $[M-H]^-$ at m/z 775.2446 (calcd. For $C_{37}H_{43}O_{18}^-$, 775.2449) in the UPLC-ESI-Q-TOF-MS/MS, implying 16 degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **3** was resemble of **1**, except for the lacking of a rhamnose. Thus, the structure of compound **3** was assigned as kaempferol-3-O-*β*-D-glucosyl-7-O-{2-O-[(2*E*)-2,6-dimethyl-6-hydroxy −2,7-octadienoyl]}-*β*-D-glucoside.

Compounds **4–13** were identified by compared with literature data, and the ten known compounds were identified as kaempferol-3-O-*β*-D-sophoroside-7-O-*α*-L-rhamnoside (**4**) [14,15], kaempferol-3-O-[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl(1 → 6)]-*β*-D-glucoside(1 → 2)-*β*-D-glucoside-7-O-*α*-L-rhamnoside (Hippophin K; **5**) [6], kaempferol-3-O-*β*-D-sophoroside − 7-O-{[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl(1 → 2)]}-*α*-L-rhamnosider(**6**) [6], kaempferol − 3-O-*β*-D-sophoroside-7-O-{[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl(1 → 3)]}-*α*-L-rhamnoside (**7**) [6], kaempferol-3-O-(6-O-3,4,5-trimethoxycinnamoyl)-*β*-D-glucoside(1 → 2)-*β*-D-glucoside-7-O-*α*-L-rhamnoside (Hippophin M; **8**) [8], kaempferol-3-O-(3-O-trans-sinapoyl)-*β*-D-glucoside(1 → 2)-*β*-D-glucoside-7-O-*α*-L-rhamnoside (**9**) [8], kaempferol-3-O-(6-O-*E*-feruloyl)-*β*-D-glucosyl(1 → 2)-*β*-D-glucoside-7-O-[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl(1 → 2)]-*α*-L-rhamnoside (Hippophin F; **10**) [7], kaempferol-3-O-(6-O-*E*-sinapoyl)-*β*-D-glucosyl(1 → 2)-*β*-D-glucoside − 7-O-[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl (1 → 2)]-*α*-L-rhamnoside (Hippophin D; **11**) [7], kaempferol-3-O-(6-O-*E*-feruloyl)-*β*-D-glucosyl(1 → 2)-*β*-D-glucoside-7-O-[(2*E*)-2,6-dimethyl − 6-hydroxy-2,7-octadienoyl(1 → 3)]-*α*-L-rhamnoside (Hippophin E; **12**) [7] and kaempferol-3-O-(6-O-*E*-sinapoyl)-*β*-D-glucosyl(1 → 2)-*β*-D-glucoside-7-O-[(2*E*)-2,6-dimethyl-6-hydroxy-2,7-octadienoyl(1 → 3)]-*α*-L-rhamnoside (Hippophin C; **13**) [7]. The ^{1}H NMR and ^{13}C NMR data of these compounds were given at supplementary data.

Though we have discovered a series of flavonoids acylated with one monoterpenic acid or phenylpropionic acid in *H. rhamnoides*, **1–3** were first found as different sugar-linked configuration and their MS^2 spectra were also different. The negative ion peak at m/z 737 or 767 ([M–H-184]$^-$) in MS^2 spectra of **1–2** resulted from a loss of monoterpenyl moiety, indicating the presence of McLafferty Rearrangement reaction. Fragments at m/z 593 or 623 ([M–H-184-144]$^-$) and m/z 285 or 315 ([M–H–184–144 -308]$^-$) indicated the loss of sugars. While, MS^2 of the other compounds were similar to **6**, which observed fragmentation [M–H -166]$^-$, [M–H–166-146]$^-$ and [M–H-166–146-324]$^-$. (Fig. 5).

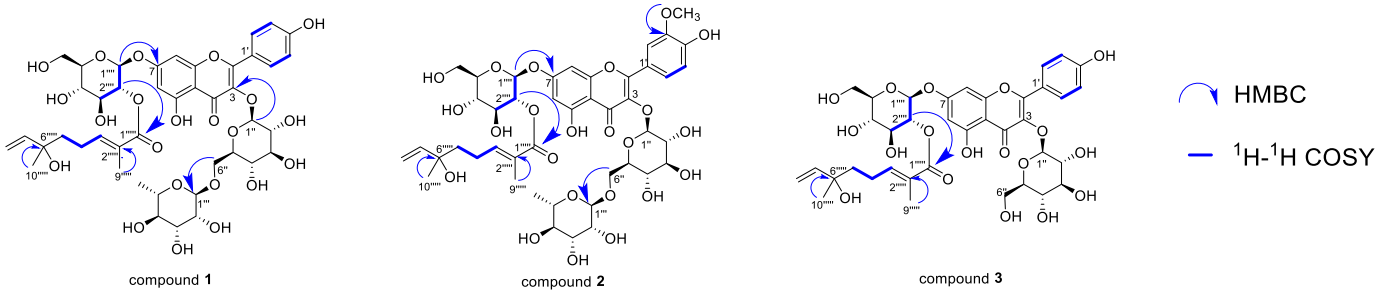

Fig. 4. Key HMBC and ^{1}H–^{1}H COSY correlations for compounds **1–3**.

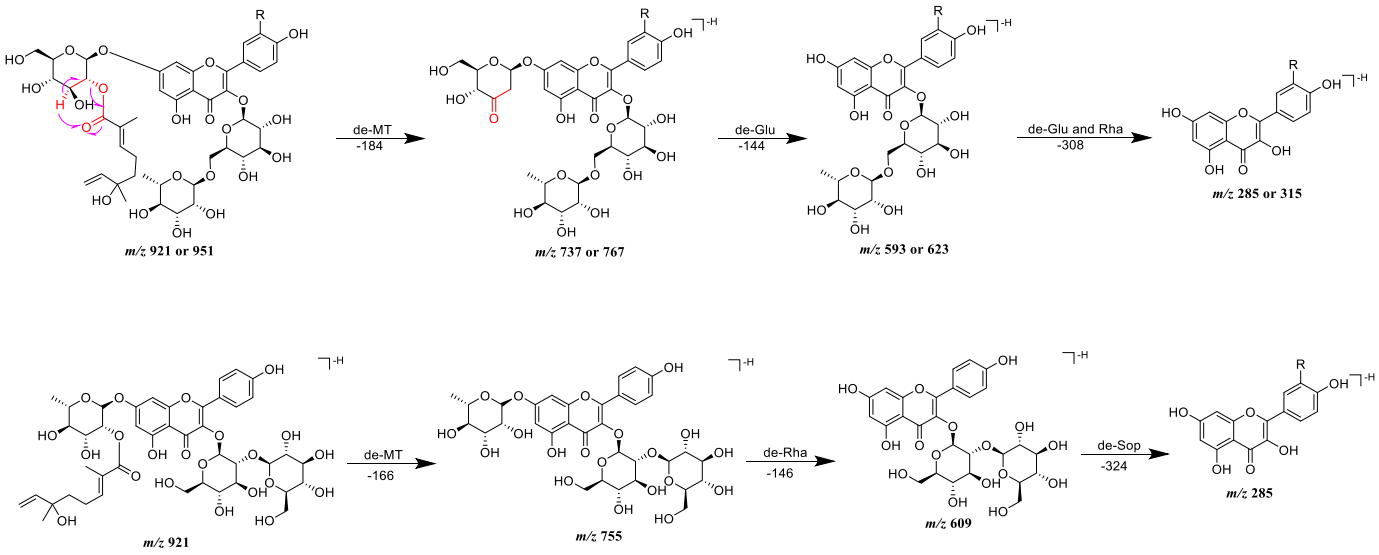

Fig. 5. Key MS fragmentation pathway of compounds **1**, **2** and **6**.

Table 3
α-Glucosidase inhibitory activity of extract and compounds **1–13**.

Compound	IC_{50}	
	(mg/mL)	(μM)
1	> 5	> 5000
2	> 5	> 5000
3	> 5	> 5000
4	> 5	> 5000
5	0.10	112.11 (C.I. 68.19–181.39)
6	0.049	53.20 (C.I. 33.37–84.40)
7	0.095	103.36 (C.I. 73.64–144.31)
8	0.0081	8.30 (C.I. 4.60–13.78)
9	0.086	89.74 (C.I. 54.39–147.78)
10	> 5	> 5000
11	> 5	> 5000
12	> 5	> 5000
13	> 5	> 5000
kaempferol	0.0069	23.98 (C.I. 12.33–45.48)
70% elution fraction	0.00062	–
acarbose	1.11	1727.07 (C.I. 1365.09–2184.01)

IC_{50} was afforded with confidence interval (n = 3); C.I.: 95% confidence interval.
Positive control: acarbose.

3.2. The α-glucosidase inhibition acssay of compounds 1–13

The *α*-glucosidase inhibitory activities were evaluated by using spectrophotometric in vitro (Table 3.). We tested the *α*-glucosidase inhibition assay in vitro of compounds **1–13**, kaempferol, 70% ethanol elution fractions and acarbose. Kaempferol, aglycone of these flavonoids, was considered as the one of the active compounds to treat diabetes [5,16]. Results showed that the 70% ethanol elution fraction was the most active sample of *α*-glucosidase inhibition (IC_{50} value of 0.62 μg/mL). Compounds **5–9** and kaempferol showed moderate *α*-glucosidase inhibitory activities (IC_{50} values of 112.1, 53.2, 103.36, 8.30, 89.7 μM, correspondingly), while other compounds showed no effect at dosage up to 5 mM.

Structure-activity relationship of these flavonoid glycosides suggested that the *α*-glucosidase inhibiting activity might relate to the side chain groups. Compounds **5–9**, which had only one side chain group, showed the moderate inhibitory effect of *α*-glucosidase. While, compounds **10–13**, which had two side chain groups, showed no activities, perhaps because they had better steric hindrance to weak the activity. Besides, compounds **1–3** showed no inhibitory activities, suggesting that different connections of sugars may not be negligible. The above structure-activity relationship discussion was preliminary, and the relationship remains to be examined further and verified.

Thus, three novel compounds (**1–3**) and ten known compounds (**4–13**) were isolated from seeds of *H. rhamnoides*. Their structures were elucidated by spectroscopic and chemical methods. *α*-Glucosidase inhibition assay revealed that 70% ethanol elution, compounds **5–9** and kaempferol showed moderate effect.

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Conflict of interests statement

The authors declare that there are no conflicts of interest associated with this manuscript.

Appendix A. Supplementary data

HRESI-MS, 1D and 2D NMR spectra for compounds **1–3** and the spectra data of compounds **4–13** are available in the Supplementary data. Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.104248.

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