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Antioxidant phenolic constituents from *Fagopyrum dibotrys*

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Abstract

Fagopyrum dibotrys (D. Don.) Hara. is an erect perennial Polygonaceous herb. In China, its rhizome was used as folk medicine for the treatment of lung diseases, dysentery and rheumatism. The crude aqueous acetone extract from the rhizomes of this plant exhibited high antioxidant activity (SC_{50} = 10.95 μg/mL) in 1,1-diphenyl-2-picryldydrazyl (DPPH) radical scavenging assay. Detailed chemical investigation on the extract led to the isolation of two new phenols (**1** and **2**), together with 14 known antioxidant phenolic compounds (**3**–**16**). Their structures were determined by detailed spectroscopic analysis. The two new compounds were characterized as 3-methyl-gossypetin 8-*O*-β-D-glucopyranoside (**1**) and 1,3,6′-tri-*p*-coumaroyl-6-feruloyl sucrose (diboside A, **2**). The radical scavenging activity of all the isolated compounds was also described.

Keywords: *Fagopyrum dibotrys*; Antioxidant phenolics; Diboside A; Flavonol glycoside

1. Introduction

The genus *Fagopyrum* (Polygonaceae), comprising of 15 species, is mainly distributed in the North Temperate Zone. Eight species, including some common crops and medicinal plants occur in China: buckwheat (*Fagopyrum esculentum* Moench.), tartary buckwheat (*Fagopyrum tartaricum* (L.) Gaertn.), *Fagopyrum urophyllum* (Bur. Et Franch.) H. Gross., etc. *Fagopyrum dibotrys* (D. Don.) Hara. is an erect perennial herb, growing mainly in China, India, Vietnam, Thailand and Nepal. Its rhizome was used as folk medicine in China for the treatment of lung diseases, dysentery and rheumatism (Editorial Board of Zhong Hua Ben Cao (China Herbal), State Administration of Traditional Chinese Medicine, 1999). Previous chemical studies showed the presence of some phenolics and other compounds in the rhizome, such as hecogenin, β-sitosterol, *p*-coumaric acid, ferulic acid and shakuchirin (Liu et al., 1983; Yao et al., 1989; Zhang et al., 1994), but there are no reports on their antioxidant activity. Our preliminary experiment showed that the 60% aqueous acetone extract of the rhizomes of *Fagopyrum dibotrys* exhibited considerable antioxidant activity on 1,1-diphenyl-2-picryldydrazyl (DPPH) radical scavenging assay. This observation propelled us to perform a detailed bioassay-guided chemical investigation on this plant, which led to the isolation of a new flavonol glycoside methyl ether (**1**) and a new phenylpropanoid ester sucrose (**2**), together with 14 known compounds. This paper describes the structure elucidation of the new compounds (**1**–**2**) on the basis of spectroscopic method and the antioxidant activity of these isolated compounds on DPPH radical scavenging assay.

2. Materials and methods

2.1. Plant material

The dried rhizome of *Fagopyrum dibotrys* was bought from Kunming herbal medicinal market and identified by Prof. C. R. Yang, Kunming Institute of Botany, Chinese Academy of Sciences.

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2.2. General experimental procedures

$[\alpha]_D$ was carried out on a JASCO-20 polarimeter. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were recorded on a UV 210A Shimadzu spectrometer. 1D- and 2D-NMR spectra were run on Bruker AM-400 and DRX-500 instruments with TMS as internal standard. The MS data were recorded on a VG Auto Spec-3000 spectrometer. DPPH radical (Aldrich Chemical Co.) scavenging assay was performed on Emax precision microplate reader. Column chromatography was performed on Diaion HP20SS (Mitsubishi Chemical Co.), MCI-gel CHP-20P (75–150 μm, Mitsubishi Chemical Co.), Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co. Ltd.), Chromatorex ODS (100–200 mesh, Fuji Silysia Chemical Co. Ltd.), and Toyopearl HW-40F (37–70 μm, Tosoh Co.). TLC was carried out on silica gel G precoated plates (Qingdao Haiyang Chemical Co.) with benzene–ethyl formate–formic acid (3:6:1). Spots were detected by spraying with ferric chloride ($FeCl_3$) and 10% sulfuric acid reagents followed by heating.

2.3. Extraction and isolation

The dried rhizome (6.0 kg) of *Fagopyrum dibotrys* was extracted three times with 15 L of 60% aqueous acetone at room temperature. The combined extract was concentrated under reduced pressure to give a residue (600 g), which showed SC_{50} value as 10.95 μg/mL on the DPPH assay. Accordingly, further isolation was carried out.

The crude extract was applied to Diaion HP20SS column eluted with H_2O–MeOH (1:0–0:1) and then 50% acetone to give nine fractions (A_1–A_9). Fraction A_7 was subjected to column chromatography on Sephadex LH-20 eluted with H_2O–MeOH (1:0–0:1) and EtOH to give **1** (10 mg), **4** (11 mg) and **5** (14 mg). A_9 was separated by column chromatography on Sephadex LH-20 (EtOH) and silica gel (CH_3Cl–MeOH–H_2O, 10:1:0.1, lower layer) to give **2** (13 mg) and **3** (11 mg). Fraction A_5 was chromatographed on Sephadex LH-20 and Chromatorex ODS eluted with H_2O–MeOH (1:0–0:1) to give **7** (150 mg), **15** (50 mg) and **16** (70 mg). A_8 was subjected to column chromatography on Sephadex LH-20 eluted with H_2O–MeOH (1:0–0:1) and EtOH to give **6** (12 mg) and **8** (8 mg). A_1 was separated by column chromatography on Sephadex LH-20 eluted with EtOH–H_2O (1:0–1:1) and Chromatorex ODS eluted with H_2O–MeOH (1:0–0:1) to give **9** (100 mg) and **10** (20 mg). A_3 was repeatedly chromatographed on Sephadex LH-20, Chromatorex ODS, MCI-gel CHP-20P and Toyopearl HW-40F eluted with H_2O–MeOH (1:0–0:1) to give **11** (20 mg), **12** (17 mg), **13** (10 mg) and **14** (11 mg).

2.3.1. 3-Methyl-gossypetin 8-O-β-D-glucopyranoside (1)

Yellow amorphous powder, $[\alpha]_D^{21} + 31.58^\circ$ (0.095, DMSO); UV (MeOH) λ_{max} (nm) ($\log \varepsilon$): 203 (4.33), 278 (3.78); IR (KBr) ν_{max} 3441, 2924 and 1629 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) see Table 1; negative ion HRFAB-MS *m/z*: 493.0977 $[M-H]^-$, calcd. for $[C_{22}H_{21}O_{13}]^-$: 493.0982.

Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of **1** in DMSO-d_6

No.	C	H (*J* in Hz)
2	155.4 (s)	
3	137.5 (s)	
4	177.5 (s)	
4a	103.3 (s)	
5	157.0 (s)	12.55 (s, OH-5)
6	99.3 (d)	6.19 (1H, s)
7	157.0 (s)	
8	125.6 (s)	
8a	148.8 (s)	
1′	121.6 (s)	
2′	115.5 (d)	7.74 (1H, d, 2.00)
3′	145.0 (s)	
4′	148.5 (s)	
5′	115.8 (d)	7.72 (1H, d, 8.15)
6′	121.1 (d)	6.90 (1H, dd, 2.00, 8.15)
3-OCH_3	59.6 (q)	3.78 (3H, s)
1″	106.7 (d)	4.59 (1H, d, 7.75)
2″	74.2 (d)	
3″	76.2 (d)	
4″	69.3 (d)	
5″	77.2 (d)	
6″	60.6 (t)	3.15–3.72 (2H, m, overlapped)

2.3.2. Diboside A (2)

White amorphous powder, $[\alpha]_D^{21} + 15.56^\circ$ (0.68, MeOH); UV (MeOH) λ_{max} (nm) ($\log \varepsilon$): 228 (4.16), 315 (4.40); IR (KBr) ν_{max} 3400, 1700, 1630, 1515, 1268 and 1170 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data see Table 2; negative FAB-MS *m/z*: 955 $[M-H]^-$, 809 $[M-H-146(coumaroyl)]^-$, 779 $[M-H-176(feruloyl)]^-$, 663 $[M-H-146(coumaroyl)-146(coumaroyl)]^-$, 517 $[M-H-146(coumaroyl)-146(coumaroyl)-146(coumaroyl)]^-$, 341 $[M-H-146(coumaroyl)-146(coumaroyl)-146$ $(coumaroyl)-176(feruloyl)]^-$; negative ion HRFAB-MS *m/z*: 955.2706 $[M-H]^-$, calcd. for $[C_{49}H_{47}O_{20}]^-$: 955.2660.

2.4. DPPH radical scavenging assay

The DPPH assay was performed as described (Yoshida et al., 1989). In this assay, ascorbic acid was used as positive control, reaction mixtures containing an ethanolic solution of 200 μM DPPH (100 μL) and two-fold serial dilutions of sample (dissolved in 100 μL ethanol, with amounts of sample ranging from 2 to 1000 μg/mL) were placed in a 96-well microplate and incubated at 37 °C for 30 min. After incubation, the absorbance was read at 517 nm by an Emax precision microplate reader and mean value was obtained from three duplicated readings. Scavenging activity was determined by the following equation: % scavenging

Table 2
^{1}H (500 MHz) and ^{13}C (100 MHz) NMR spectral data of **2** in CD_3OD

No.	^{1}H (J in Hz)	^{13}C
Fructose		
1	4.35 (2H, m)	66.3 t
2		103.4 s
3	5.67 (1H, d, 8.76)	79.3 d
4	4.74 (1H, m)	74.1 d
5	4.23 (1H, m)	81.0 d
6	4.57 (2H, m)	65.5 t
Glucose		
1′	5.59 (1H, d, 3.83)	92.9 d
2′	3.50 (1H, m)	72.9 d
3′	3.69 (1H,m)	74.9 d
4′	3.30 (1H, m)	72.2 d
5′	3.32 (1H, m)	72.4 d
6′	4.33 (1H, m), 4.73 (1H, m)	65.6 t
Phenylpropanoids		
Glu-6′-*O*-*p*-coumaroyl		
1″		127.1 s
2″	7.30 (1H, d, 8.67)	131.1 d
3″	6.76 (1H, d, 8.67)	116.1 d
4″		161.3 s
5″	6.76 1H, d, 8.67)	116.1 d
6″	7.30 (1H, d, 8.67)	131.1 d
7″	7.55 (1H, d, 16.00)	146.8 d
8″	6.23 (1H, d, 16.00)	114.8 d
9″		169.3 s
Fru-1-*O*-*p*-coumaroyl		
1‴		127.4 s
2‴	7.44 (1H, d, 8.80)	131.5 d
3‴	6.79 (1H, d, 8.80)	116.7 d
4‴		161.0 s
5‴	6.78 (1H, d, 8.80)	116.7 d
6‴	7.44 (1H, d, 8.80)	131.5 d
7‴	7.70 (1H, d, 15.90)	148.2 d
8‴	6.43(1H, d, 15.90)	114.3 d
9‴		168.4 s
Fru-3-*O*-*p*-coumaroyl		
1⁗		127.2 s
2⁗	7.38 (1H, d, 8.70)	131.2 d
3⁗	6.77 (1H, d, 8.70)	116.3 d
4⁗		161.2 s
5⁗	6.77 (1H, d, 8.70)	116.3 d
6⁗	7.38 (1H, d, 8.70)	131.2 d
7⁗	7.63 (1H, d, 8.70)	147.1 d
8⁗	6.30 (1H, d, 8.70)	114.7 d
9⁗		168.4 s
Fru-6-*O*-feruloyl		
1⁗′		127.7 s
2⁗′	7.16 (1H, d, 1.76)	111.7 d
3⁗′		149.2 s
4⁗′		150.4 s
5⁗′	6.79 (1H, d, 8.20)	116.3 d
6⁗′	6.99 (1H, dd, 1.76, 8.20)	124.4 d
7⁗′	7.59 (1H, d, 15.82)	147.1 d
8⁗′	6.46 (1H, d, 15.82)	115.9 d
9⁗′		168.9 s
(O-Me)	3.80 (3H, s)	56.5 q

activity = $100 \times (A_{control} - A_{sample})/A_{control}$. The SC_{50} value was obtained through extrapolation from linear regression analysis and denoted the concentration of sample required to scavenge 50% of DPPH radicals.

3. Results and discussion

The 60% aqueous acetone extract of air-dried rhizomes of *Fagopyrum dibotrys* exhibited obvious antioxidant activity (SC_{50} = 10.95 μg/mL) on DPPH radical scavenging assay. The crude extract was further fractionated and purified over Diaion HP 20SS, Sephadex LH-20, Chromatorex ODS, MCI-gel CHP-20P and silica gel to give two new compounds **1** and **2**, as well as 14 known compounds (**3**–**16**). The known ones were identified as lapathoside A (**3**) (Takasaki et al., 2001), quercetin (**4**) (Wenkert and Gottlieb, 1977), 3-methylquercetin (**5**) (Bacon et al., 1978), 3,5-dimethylquercetin (**6**), rutin (**7**) (Wenkert and Gottlieb, 1977), emodin-8-*O*-β-D-glucopyranoside (**8**) (Steglich and Losel, 1969), gallic acid (**9**), 6-*O*-galloyl-D-glucose (**10**) (Nonaka and Nishioka, 1983), (+)-catechin (**11**) (Nonaka et al., 1983a), (−)-epicatechin (**12**) (Nonaka and Nishioka, 1982), procyanidin B-1 (**13**) (Nonaka et al., 1981), procyanidin B-2 (**14**) (Nonaka et al., 1981), 3,3′-di-*O*-galloyl-procyanidin B-2 (**15**) (Nonaka et al., 1981) and 3′-*O*-galloyl-procyanidin B-2 (**16**) (Nonaka et al., 1983b) by the spectroscopic evidences and comparing with literature data (Fig. 1).

Compound **1** was obtained as a yellow amorphous powder. Its molecular formula was assigned as $C_{22}H_{22}O_{13}$ on the basis of the negative ion high-resolution (HR) FAB mass spectrum (MS) (*m*/*z*: 493.0977 $[M-H]^-$, calcd. 493.0982) and the ^{13}C DEPT NMR spectrum. The ^{13}C NMR spectrum showed the presence of 15 carbon signals due to the flavonol skeleton, a set of signals arising from a glucopyranosyl moiety [anomeric H: δ 4.59 (J = 7.75 Hz), anomeric C: δ 103.4 (d)] and a methoxyl group [δ_H 3.78 (3H, s), δ_C 59.6 (q)]. The ^{1}H NMR spectrum exhibited a characteristic proton signal at δ 12.55 corresponding to a free hydroxyl at C-5, a singlet proton signal at δ 6.19 arising from H-6 of ring A and an ABX coupling system [δ 7.74 (1H, d, J = 2.00 Hz), 7.72 (1H, d, J = 8.15 Hz), 6.90 (1H, dd, J = 2.00, 8.15 Hz)] referring to the 3′,4′-dihydroxylated ring B. These ^{1}H and ^{13}C NMR spectral features were closely related to those of gossypetin 8-*O*-glucoside (Nawwar and Buddrus, 1981), except for the appearance of the additional methoxyl signal. In the HMBC spectrum (Fig. 2), the methoxyl proton signal at δ 3.78 was correlated with δ 137.5 (C-3), indicating that the methoxyl group was linked at C-3 of the flavonol skeleton. In addition, HMBC correlations of the 5-OH proton at δ 12.55 with δ 99.3 (C-6), 100.3 (C-4a) and 157.0 (C-5), and the glucose anomeric proton signal at δ 4.59 with δ 125.6 (C-8) were also observed, which confirmed the location of the glucopyranosyl moiety on C-8 position. On the basis of the above evidences, compound **1** was determined to be 3-methyl-gossypetin 8-*O*-β-D-glucopyranoside.

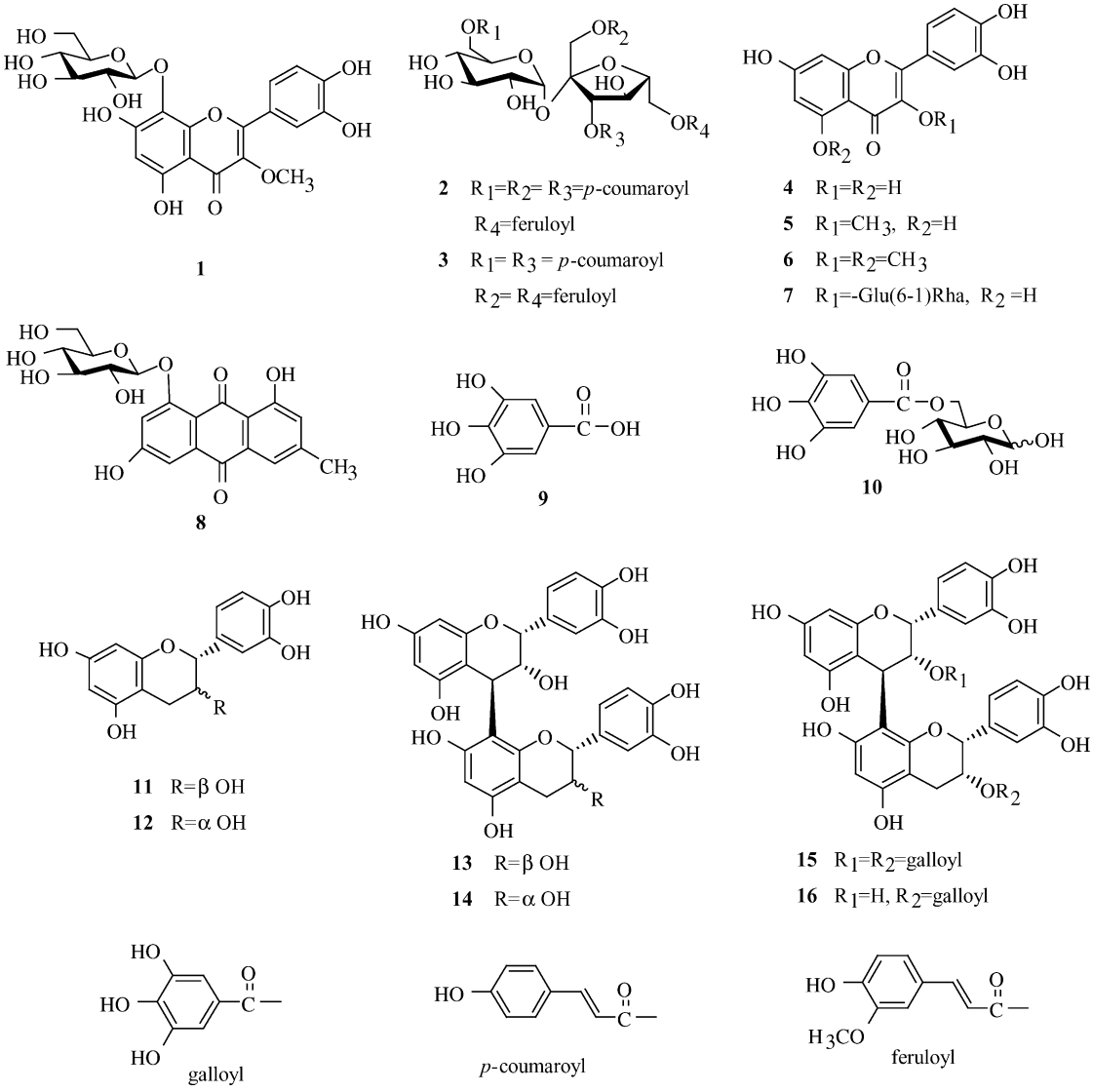

Fig. 1. Structures of phenolic antioxidants from *Fagopyrum dibotrys*.

The molecular formula of compound **2** was determined to be $C_{49}H_{48}O_{20}$ on the basis of negative ion HRFAB-MS (*m*/*z*: 955.2706, $[M-H]^-$ calcd. 955.2660) and the ^{13}C DEPT NMR spectrum. The UV (228, 315 nm) and IR (3400, 1700, 1630, 1515, 1268 and 1170 cm^{-1}) absorptions suggested the existence of hydroxyl and α, β-unsaturated aromatic ester groups in **2**. The ^1H and ^{13}C NMR spectra exhibited typical signals rising from *p*-coumaroyl moiety, feruloyl moiety and a sucrose unit (Table 2), which were similar to those of vanicoside B (1,3,6-tri-*p*-coumaroyl-6′-feruloyl sucrose) isolated from *Polygonum pensylvanicum* (Zimmermann and Sneden, 1994). In the NOESY spectrum of **2** (Fig. 3), cross peaks between the methoxyl group at *δ* 3.80 (3H, s) and an aromatic proton at *δ* 7.16 (1H, d, *J* = 1.76 Hz) confirmed the presence of the feruloyl moiety, in which the methoxyl group was linked to C-3‴′. Characteristic fragment ion peaks in negative FAB-MS were also observed at *m*/*z* 809 $[M-H-146(\text{coumaroyl})]^-$, 779 $[M-H-176(\text{feruloyl})]^-$, 663 $[M-H-146(\text{coumaroyl})\text{-}146(\text{coumaroyl})]^-$, 517 $[M-H-146(\text{coumaroyl})\text{-}146(\text{coumaroyl})\text{-}146(\text{coumaroyl})]^-$ and 341 $[M-H-146(\text{coumaroyl})\text{-}146(\text{coumaroyl})\text{-}146(\text{coumaroyl})\text{-}176(\text{ferul-}$

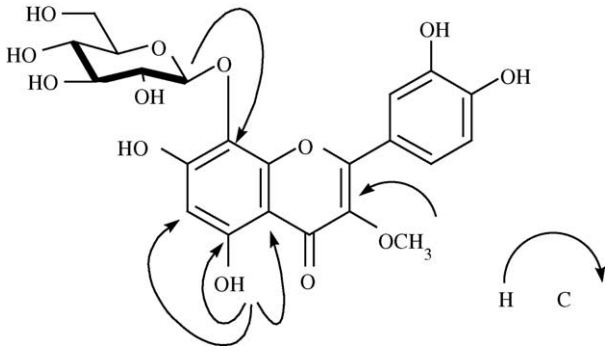

Fig. 2. Important HMBC correlations of compound **1**.

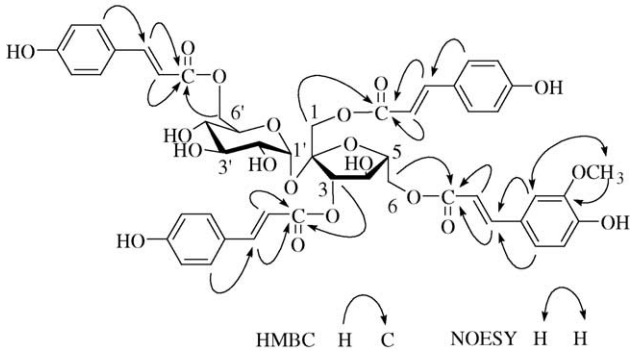

Fig. 3. Important HMBC and NOESY correlations of compound **2**.

oyl)]$^{-}$. The above observations indicated that **2** was a sucrose acylated by three *p*-coumaric acids and one ferulic acid.

Locations of *p*-coumaroyl and feruloyl moieties on sucrose were determined as follows. The proton and carbon signals of *p*-coumaroyl, feruloyl and sucrose moieties of **2** were assigned by 2D NMR techniques, including the ^{1}H–^{1}H COSY, HMQC and NOESY. In the ^{13}C NMR spectrum of **2**, signals of C-1, C-6 of fructose and C-6′ of glucose in the sucrose moiety were shifted to lower field (+2 to +3 ppm), while C-2, C-5 of fructose and C-5′ of glucose were shifted to higher field (−2 to −3 ppm), comparing with those of sucrose [^{13}C NMR data (100 MHz, in CD_3OD): δ 64.0 (C-1), 105.3 (C-2), 79.3 (C-3), 75.7 (C-4), 83.8 (C-5), 63.4 (C-6), 93.6 (C-1′), 73.2 (C-2′), 74.7(C-3′), 71.3 (C-4′), 74.41 (C-5′) and 62.2 (C-6′)]. These observations suggested that the C-1, C-6 of fructose and C-6′ of glucose were esterified. In the HMBC spectrum of **2** (Fig. 3), the long-range correlations of H-1, H-3 and H-6′ of sucrose with C-9, C-9‴ and C-9⁗ rising from three *p*-coumaroyl groups, and H-6 of sucrose with C-9⁗′ of feruloyl group indicated that the acylated positions of the three coumaroyl groups were at C-1, C-3 and C-6′ of sucrose and the feruloyl group was attached at C-6 of the sucrose. Accordingly, compound **2** was characterized as 1,3,6′-tri-*p*-coumaroyl-6-feruloyl sucrose, named diboside A.

The antioxidant activity of compounds **1**–**16** was measured in DPPH assay (Table 3). Comparing with the positive control ascorbic acid, compounds **4**, **6** and **9**–**16** displayed higher activities, while **1**–**3**, **5**, **7** and **8** showed only lower activities.

Gallic acid (**9**), the well-known natural antioxidant found widely in plants, and its derivative, **10**, exhibited higher free radical-scavenging activities than ascorbic acid. While, compounds **2**, **3** and **8**, the minor constituents from *Fagopyrum dibotrys* and without trihydroxylphenyl or *o*-dihydroxylphenyl groups existing in the molecule, showed lower activities. This result is consistent with the previous reports and suggests that three or two adjacent phenolic hydroxyl group in the molecule is a key factor for enhancing the activity (Guo et al., 1999; Okawa et al., 2001).

Table 3
DPPH radical scavenging activity of compounds **1**–**16** from *Fagopyrum dibotrys*

Compounds	SC_{50} (μM)
Ascorbic acid (CK)	30.79
1	37.99
2	199.48
3	165.52
4	13.68
5	31.23
6	24.40
7	42.49
8	61.80
9	12.10
10	25.17
11	22.64
12	17.39
13	18.40
14	15.31
15	10.12
16	7.94

SC_{50}: radical scavenging activity (concentration in μM required for 50% reduction of DPPH radical).

Flavonoids have generally been considered as important antioxidants. Eleven flavonoids, including quercetin (**4**) and its derivatives **1**, **5**–**7**, as well as the major constituents of *Fagopyrum dibotrys*, flavan-3-ols and its derivatives (**11**–**16**), were isolated from *Fagopyrum dibotrys*. The activity order of quercetin derivatives was shown to be **4** > **6** > **5** > **1** > **7**, suggesting that the presence of 3-hydroxyl group is important to their radical-scavenging capabilities. When the hydroxyl group at C-3 of quercetin was substituted with methoxyl group, it gave a negative influence for the activity. And if a bigger substituent like *O*-glycosyl was linked at C-3, the activity was greatly reduced probably for the steric hindrance (Cioffi et al., 2002; Braca et al., 2003).

All of the flavan-3-ols displayed higher radical-scavenging activities (7.94–22.64 μM) than ascorbic acid. Among the isolated compounds, **15** and **16** were the most active ones due to the molecule possessing more phenolic hydroxyl groups, particularly the galloyl and catechol groups. These main constituents may play an important role for the antioxidant activity of *Fagopyrum dibotrys*. The above results will promote the reasonable usage of this herb.

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