

Four flavonoid glycosides from the pulps of *Elaeagnus angustifolia* and their antioxidant activities

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Abstract—Four flavonoid glycosides were isolated from the pulps of *Elaeagnus angustifolia* and identified as Quercetin 3,4'-O- β -D-diglucoside (1), Isorhamnetin-3-O- β -D-galactopyranoside (2), Quercetin 3-O- β -D-Galactopyranoside-4'-O- β -D-glucopyranoside (3) and Isorhamnetin 3-O- β -D-Galactopyranoside-4'-O- β -D-glucopyranoside (4). Among which, the compounds 1, 3 and 4 were isolated for the first time from this plant. The antioxidant activities of the four compounds were evaluated by measuring their reducing power and ability to scavenge the ABTS radical, DPPH radical and to inhibit superoxide anions. The results indicated that these compounds exhibited antioxidant activities with a dose-dependent manner in all tested models, and had some structure-activity relationships. The order of potential antioxidant capacity was as follows: 1>3>2>4. Considering related reports, the flavonoid might be responsible for antioxidant activity of pulps of *E. angustifolia*.

Keywords-*Elaeagnus angustifolia*; antioxidant activity; flavonoid

I. INTRODUCTION

The genus *Elaeagnus* (Araliaceae) consists of more than 80 species, which are mainly distributed in Asia and Europe, partly in the Northern America. There are approximate 55 species in China [1]. *Elaeagnus angustifolia* L., a deciduous arbor or small arbor (5-10m high), is widely distributed in northwest China such as Xinjiang, Gansu, and other provinces. Flower, fruit, leaf and bark of the plant has been used in Traditional Chinese Medicine for the treatment of asthenia of the spleen and stomach, dyspepsia, enteritis, diarrhea and cough with yellow sputum [2]. The *E. angustifolia* fruit is an autumn fruit which is generally consumed dried, and is rich in many effective components. Modern pharmacological studies have found that *E. angustifolia* fruit has anti-fatigue [3], anti-inflammatory [4], antioxidant activities [5] and other health functions. Up to now, nine flavonoids including (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, kaempferol, quercetin, luteolin, isorhamnetin and isorhamnetin-3-O- β -D-galactopyranoside have already been isolated and identified from this plant [6, 7].

Flavonoids, a group of low molecular weight polyphenolic substances including flavone, flavonols, isoflavone, flavanol, flavanone, anthocyanin and proanthocyanidin, widely exist in fruits, vegetables. In fact, these flavonoid compounds have attracted the attention of food and medical scientists because of their strong in vitro

and in vivo antioxidant activities and their ability to scavenge free radicals, break radical chain reaction and chelate metals[8]. In this study, four flavonoid glycosides were isolated from the pulps of *E. angustifolia* and identified according to their NMR and mass spectra. Moreover, their antioxidant activities were evaluated and compared.

II. MATERIAL AND CHEMICALS

A. Chemicals

DPPH and ABTS were purchased from Sigma. All other reagents and solvents used in this study were of analytical grade.

B. Plant material

Mature fruits of *E. angustifolia* were collected in July of 2008 from Gulang town, Gansu in China (37°N and 102°E). The plant was identified by Prof. Sheng-Li Pan. A voucher specimen SM/WY/08/02 was deposited in our laboratory for future reference.

C. Extraction and isolation

Air-dried fruit pulps of *E. angustifolia* (1.5 kg) were percolated with 50% EtOH, The solvent was evaporated under reduced pressure to produce the ethanol extract (720 g). The extract was subjected to macroporous resin NKA-9 column chromatography and eluted with an EtOH-H₂O gradient. The solution that was eluted with 70% ethanol was evaporated to dryness under vacuum to afford a residue (59 g). The residue was chromatographed on silica gel with a CH₃Cl₃-MeOH gradient to provide eight fractions. The fraction eluted with CH₃Cl₃-MeOH (4:1) yield compound 1 (15 mg).

The solution that was eluted with 50% ethanol was evaporated to dryness under vacuum to afford a residue (59 g). The residue was eluted with ethyl acetate-MeOH. The fraction eluted with CH₃Cl₃-MeOH (8:1) afforded compound 2 (7 mg). The fraction eluted with CH₃Cl₃-MeOH (5:1) was subjected to preparative HPLC using the solvent system MeOH-H₂O (v/v=35:65) to afford compounds 3 (5 mg) and 4 (6 mg).

Quercetin 3,4'-O- β -D-diglucoside (**1**). Brown powder; ESI-MS *m/z*: 627.1[M+H]⁺; ¹HNMR (400MHz, CD₃OD) δ : 7.71 [1H, dd, *J*=8.6/2.2Hz, H-6'], 7.52 [1H, d, *J*=2.2 Hz, H-2'], 6.86 [1H, d, *J*=8.7 Hz, H-5'], 6.37 [1H, d, *J*=1.6Hz, H-8], 6.18 [1H, d, *J*=1.7Hz, H-6], 5.15 [1H, d, *J*=7.3Hz, H-1"], 4.73 [1H, d, *J*=7.1Hz, H-1'"]. ¹³C NMR(100MHz,

CD₃OD) data, see Table 1.

Isorhamnetin-3-*O*-β-D-galactopyranoside (**2**). Yellow powder; ESI-MS *m/z*: 479.1[M+H]⁺, 477.5[M-H]⁻; ¹H-NMR(CD₃OD, 400MHz): δ8.03(1H, d, *J*=2.0, H-2'), 7.57(1H, dd, *J*=8.4/2.0 Hz, H-6'), 6.89 (1H, d, *J*=8.8 Hz, H-5'), 6.40(1H, d, *J*=2.4, H-8), 6.20(1H, d, *J*=2.4, H-6), 5.34(1H, d, *J*=8 Hz, H-1"), 3.94(1H, s, OCH₃). ¹³C NMR(100MHz, CD₃OD) data, see Table 1.

Quercetin 3-*O*-β-D-Galactopyranoside-4'-*O*-β-D-glucopyranoside (**3**). Yellow powder; ESI-MS *m/z*: 625.4 [M-H]⁻; ¹H-NMR(CD₃OD, 400MHz): δ:7.67 [1H, dd, *J*=8.7/2.1 Hz, H-6'], 7.63 [1H, d, *J*=2.1Hz, H-2'], 7.18 [1H, d, *J*=8.7Hz, H-5'], 6.44 [1H, d, *J*=2.0, H-8], 6.21 [1H, d, *J*=2.0, H-6], 5.40 [1H, d, *J*=7.7Hz, H-1"], 4.76 [1H, d, *J*=7.1Hz, H-1"]. ¹³C NMR(100MHz, CD₃OD) data, see Table 1.

TABLE 1. ¹³C NMR SPECTROSCOPIC DATA OF COMPOUNDS 1-4

	c	1	2	3	4
Flavonol	2	158.4	158.6	158.5	158.7
	3	136.0	135.4	135.1	134.8
	4	180.1	179.6	179.9	179.5
	5	162.0	163.1	163.1	163.0
	6	100.9	99.8	99.8	99.8
	7	165.3	165.9	165.9	165.9
	8	96.8	94.6	94.7	94.6
	9	160.0	158.4	158.8	158.4
	10	106.5	105.7	105.0	105.7
	1'	124.9	123.0	123.0	123.0
	2'	118.9	114.6	116.2	114.3
	3'	145.8	148.4	145.9	148.4
	4'	149.7	150.8	148.2	150.8
	5'	117.7	115.9	112.0	115.0
	6'	123.9	123.5	117.8	123.5
	-OMe		56.9		57.1
Sugar at C-3	1"	104.5	104.4	101.9	104.2
	2"	75.9	73.1	74.9	74.7
	3"	78.0	75.0	75.4	75.2
	4"	71.9	70.0	70.0	69.4
	5"	79.7	77.2	77.9	77.7
	6"	63.1	62.2	62.3	62.4
Sugar at C-4'	1'''	102.8		101.7	101.3
	2'''	75.4		77.0	77.0
	3'''	77.2		78.1	78.1
	4'''	70.9		71.0	71.1
	5'''	78.4		80.7	79.7
	6'''	62.7		61.9	62.1

Isorhamnetin 3-*O*-β-D-Galactopyranoside-4'-*O*-β-D-glucopyranoside (**4**). Yellow powder; ESI-MS *m/z*: 641.1 [M+H]⁺; ¹H-NMR(CD₃OD, 400MHz): δ7.93(1H, d, *J* = 2.4 Hz, H-2'), 7.62(1H, dd, *J*=8.4/2.4 Hz, H-6'), 6.90 (1H, *J*=8.4 Hz, H-5'), 6.39(1H, d, *J*=2.0, H-8), 6.18(1H, d, *J*=2.0, H-6), 5.53(1H, d, *J*=7.6 Hz, H-1"), 4.77(1H, d, *J*=7.2, H-1"). ¹³C NMR(100MHz, CD₃OD) data, see Table 1.

D. Antioxidant assays

ABTS radical scavenging activity: The method as described previously [9] was used to determine the antioxidant capacity of obtained compounds. ABTS⁺ stock solution was prepared by reacting 5 ml of 7 mM ABTS water solution with 88μL of 140 mM potassium persulphate, and the mixture was away from light at room temperature for 12–16 h before use. To this end, the ABTS⁺ stock solution was diluted with 80% methanol to the absorbance of 0.70 ±

0.02 at 734 nm. For the spectrophotometric assay, 3.8 mL of the ABTS⁺ working solution and 0.2 mL of the sample solution were mixed and absorbance was measured immediately after 6 min. The percentage inhibition was calculated with the following equation: inhibition (%) = (A_{control734} - A_{sample734}) / A_{control734} × 100, in which IC₅₀ is the concentration of half-inhibition.

DPPH radical scavenging activity: Assay for DPPH free radical scavenging capacity was based on the scavenging activity of stable DPPH free radicals [10]. The reaction mixtures containing 2.5mL of test samples solution and DPPH (0.025mg/ml) were incubated for 30 min. Following that, the absorbance at 517 nm was measured and the inhibition (%) of DPPH radical formation was calculated with the following equation: inhibition (%) = (A_{control517} - A_{sample517}) / A_{control517} × 100, in which IC₅₀ is the concentration of half-inhibition.

Superoxide anion radical scavenging activity: The superoxide anion radical scavenging activity was performed using the previously reported method developed by Zhang, et al., 2011[11]. A pyrogallol solution (3 mM) was added to a tube containing a sample that had been dissolved in Tris-HCl-EDTA buffer (0.1 M, pH 8.0). The UV absorbance was in triplicate measured at 320 nm. The antioxidant activity was determined as the percentage of inhibited pyrogallol autoxidation and was calculated as follows: (A_{control320} - A_{sample320}) / A_{control320} × 100, in which IC₅₀ is the concentration of half-inhibition.

Reducing power: The reducing power was determined by the method described previously[12] with little modification. The samples with different concentrations were mixed with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. After incubation, trichloroacetic acid (2.5 mL, 10%) was added to the reaction mixture, which was subsequently centrifuged at 6000 rpm for 10 min. The upper layer solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride solution (0.1%, 0.5 mL), and its absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

E. Statistical analysis

Data were expressed as the mean values ± standard deviation (SD) for each measurement. All analysis was performed with SPSS 13.0.

III. RESULTS AND DISCUSSION

Our previous studies revealed that the 50% ethanol extract of the pulps of *E. angustifolia* had antioxidant activity. These fractions eluted with 70%, 50% and 30% when the ethanol extract was subjected to macroporous resin NKA-9 column chromatography, showed significantly antioxidant effect. In the present study, four compounds from pulp of *E. angustifolia* were isolated, and identified by spectra methods [13-15], as Quercetin 3,4'-*O*-β-D-diglucoside (**1**), Isorhamnetin-3-*O*-β-D-galactopyranoside (**2**), Quercetin 3-*O*-β-D-Galactopyranoside-4'-*O*-β-D-glucopyranoside (**3**) and Isorhamnetin

3-O-β-D-Galactopyranoside-4'-O-β-D-glucopyranoside (4). Among which, the compounds 1, 3 and 4 were isolated for the first time from this plant. In addition, the antioxidant

activity of four flavonoid glycosides were evaluated by four classic antioxidant methods.

TABLE 2. THE PERCENTAGE INHIBITION OF ABTS AND DPPH FREE RADICAL, AND THE ABSORBANCE IN REDUCING POWER TEST FROM DIFFERENT CONCENTRATIONS OF COMPOUNDS 1-4 AND VC

Concentration (µg/mL)	VC	1	3	Concentration (µg/mL)	2	4
ABTS free radical scavenging test						
20	27.19±0.229	7.31±0.180	14.49±0.377	20	0.95±0.071	9.22±0.121
40	55.45±0.294	25.89±0.290	16.92±0.415	40	5.61±0.211	12.77±0.289
60	72.29±0.191	33.00±0.382	25.88±0.272	60	13.51±0.255	18.80±0.335
80	99.72±0.042	42.53±0.334	34.82±0.458	80	20.83±0.275	25.04±0.185
120	99.82±0.031	66.44±0.315	44.83±0.385	120	32.48±0.269	30.66±0.273
160	99.89±0.035	87.75±0.446	53.65±0.383	160	48.51±0.285	44.56±0.248
DPPH free radical scavenging test						
0.4	10.42±0.161	7.57±0.337	10.41±0.258	4	11.21±0.405	3.35±0.093
0.8	27.80±0.335	14.88±0.417	14.97±0.149	8	23.98±0.243	6.33±0.143
1.6	53.44±0.419	30.97±0.171	21.94±0.370	16	34.65±0.642	12.06±0.213
2.4	85.58±0.432	50.32±1.445	33.48±0.351	24	63.77±1.271	20.77±0.377
3.2	87.62±0.378	64.06±0.396	41.49±0.291	32	75.17±0.480	26.09±0.211
4.0	90.42±0.354	78.66±0.704	56.50±0.391	40	88.50±0.863	32.40±0.414
Reducing power test						
40	1.265±0.019	0.071±0.001	0.050±0.001	1000	0.747±0.008	0.062±0.002
80	1.601±0.011	0.308±0.006	0.257±0.005	1200	1.023±0.009	0.103±0.004
120	1.737±0.031	0.570±0.008	0.538±0.006	1400	1.232±0.017	0.127±0.005
160	1.801±0.006	0.913±0.010	0.754±0.005	1600	1.455±0.224	0.168±0.004
200	1.822±0.019	1.146±0.016	0.849±0.039	1800	1.591±0.002	0.215±0.009
240	2.221±0.066	1.444±0.073	1.143±0.073	2000	1.780±0.015	0.258±0.005

TABLE 3. THE PERCENTAGE INHIBITION OF SUPEROXIDE ANION RADICAL OF DIFFERENT CONCENTRATIONS OF COMPOUNDS 1-4 AND VC

Concentration (µg/ml)	1	2	3	4	Concentration (µg/ml)	VC
36	37.97±0.913	34.47±1.186	37.94±0.892	6.27±0.331	2	10.42±0.327
44	49.61±1.013	43.36±0.888	46.29±1.377	10.09±0.542	4	21.49±0.483
52	62.87±1.012	51.33±1.043	51.52±0.628	15.83±1.087	6	41.31±0.688
60	76.51±1.200	63.40±0.399	65.69±1.392	20.46±1.232	8	58.09±0.991
68	85.11±1.005	74.50±1.165	76.57±1.364	25.49±1.135	10	75.02±1.003
76	99.27±1.451	85.02±1.644	87.67±1.186	32.92±0.956	12	87.74±1.290

TABLE 4. IC₅₀ VALUES OF COMPOUNDS 1-4 AND VC (µG/ML)

Assay	VC	1	2	3	4
RSA (O ₂ ^{•-})	7.17±0.044	44.06±0.114	50.03±0.466	48.28±0.162	108.21±0.614
RSA (ABTS)	38.52±0.118	93.59±0.359	175.84±1.064	144.5±0.091	192.84±1.201
RSA (DPPH)	1.45±0.008	2.51±0.018	20.87±0.235	3.69±0.024	61.07±0.373
EC ₅₀ (Reducing power)	19.25±0.723	105.67±0.619	714.37±11.170	119.35±1.496	3175.33±22.805

The results from table 2 and table 3 showed that these compounds had antioxidant activity in a concentration-dependent manner. The ABTS⁺ radicals scavenging activity of compounds **1**, **2**, **3** and **4** were respective with the IC₅₀ values of 93.59±0.359, 175.84±1.064, 144.5±0.091 and 192.84±1.20 µg/mL (Table 4). Among which compound **1** have higher antioxidant activity than other three compounds. The IC₅₀ of compounds **1** and **3** on DPPH radical scavenging (2.51±0.018 and 3.69±0.024) was very close, and lower than that of compounds **3** and **4** (20.87±0.235 and 61.07±0.373). In addition, the IC₅₀ of compounds **1**, **2** and **3** on superoxide anion radical Scavenging was approximative (44.06±0.114, 50.03±0.466 and 48.28±0.162), and far less than that of compound **4** (108.21±0.614), showing stronger antioxidant activity. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. In the test, compounds **2**, **4** didn't show antioxidant activity when their concentrations wasn't higher than 240 µg/mL; However, compounds **1**, **3** showed different levels of antioxidant activity at these concentrations. Compounds **1** and **3** had higher activity than other two compounds. The results obtained from this study showed that multiple in vitro methods targeting different radical species and reducing power were important for testing antioxidant potential of compounds. Employment of more than one test method gave a better estimate of comparative antioxidant potential of these compounds. According to these results, the order of antioxidant activity of these flavonoids is as follows: **1**>**3**>**2**>**4**.

IV. CONCLUSION

In conclusion, the four compounds from the pulps of *E. angustifolia* exhibited different levels of antioxidant activity in all tested models. Considering related reports [6], the flavonoid might be responsible for antioxidant activity of pulp of *E. angustifolia*. Further investigation should be focused on the isolation of more individual compounds to complement and confirm the results of the antioxidant action of flavonoids and their glycosides.

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