

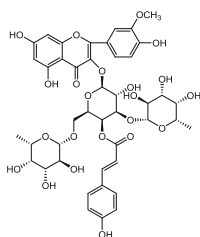
Three flavonoids from the leaves of *Astragalus membranaceus* and their antifungal activity

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Abstract A new flavonoid and two known flavonoids were isolated from the EtOAc extract of the leaves of *Astragalus membranaceus* (Fisch) Bge. var. *mongholicus* (Bge) Hsiao (*A. membranaceus*). This is the first report on the structure elucidation of the new compound based on spectroscopic methods including UV, IR, ESI-MS, 1D NMR, and 2D NMR techniques. The isolated compounds exhibited antifungal activity against different *Fusarium oxysporum* f.sp. *dianthi* pathotypes.

Graphical abstract



Keywords *Astragalus membranaceus* · Flavonoid · Spectroscopic methods · Antifungal

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Introduction

Astragalus membranaceus is a member of the family *Ranunculaceae*, genus *Paeonia* and distributed throughout Inner Mongolia, Shanxi, Xinjiang of China. The roots of *A. membranaceus* are the most important tonic traditional Chinese medicine, and promote the discharge of pus and the growth of new tissue [1]. Polysaccharides [2], saponins [3–5], and flavonoids [6–12] have been isolated from the roots of *A. membranaceus*.

However, flavonoids are a class of secondary metabolites generally located in plant leaves as water-soluble glycosides in the vacuoles of epidermal cells [13]. These compounds are not only present in plants as constitutive agents but are also accumulated in plant tissues in response to microbial attack [14, 15]. The secondary metabolites often differ in different organs of the same plant. For aiming to discover structurally unique and bioactive compounds, a new flavonoid with two known flavonoids was isolated from the leaves of *A. membranaceus*.

The flavonoids have been subjected to antifungal tests on different Fod pathotypes to evaluate the possible involvement of *A. membranaceus* flavonoids in resistance to pathogen attack.

Results and discussion

Structure elucidation

The 95 % ethanol extract of *A. membranaceus* was suspended in water, and then partitioned with petroleum ether, CHCl₃, EtOAc, and *n*-BuOH. The EtOAc-soluble fraction was separated by chromatography and afforded a new flavonoid with two known flavonoids which are isolated from this plant

for the first time (Fig. 1). The structures of the known compounds were identified by comparing their spectroscopic data with those reported in the literature [16, 17].

Compound **1** was obtained as a yellow powder. The positive reactions to the Molish and HCl–Mg tests suggested that the compound was a flavonoid glycoside. The molecular formula was determined to be $C_{43}H_{48}O_{22}$ by HR-ESIMS at $m/z = 915.2550 [M-H]^-$. The 1H NMR spectrum of **1** (Table 1) showed two olefinic hydrogens at $\delta = 7.66$ ppm (1H, d, $J = 16.0$ Hz) and 6.37 ppm (1H, d, $J = 16.0$ Hz), which suggested the presence of a trans-olefinic group in compound **1**. Furthermore, the signals of nine aromatic protons at 7.64 ppm (1H, dd, $J = 8.0, 1.5$ Hz), 6.98 ppm (1H, d, $J = 8.0$ Hz), 8.10 ppm (1H, d, $J = 1.5$ Hz), 6.19 ppm (1H, d, $J = 1.5$ Hz), and 6.41 ppm (1H, d, $J = 1.5$ Hz) indicated the presence of an ABC system for B ring and an AB system for A ring of a flavone. The remaining aromatic signals at 7.53 ppm (2H, d, $J = 8.0$ Hz) and 6.88 ppm (2H, d, $J = 8.0$ Hz) were assigned to a benzene ring of coumaroyl acid.

A detailed analysis of the ^{13}C NMR spectral data (Table 1) and correlations observed by HSQC, HMBC, DEPT, and 1H – 1H COSY experiments provided evidence for a flavone and a benzoic acid unit including an isorhamnetin unit confirmed the HMBC correlations (Fig. 2) from 7.43 ppm (H-6') to C-2 (157.2 ppm), C-2' (113.2 ppm) and C-4' (149.4 ppm), and 8.01 ppm (H-2') to C-2 (157.2 ppm), C-6' (122.3 ppm) and C-4' (149.4 ppm), and 6.98 ppm (H-5') to C-3' (146.9 ppm) and C-1' (121.1 ppm), and 6.19 ppm (H-6) to C-8 (93.4 ppm) and C-10 (104.5 ppm), and 6.41 ppm (H-8) to C-6 (98.4 ppm) and C-10 (104.5 ppm), 4.06 ppm (OCH₃) to C-3' (146.9 ppm); a coumaroyl acid unit confirmed the HMBC

correlations from 7.53 ppm (H-2''',6''') to C-4''' (160.0 ppm) and C-7''' (146.2 ppm), and 6.88 ppm (H-3''',5''') to C-1''' (125.7 ppm) and C-4''' (160.0 ppm).

The ^{13}C NMR spectrum of **1** (Table 1) also showed the presence of 18 carbon signals except for the aglycone carbons. The presence of a D-galactopyranose (99.4, 72.4, 76.8, 70.7, 72.5, and 65.4 ppm) was confirmed via sugar analysis [18]. The anomeric proton appearing at 5.83 ppm (1H, d, $J = 7.5$ Hz) and their corresponding carbons resonating at 99.4 ppm (C-1'') from the HSQC experiment also suggested the presence of a β -D-galactopyranose. The remaining signals at 101.6, 70.9, 70.8, 72.5, 68.6, and 16.2 ppm, and 100.8, 70.7, 70.6, 72.3, 68.5, and 16.6 ppm belong to two L-fucopyranoses, which was confirmed by comparing their spectral data with those reported in the literature [19].

The HMBC correlations from H-1'' (5.83 ppm) to C-3 (132.9 ppm), and H-4'' (5.41 ppm) to C-9''' (167.3 ppm) indicated that the β -D-galactopyranose and a coumaroyl acyl were attached to C-3 and C-4'', respectively. In addition, the HMBC correlations of H-1'''' (5.18 ppm) to C-3'' and H-1'''' (4.52 ppm) to C-6'' confirmed that the two L-fucopyranoses were linked at C-3'' and C-6'', respectively. The anomeric configuration in the L-fucopyranoses was determined as α according to the singlet peak. Thus, the structure of compound **1** was elucidated as isorhamnetin-3-O-(4-O-[E]-coumaroyl-3,6- α -L-O-fuco-pyranosyl)- β -D-galactopyranoside.

Biological assays

The isolated flavonoids **1–3** and a commercial sample of rutin have been tested to evaluate their ability to act as fungitoxic agents towards three main races of Fod affecting

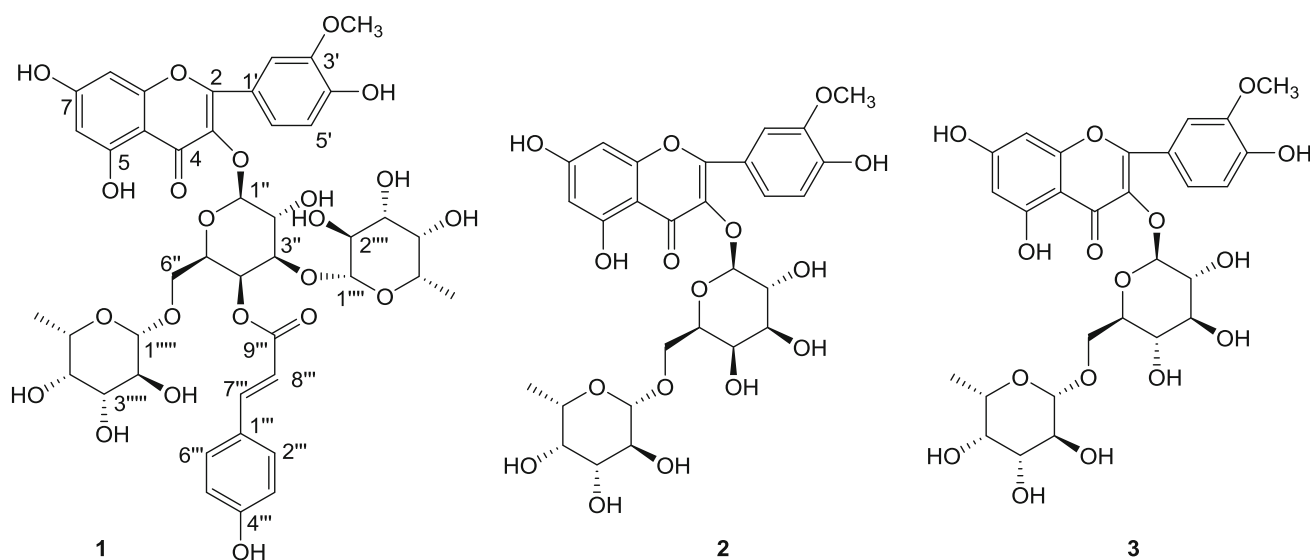
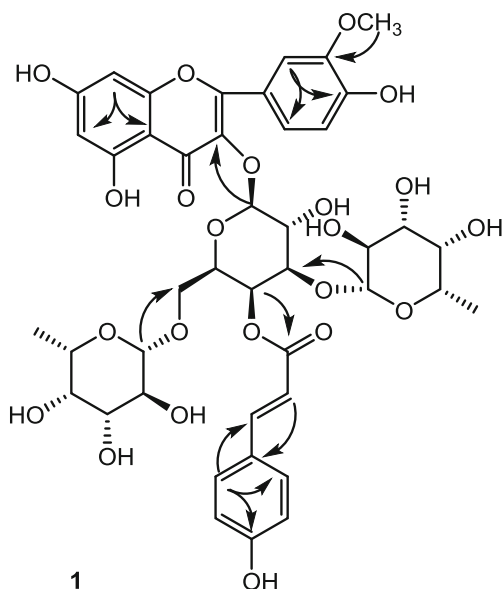


Fig. 1 Structures of compounds **1–3**

Table 1 ^1H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **1** in CD_3OD

No.	$\delta_{\text{C}}/\text{ppm}$	$\delta_{\text{H}}/\text{ppm}$, J/Hz	No.	$\delta_{\text{C}}/\text{ppm}$	$\delta_{\text{H}}/\text{ppm}$, J/Hz
2	157.2		1'''	125.7	
3	132.9		2'''	130.0	7.53 d (8.0)
4	177.7		3'''	115.6	6.88 d (8.0)
5	161.7		4'''	160.0	
6	98.4	6.19 d (1.5)	5'''	115.6	6.88 d (8.0)
7	164.4		6'''	130.0	7.53 d (8.0)
8	93.4	6.41 d (1.5)	7'''	146.2	7.66 d (16.0)
9	156.9		8'''	113.3	6.37 d (16.0)
10	104.5		9'''	167.3	
1'	122.1		1''''	101.6	5.18 s
2'	113.2	8.10 d (1.5)	2''''	70.8	3.84 m
3'	146.9		3''''	70.9	4.03 m
4'	149.4		4''''	72.5	3.38 m
5'	114.7	6.98 d (8.0)	5''''	68.6	3.40 m
6'	122.3	6.94 dd (8.0, 1.5)	6''''	16.2	1.03 d (6.0)
1''	99.4	5.83 d (7.5)	1''''''	100.8	4.52 s
2''	72.4	3.95 m	2''''''	70.7	3.54 m
3''	76.8	4.00 m	3''''''	70.6	3.62 m
4''	70.7	5.41 brs	4''''''	72.3	3.24 m
5''	72.5	4.02 m	5''''''	68.5	4.18 m
6''	65.4	3.54 m, 3.30 m	6''''''	16.6	1.06 d (6.0)
OCH_3	55.8	4.06 s			

**Fig. 2** Some key HMBC correlation of **1**

A. membranaceus, pathotypes 2, 4, and 8 [20, 21]. The biological tests (Table 2) showed that flavonoids **1–3** exhibited appreciable inhibition of mycelial growth with variable activities depending on Fod race. In particular, lowest inhibition percentages were observed on race 2,

where flavonoid **3** was the most effective treatment (36 %). Flavonoids **1–3** and rutin exhibited a good activity towards Fod race 4 with an average value of growth inhibition approximately 54 % at the highest dose used (Table 2). Pathotype 8 was inhibited mostly by flavonoids **1–3**, though Rutin was scarcely or not effective on races 2 and 8. Flavonoids are abundant widespread in many other plants. Although the mechanism of action of such compounds against fungi is still unknown, their efficacy, availability at low cost, and low toxicity to humans give the flavonoids potential as natural fungicides.

Conclusions

A new flavonoid and two known flavonoids were isolated from the EtOAc extract of the leaves of *A. membranaceus*. Flavonoids **1–3** exhibited appreciable inhibition of mycelial growth with variable activities depending on Fod race.

Experimental

The HR-ESI-MS spectra were measured on Bruker Daltonics Micro TOFQ (Bruker, Germany). NMR spectra were measured on a Bruker AV-500 spectrometer

Table 2 Fungitoxic activity of compounds **1–3** and rutin at different concentrations towards Fod races

Pathotype	Conc/ μ M	Rutin	1	2	3
Fod race 2	35	-10 ± 3.1^a	1 ± 0.5^b	$15 \pm 2.3^{c,d,e,f}$	$19 \pm 2.5^{b,g}$
	350	-5 ± 2.0^b	$9 \pm 1.4^{b,c,d,e}$	$20 \pm 2.2^{d,e,f,g}$	$29 \pm 3.2^{f,g}$
	700	$12 \pm 2.9^{b,c,d}$	$11 \pm 2.0^{b,c,d,e}$	$23 \pm 4.2^{e,f,g}$	36 ± 2.7^g
Fod race 4	35	$15 \pm 2.4^{a,b}$	$32 \pm 1.3^{c,d}$	$26 \pm 4.1^{b,c}$	$27 \pm 6.5^{b,c}$
	350	$27 \pm 5.9^{b,c}$	$50 \pm 8.5^{e,f}$	$45 \pm 6.2^{e,f}$	$44 \pm 6.3^{d,e}$
	700	$53 \pm 6.8^{e,f}$	$57 \pm 5.5^{e,f}$	$51 \pm 4.6^{e,f}$	$56 \pm 4.3^{e,f}$
Fod race 8	35	1 ± 1.7^a	22 ± 4.6^c	2 ± 0.5^a	$9 \pm 2.7^{a,b,c}$
	350	$10 \pm 5.3^{a,b,c}$	$45 \pm 3.1^{d,e}$	$43 \pm 6.5^{d,e}$	$21 \pm 7.9^{b,c}$
	700	$7 \pm 3.3^{a,b}$	$48 \pm 7.2^{d,e}$	$55 \pm 5.9^{d,e,f}$	43 ± 4.0^d

Data indicated mycelial growth inhibition percentages with respect to untreated control (0 % inhibition). Values are means of four replicates \pm standard error. For each race of Fod, values with the same letter are not significantly different for $P = 0.05$ according to the Student–Newman–Keuls test. Values were arcsine transformed before the analysis

(Bruker, Germany) with tetramethylsilane (TMS) as the internal reference, and chemical shifts are expressed in δ (ppm). Semi-preparative HPLC (Shimadzu, Japan) was performed using a Japanese liquid chromatograph equipped with a EZ0566 column. The UV spectra were recorded on a Shimadzu UV-2201 spectrometer (Shimadzu, Japan). The IR spectra were recorded in KBr discs on a Thermo Nicolet 200 double-beam spectrophotometer (Shimadzu, Japan). Column chromatography was performed using silica gel (200–300 mesh, Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Fractions were monitored by TLC (silica gel GF₂₅₄ 10–40 μ m, Marine Chemical Factory, Qingdao, China), and spots were visualized by heating silica gel plates sprayed with 10 % H₂SO₄ in EtOH.

Plant material

The leaves of *A. membranaceus* were collected in Xinganling, Inner Mongolia of China, in August 2013, and identified by Prof. Buhebateer (Inner Mongolia University for Nationalities). A voucher (No. 20130628) has been deposited in the School of Traditional Mongolian Medicine of Inner Mongolia University for Nationalities.

Extraction and isolation

The leaves of *A. membranaceus* (3.0 kg) were powdered and extracted twice under reflux with 30 dm³ 95 % EtOH. Evaporation of the solvent under reduced pressure delivered the 95 % EtOH extract. The extract was partitioned with petroleum ether (P.E.), CHCl₃, EtOAc, and *n*-BuOH. The EtOAc-soluble fraction (28.0 g) was isolated by column chromatography on silica gel and gradiently eluted with CHCl₃-CH₃OH (60:1–10:1) to give five fractions

(fractions 1–5). Fraction 4 (200 mg) was further chromatographed on Sephadex LH-20 column eluting with MeOH, and then separated by semi-preparative HPLC (CH₃OH–H₂O, 39:61) yielding **1** (28 mg), **2** (32 mg), and **3** (40 mg).

Biological assays

Fungitoxic activity of flavonoids **1–3** and commercial rutin (Sigma-Aldrich, MO) was evaluated by means of the poisoned medium technique [22]. Three different pathotypes of Fod, obtained by Prof. Burie (Inner Mongolia University for Nationalities, China), were employed in this trial: Fod race 2 (isolate 2–75), Fod race 4 (isolate 1.06.04), and Fod race 8 (276). Weighted amounts of each tested compound were aseptically added, after dissolution in 200 cm³ of sterile H₂O and ultra-filtration (0.1 μ m), to flasks containing 20 cm³ of sterilized potato dextrose agar (PDA), when still molten, to reach the final concentrations of 700, 350, and 35 mM. The medium was then poured into 35 mm i.d. Falcon culture dishes and allowed to solidify. A mycelial disc (2 mm i.d.) taken from 7-day-old culture was inoculated to each Petri dish. Plates containing non-poisoned medium served as control. Mycelial diameters, in control as well as in treatment sets, were recorded after incubation for 72 h at 26 °C. The data obtained on mycelial growth were pooled from four replicates and subjected to one-way ANOVA, after arcsine transformation. Treatments means were compared using critical difference at $P = 0.05$; similarity groups were determined using the Student–Newman–Keuls post hoc test.

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