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Flavonol glycosides from the leaves of *Astragalus microcephalus*

Fatemeh Fathiazad[1*], Ali Movafeghi[2], Mohammad karim Khosropanah[2,3]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran*

[2]*Department of Plant Biology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran*

[3]*Department of Biology, Faculty of Science, Islamic Azad University of Sanandaj, Sanandaj, Iran*

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Abstract

Four new flavonol glycosides were isolated from the leaves of *Astragalus microcephalus*. The structures of the new compounds were established as Isorhamnetin-3-O- [β-D-apiofuranosyl (1→3)]-β-D-glucopyranoside (Microcephalin I), Isorhamnetin-3-*O*- [α-L-rhamnopyranosyl (1→3)]-β-D-glucopyranoside (Microcephalin **II**), Their strucrures were totally elucidated based on spectroscopic methods, 1D- (1H, 13C, 13C DEPT) and 2D- NMR experiments as well as UV spectra analysis..

*Corresponding Author: Fatemeh Fathiazad ✉ fathiazad@tbzmed.ac.ir

Introduction

Astragalus L. is the largest genus in the Leguminosae family and one of the largest genera in the vascular plants, comprising about 2500 species of herbs or shrubs, mostly perennial, widely distributed throughout the temperate region of the world.). Iran is one of the largest centers of diversity for the genus. It has nearly 750 species and an endemism rate of nearly 50% (Maasoumi, 1998; Podlech, 2001; Ghahreman et al., 2002; Podlech *et al.*, 2002; Podlech *et al.*, 2003).

Astragalus microcephalus, another example of the "tragacanthic" astragali of the Old World and representative of subgenus *Tragacantha* Bunge, is endemic to the eastern Mediterranean region and the Middle East. The tragacanths are characterized by few, sessile flowers and inflorescences, generally unilocular fruits, and thorns consisting of persistent petioles and rachises. "Gum tragacanth", an exudate of branches and roots used in the manufacture of food and industrial products because of its valuable hydrophilic and colloidal properties, is obtained from several tragacanthic species of *Astragalus* occurring principally in the mountain regions of Iran, Iraq, Syria, and Turkey.

In recent years flavonoids have drawn attention the interest of researchers because they show promise of being powerful antioxidants that can protect the human body from free radicals for their hydrogen radical donating abilities (Cao et al., 1997; Lemańska et al., 2001). Flavonoids are an important group of secondary metabolites, which are synthesized by plants as a result of plant adaptation to biotic and abiotic stress conditions (infection, wounding, water stress, cold stress, high visible light) (Harborne *et al.,* 2000). Mostly flavone and flavonol glycosides and their aglycones displaying different biological activities were isolated from Astragalus spp. The most interesting properties of flavonoids are their antioxidative, vasodilatory and antimicrobial traits (Cook *et al.,* 1996).

Considerable information is available on the flavonoids of Astragalus (Norris *et al.,* 1970; Cui *et al.,* 1991; Benbassat *et al.,* 1995) and these compounds are implicated in pharmacological activity (Marichcova et al., 1984). As A. microcephalus growth widely in Iran, it was thus interesting to study the flavonoid content of this species.

Previous phytochemical study on this genus led to the isolation and characterization of a series of cycloartane- type triterpenic saponins from roots of *Astragalus caspicus* Bieb. (Fathiazad *et al.,* 2010). In our continuing studies on the chemistry of *Astragalus* species, we investigated the flavonoids from the leaves of Astragalus microcephalus, endemic perennial herb in suburb of Tabriz (Iran). From a phytochemical point of view, according to literatures, the flavonoids in the genus Astragalus have been extensively studied. Previous studies showed a large diversity of flavonol glycosides based on kaempferol, kaempferide, quercetin, rhamnocitrin, rhamnetin, myricetin (Semmar et al., 2005) and isorhamnetin (Ozipek *et al.,* 2003) as flavonol derivatives (R1,2), apigenin and its glycosides as flavone derivatives, daidzein, daidzin, ononin, calycosin, pseudobaptigenin, genistein, genistin and pratensein as isoflavone derivatives and makiain as pterocarpan derivative (Pistelli *et al.,* 2003).

In this study for the first time we report the isolation and structure elucidation of four flavonoids from the leaves of A. microcephalus. Total flavonoid content was to be 0.72% in the leaves.

Materials and methods

General

All solvents used for column chromatography were of analytical grade from Merck. The solvents used for HPLC were of HPLC grade from Merck and Methanol-d_4 was from Merck. 1H, 13C-NMR were recorded on Bruker (DRX-500 Avance) NMR spectrometer.

Plant material

Leaves of *Astragalus microcephalus* were collected from Payam area, East Azerbaijan province of Iran and authenticated by Dr.Movafeghi, professor of Botany. Voucher specimens have been deposited at the herbarium of the Department of Botany, Faculty of Natural Sciences, University of Tabriz, Iran.

Extraction and Isolation

The air-dried and powdered leaves (300g) were exhaustively extracted (three times) with hexane, dicloromethane and methanol (48 h, 1l each) successively by maceration at room temperature. Solvents were evaporated at 40 °C under reduced pressure and affording the hexane extract (1g), dicloromethane extract (2g) and methanol extract (27g).

Compounds 1 and 2 were obtained from methanol extract. The methanol extract (2g) was fractioned by SPE using C18 cartridge (Sep-Pak, Waters, Ireland) as the stationary phase eluting with step-gradient H2O-MeOH mixtures (9:1, 8:2, 6:4, 4:6, 2:8, each 200 ml) and MeOH (300 ml) to gives five fractions (A-F). Compounds 1 was purified from fraction C using preparative HPLC. At first, fraction C was further fractionated with preparative HPLC. Preparative HPLC analyses were performed on a Shimadzu LC-8A liquid chromatograph (Shimadzu, Japan), consisting of a dual pump, RP-18 column (20mm×250mm I.D., 5 µm, Shimadzu, Japan), a photodiode-array ultraviolet detection (DAD) system at 220nm, 280nm and 330nm, the mobile phases consisted of water (A) and methanol (B) using a linear gradient elution of 10–45% B at 0–50 min and flow rate 20 ml/min. Three fractions were collected: F-1, F-2 and F-3 according to chromatogram each consists of peaks with retention times of 1 to 15min, 15 to 20min and 20 to 40min respectively. Compound 1 was purified from subfraction F-2 by further preparative HPLC using the same column. The elution was performed by linear gradient combining a solvent A (water) and a solvent B (MeOH):15–25% B from 0 to 50 min and flow rate was 20 ml/min. A peak fraction (Rt=26min) was manually collected and solvents were evaporated *in vacuo* to yield compound 1(11mg).

Compounds 2 was purified from methanol extract by Si-gel CC with $CHCl_3$: EtOAc: MeOH (80:20:10 to 10:90:17) to yield eight fractions (F_1-F_8). F_6 and F_7 were combined and further submitted to preparative TLC using EtOAc: MeOH: H_2O (75:15:10) as developing system to give compound 2 (10mg) with R_f value of 0.28. The bands were revealed under UV by quenching at 254nm.

Results and discussion

Identification of compounds

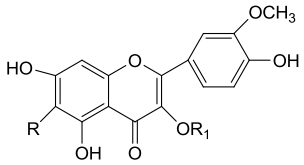
In this study, for the first time we investigated the flavonoids of the leaves of Astragalus microcephalus endemic in North West of Iran.

Methanol extract of leaves of this plant was fractionated with RP-18 Sep-pak cartridge and Si-gel column chromatography and then purified by preparative HPLC and preparative TLC resulted in isolation of four flavonoids.

Compound 1 was obtained as a white amorphous powder. The UV absorbtions (UV λ_{max} 254, 268 sh, 353) suggested 1 to be a flavonol. The UV spectrum and its changes in the presence of diagnostic shift reagents (Mabry et al., 1970; Markham et al., 1975) pointed to the presence of free hydroxyl groups at C5, C7 and C4' of a 3-substituted flavonoid glycoside framework. In the ^{1}H NMR spectrum of 1 the two meta-coupled doublets at δ 6.23(J=2.0Hz) and δ 6.41(J=2.0Hz) in the aromatic region indicated H-6 and H-8 respectively. The ^{1}H NMR resonances at δ 7.95(d, J=2.0Hz), δ 7.6(d, J=8.0/2.0Hz) and ^{1}H δ 7(d, J=8.0Hz) were attributed to H-2′, H-6′ and H-5′, respectively. Additionally, the singlet at δ 3.98 in the ^{1}H NMR spectrum was indicative for the presence of a methoxy group which showed a correlation with C-3' at

δ149.59. Additionally, the ^{1}H NMR spectrum revealed two anomeric proton signals at 5.46 ppm (s) and 5.71 (d, *J* =5Hz) indicative of two β-linked sugar units. These were respectively correlated to the carbons at δ 109.54 and 100.06. This observation was supported by the ^{13}C NMR spectra data. The ^{13}C NMR spectrum contained 26 signals, 15 of them were attributed to the flavonol aglycone and verified the isorhamnetin structure, while the remaining 11 resonances were due to a pentosyl and a hexosyl units (Table 2). Two anomeric carbons at δ 109.54 and 100.06, indicating the presence of two sugar moieties. The chemical shifts of the signals assigned to the sugar moieties revealed the presence of glucose and an apiose unit. In the ^{13}C NMR spectrum the carbon signal at 109.54 ppm ppm and the CH2 signal (DEPT) resonated at 65.48 ppm were assigned to the anomeric and C-4 of the apiose moiety, respectively, (Tables 1 and 2). The carbon signal at 100.06 ppm was assigned to the anomeric carbon of the glucose moiety. The rest of the sugar carbons were assigned by comparison with the published data (Markham and Mabry, 1975; Markham *et al.,* 1982).

According to the findings, compound 1 was designated as Isorhamnetin-3-O- [β-D-apiofuranosyl (1→3)]-β-D-glucopyranoside and named microcephalin I (Fig. 1). The striking similarity of UV and ^{1}H, ^{13}C NMR between 1 and 2 suggested a close similarity in their structure.



1 R=H R_1=(3'-O-apiosyl) glucose
2 R=H R_1=(3'-O-rhamnosyl) glucose

Fig. 1. Chemical structure of compounds 1 and 2.

Compound 2 (microcephalin II) was isolated as a pale yellow solid. Its UV spectrum exhibited characteristic absorbance bands of flavonols at 352, 254 and 268 (sh) nm. Its changes in the presence of diagnostic shift reagents (Mabry and Markham et al., 1970; Markham and Mabry, 1975) revealed the presence of a flavonol skeleton with free hydroxyl groups at 5, 7, 4' and 3-substituted flavonoid glycoside.

Table 1. ^{1}H NMR assignments of aglycon moieties of compounds **1-2** (500MHz, δ ppm, in CD_3OD).

Position	Compound	
	1	**2**
2	-	-
3	-	-
4	-	-
5	-	-
6	6.23 S	6.21 (d)
7	-	-
8	6.41 S	6.42 (d)
9	-	-
10	-	-
2′	8.1 (d)	8.1 (d)
3′	-	-
4′	-	-
5′	6.94 (d)	6.95
6′	7.61 (dd)	7.55 (d)
OCH_3	3.97	3.9

The ^{1}HNMR spectrum of 2 showed signals for five aromatic protons (δ 8, d, J=2Hz, H-2'; δ 7.55, dd, J=8/2Hz, H-6'; δ 6.95, d, J=8Hz, H-5'; δ 6.42, d, J=2Hz, H-8; δ 6.21, d, J=2Hz, H-6). Also present in this spectrum was a signal for the protons of a methoxy group at δ 3.9 ppm (3H, s) which showed a correlation with the carbon resonance at δ 149 (C-3') in the HMBC spectrum. These data clearly confirmed the characteristic pattern of isorhamnetin as aglycone. In addition, two anomeric protons were also confirmed by two signals at δ 5.8 (1H, d, J=8Hz, H-1") and δ 5.6 (1H, d, J=8Hz, H-1'").The identification of the sugars moieties as β-D-glucopyranose and α- L-

rhamnopyranose were determined from the chemical shifts, multiplicity of the signals and absolute values of the coupling constants in the ^{1}H NMR as well as 13C NMR. A methyl signal, as a doublet at δ 1.2 ppm (J=6.5, 3-H) was visible in the proton NMR spectra of 2, which is therefore assigned to a 6-deoxy sugar (rhamnose). In HMBC cross peaks of H-1" (δ 5.8)/C-3 (δ 133.45) and H-1"' (δ 5.6)/ C-3" (δ 74.60) were detected, suggesting the connectivity of C-1"' rhamnopyranose moiety to C-3" of the glucopyranose moiety and C-1" to C-3, respectively. The structure of **2** was unambiguously elucidated to be Isorhamnetin-3-*O*- [α-L-rhamnopyranosyl (1→3)]-β-D-glucopyranoside.

Table 2. ^{13}C NMR assignments of aglycon moieties of compounds 1-2 (500MHz, δ ppm, in CD_3OD).

Position	Compound	
	1	**2**
2	157.28	160.3
3	133.74	133.45
4	178.31	178.43
5	163.0	162.16
6	99.5	99.78
7	165.0	164.76
8	98.3	93.67
9	157.16	157.4
10	105.0	104.0
1′		
2′	113.5	113.6
3′	149.59	149.0
4′	147.43	147.46
5′	114.9	115.19
6′	122.3	122.25
OCH3	56.07	56.9

Table 3. ^{1}H NMR and ^{13}C NMR data of the sugar proton of compounds 1-2.

	Compound			
Position	**1**		**2**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1″	5.71	100.06	5.8	101.77
2″	3.7	74.45	3.7	76.83
3″	3.6	100.06	4.0	74.6
4″	3.7	69.45	3.7	68.1
5″	3.9	76.96	3.7	76.5
6″	4.2,3.8	61.26	3.8,4.0	66.1
1‴	5.46	109.5	5.65	100.87
2‴	4.2	76.0	4.6	72.8
3‴	1.7	79.96	4.6	71.43
4‴	3.7,4.0	75.5	4.1	74.2
5‴	3.5,3.7	65.48	4.0	69.54
6‴			0.9	16.37

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