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Original article

Secondary metabolites from the roots of *Astragalus maximus*

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Abstract

Background and objectives: *Astragalus* is one of the most abundant genera of flowering plants in Iran. There are a few reports on phytochemical investigation of this valuable genus. Saponins, flavonoids and polysaccharides have been reported as the most important metabolites in *Astragalus* species. In the present research, we aimed to identify the foremost constituents of *Astragalus maximus*. **Method:** Phytochemical analysis of the ethyl acetate (EtOAc) fraction of *Astragalus maximus* roots was performed using different methods of chromatography such as HPLC, SPE and preparative TLC. The structures of the isolated compounds were elucidated on the basis of extensive spectral evidence from 1D and 2D NMR including DQF-COSY, HSQC, HMBC, and DEPT, in comparison with reported values in the literature. **Results**: Analysis of the extract yielded three flavonoids namely liquiritigenin, formononetin, isoquercitrin and one acylated cycloartane-type saponin, astragaloside I. **Conclusion**: According to the results of our study, cycloartane-type saponin and flavonoids were the important metabolites in *A. maximus*.

Keywords: *Astragalus maximus*, cycloartane-type saponins, flavonoids, isoquercitrin, liquiritigenin

Introduction

Astragalus maximus belongs to the family Fabaceae (also called Leguminosae). *Astragalus* L. is the most abundant genus of the family comprising about 2000–3000 species of herbaceous and shrub species, mostly perennial, with more than 250 taxonomic sections in the world [1,2]. This genus is widely distributed throughout the temperate region of the world. Till date, about 800 species of *Astragalus* L. have been identified in rangelands and mountainous regions of Iran [3]. Numerous *Astragalus* species have been long used in traditional medicine particularly in Asia in variety of disorders. *Astragalus radix*, the dried roots of *Astragalus* species is a well-known tonic or adaptogenic herbal remedy that is widely applied to promote general health [4]. The plants have also been used for treatment of diabetes, nephritis, leukemia, stomach ulcers, hypertension and chronic bronchitis [5].

The biological functions of various phytochemicals from *Astragalus* L. have been extensively studied by many researchers [6-8]. The principal active ingredients were proved to be saponin glycosides, flavonoids and polysaccharides [9-13]. According to the systematic review by a Chinese scientists on the chemical constituents of the plants (genus

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Astragalus L.) more than 140 cycloartane-type triterpene glycosides, 60 flavonoids and 18 different polysaccharides have been identified so far [14].

In pharmacological studies, such ingredients as well as crude extracts of *Astragalus* species have demonstrated anti-cancer [15], immunostimulant [16], antihypertensive [17], neuroprotective [18], hepatoprotective [19], antimicrobial [20-22], antiviral [23], cardioprotective [24,25] and antiaging activities [7,26].

Despite the vast distribution of *Astragalus* species in Iran, there have been few investigations on the chemical constituents of the plants. In the course of our study on *Astragalus* genus we previously reported four saponins from *A. caspicus* and two flavonoids from *A. microcephalus* [26,27]. Herein, we have now reported the isolation and structure elucidation of the major saponin and flavonoids from *Astragalus maximus* roots native to northwest of Iran. It is worth to mention that this is the first report on phytochemical study on *A. maximus*.

Experimental

Materials and equipment

1D and 2D NMR spectra were acquired on a Bruker avance spectrometer (^{1}H, 400 MHz and ^{13}C, 100 MHz) at ambient temperature using a Topspin software package, chemical shifts have been given in δ (ppm), and coupling constants (J) have been reported in Hz. Analytical and preparative (0.9 mm thickness) TLCs were carried on pre-coated silica gel 60 F_{245} (Merck, Germany); VLC was performed on silica gel 60 (Merck, Germany), SPE was performed on Sep-Pak C_{18} cartridge (10g, Waters, Ireland). HPLC consisted of two pumps, DAD (Shimadzu, LC 20A, Japan) and reversed-phase preparative column (Ship-pack, CRP-C18, I.D.250×20 mm, particle size 10µm). All solvents were of analytical grade. Acetonitrile was HPLC grade from Merck (Germany).

Plant material

According to the previously published papers [28,29], roots of the plant were harvested in late July 2014 from Arasbaran area in East Azerbaijan, Iran and were authenticated at the Herbarium of Agriculture and Natural Resources Research and Education Center, Tabriz, Iran, where a voucher specimen was deposited (Registeration No. 8300).

Extraction and isolation

Air-dried powdered roots of *Astragalus maximus* (500 g) were defatted with petroleum benzene and extracted with 70% MeOH (2 L×3) by maceration at room temperature. After evaporation of MeOH *in vacuo*, the resultant aqueous residue was partitioned with EtOAc followed by *n*-butanol to give the respective residues (15.6 g, 76.5 g). The EtOAc extract (2 g) was subjected to vacuum liquid chromatography (VLC) on silica gel eluted with $CHCl_3$:MeOH (95:5) to $CHCl_3$:MeOH (5:95) each 200 mL and MeOH 300 mL, to give 18 fractions, the process was done in triplicate to acquire sufficient amounts of fractions. Fraction 5 (178 m) eluted with $CHCl_3$:MeOH (75:25), yielded a mixture of two compounds which was further subjected to Sephadex LH 20 and eluted by MeOH to afford pure compounds **1** (9 mg) and **2** (24 mg). Fraction 7 (480 mg) was partitioned on Sep-Pak (RP-18, 10 g, Waters, Ireland) eluted with step-wise gradient of MeOH:H_2O (2:8 to 10:0) each 200 mL to give five subfractions. Compound **3** was purified from subfraction 2 by preparative-HPLC using acetonitrile (A) and water (B) as eluents. HPLC was performed on C18 column (250×20 mm, 10 µm) using a gradient elution of 20% to 55% solvent A in B at 0-50 min and flow rate of 15 mL/min. Detection wavelength was either 260 or 330nm. The peak at Rt= 19.3 min was collected. The solvent was removed under vacuum to furnish pure compound **3** (16 mg).

Fraction 14 (571 mg) eluted with $CHCl_3$:MeOH (30:70) was qualified as a saponin-rich fraction on TLC using pre-coated plate of silica gel 60 F_{254} and $CHCl_3$:MeOH (13:7) as eluent. Compound **4** (18 mg) with R_f value of 0.64 was isolated from this fraction through preparative TLC of fraction 14 on silica gel 60 plates (0.9 mm thickness) eluted with $CHCL_3$:MeOH: H_2O (13:7:1) as the mobile phase. Detection was performed with spraying ethanolic H_2SO_4 10%

solution on uncovered part of the plates. The saponin corresponding spot was scraped off from covered parts of the plates according to the position of colored spot and extracted with methanol to afford compound **4** after filtering and evaporating to dryness.

Results and Discussion

EtOAc extract of *A. maximus* was investigated to identify the foremost secondary metabolites. The extract was analyzed with different chromatographic methods to give compounds **1-4**. Three flavonoids and one cycloartane-type saponin were isolated from EtOAc extract.

Compound **1** was obtained as a white amorphous powder. The molecular formula of $C_{15}H_{12}O_4$ was referred from ^{13}C NMR. The UV spectrum that showed λ_{max} at 276 nm (315 nm, shoulder) in conjunction with ^1H NMR spectrum confirmed it to be a flavonoid. This suggestion was confirmed with HMBC, HSQC, ^1H-^1H COSY and DEPT spectrum of compound **1**. In DEPT 135 a negative signal at δ 43.55 was indicative of CH_2 at position 3. The correlation between H-3a and H-3b with C-4 was clearly observed in HMBC spectrum. According to all these data and in comparison with the reported literature values the structure of liquiritigenin [30] was proved for compound **1.** With reference to Li *et al.*, liquiritigenin has been isolated from *A. membranaceous* revealing its inhibitory activity on lipopolysaccharide-stimulated bone marrow-derived dendritic cells with IC_{50} of 6.1 μM [8].

Compound **2** was a pale yellow amorphous powder. The molecular formula was determined as $C_{16}H_{12}O_4$ according to ^{13}C NMR. The UV spectrum displayed isoflavone characteristic maximum absorption at 253 nm and a shoulder at 298 nm. The isoflavone structure of this compound was elucidated with NMR data (table 1). The structure of compound **2** was suggested to be formononetin which was in agreement with literature [31]. In a number of previously published papers, formononetin was isolated and reported from the roots of *A. membranaceus, A. taipaishanensis* and *A. mongholicus*; in addition, the potential antioxidant activity of formononetin had been attained in several different methods via DPPH, ABTS, FRAP and lipid peroxidation inhibition assays [8,31,32].

Compound **3** was obtained as a yellow powder with molecular formula of $C_{21}H_{20}O_{12}$ which was established based on ^{13}C NMR. The UV spectrum showed absorption maxima at 252 nm and 361 nm. The ^1H NMR spectrum displayed typical signals corresponding to a di-substituted B-ring flavonol glycoside. The ^1H NMR also exhibited a meta- coupled signals at δ 6.22 and δ 6.39 represent H-6 and H-8 of ring A. This hypothesis was further proved with HSQC, COSY, HMBC and DEPT spectra. The ^1H NMR indicated a doublet signal at δ 5.27 for anomeric proton which was subsequently confirmed with HSQC in which the correlation of this signal with C-1 signal (δ 102.9) of sugar moiety was evidenced. In HMBC spectrum, correlation between anomeric proton signal with C-3 at δ 134.2 indicated that the sugar was attached to C-3 of the aglycon. Assignments of the whole carbons and protons were deduced from 2D NMR spectra, and therefore the structure of compound **3** was determined as Quercetin-3-*O*- glucoside (isoquercitrin) which was in agreement with published data [33]. Having reviewed phytochemicals in *Astragalus* genus, it was found that isoquercitrin had been previously isolated from the aerial parts of *A. asper* and *A. corniculatus* [34,35].

The structure of compound **4** was evidenced based on comprehensive 2D NMR spectra. Data from 1H NMR and ^{13}C NMR have shown in table 2 and 3. The molecular formula of $C_{45}H_{72}O_{16}$ which was secured from ^{13}C NMR and COSY indicated the characteristic features of a cycloastragenol triterpene as aglycon. The data from ^{13}C NMR revealed 45 carbons, 30 of which were assigned for cycloartane-type aglycone, 11 signals were attributed to two sugar moieties including one pentose and one hexose and 4 resonances were assigned for two acetyl groups (δ 19.43, 19.58, 170.75, 170.07). The ^1H NMR spectrum displayed seven tertiary methyl groups at δ 15.17, 18.82, 19.98, 25.25, 26.23, 26.69 and 27.12 and also two characteristic AX system protons of cyclopropane-methylene (H_2-19) signals at δ 0.6 (*d*, J=4 Hz) and δ 0.29 (*d*, J=4

Table 1. ^{1}H and ^{13}C NMR data of compounds **1-3** in CD_3OD purified from *Astragalus maximus*; (δ in ppm, J in Hz)

Position	Compound 1		Compound 2		Compound 3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	5.40 *dd* (J=2.8, 13.2)	79.65	8.19s	153.44	-	157.10
3a **3b**	2.71 *dd* (J=2.8, 17.2 3.08 *dd* (J=13.2,17.2)	43.55	-	-	-	134.20
5	7.75 *d* (J=8.8)	128.48	8.09 *d* (J=8.8)	124.30	-	161.63
6	6.52 *dd* (J=1.2, 8.8)	110.40	6.97 *dd* (J=2.4, 8.8)	115.14	6.22 *d* (J=2.4)	98.62
7	-	165.48	-	163.43	-	165
8	6.37 *d* (J=1.2)	102.44	6.88 *d* (J=2.4)	101.86	6.39 *d* (J=2.4)	93.4
9	-	164.19	-	158.44		157.58
10	-	113.55	-	116.76		104.19
1´	-	129.95	-	124	-	121.66
2´	7.35 *d* (J=8.4)	127.63	7.49 *d* (J=8.6)	129.99	7.74 *d* (J=2.4)	116.13
3´	6.84 *d* (J=8.4)	114.93	7.02 *d* (J=8.6)	113.44	-	144.53
4´	-	157.57	-	159.74	-	148.48
4´-OCH_3			3.85	54.3	-	-
5´	6.84 *d* (J=8.4)	114.93	7.02 *d* (J=8.6)	113.44	6.89 *d* (J=4.8)	114.61
6´	7.35 *d* (J=8.4)	127.63	7.49 *d* (J=8.6)	129.99	7.62 *dd* (J=2.4,4.8)	121.80
-C=O	-	192.19	-	176.68		178.06
3-o-β-glucopyranosyl						
1					5.27 *d* (J=7.6)	102.90
2					3.5	74.31
3					3.45	76.70
4					3.35 *t*	69.80
5					3.25 *m*	76.99
6					3.59 *dd*, 3.74 *dd*	61.12

Hz). Additionally, two protons at δ 4.34 *d* (J=8Hz, H-1´´) and δ 4.44 *d* (J=8.4Hz, H-1´) correlated with carbon signals at δ 103.29 and δ 103.55 were considered as anomeric protons of two β-linked sugars.Moreover, inspection of COSY spectrum in conjunction with HSQC and HMBC spectra indicated two sugars to be D-xylopyranose and D-glucopyranose. Long-range correlation of H-1´ and H-1´´ with carbon signals at δ 88.93 and δ 78.71 unambiguously revealed the attachment positions to be at C-3 and C-6 of aglycon. Acylation of xylopyranosyl unit was apparent by the downfield shifts of C-2´ and C-3´ signals relative to the unacylated xylopyranosyl moiety. Thus, the main saponin component of the extract was characterized as astragaloside I [36]. Likewise, astragaloside I had been detected in *A. wiedemannianus*, *A. hareftae* and root cultures of *A. membranaceus* [36-38].

The structure of compounds **1-4** have shown in figure **1.**

Previous studies on chemical constituents of various species of *Astragalus* have proved that saponins, flavonoids and polysaccharides are the most important bioactive secondary metabolites. Cycloartane-type saponins were first identified in *Astragalus* genus and about 140 cycloartane saponins have been reported to date. Nowadays, *Astragalus* species are well-known as rich sources of saponins from which astragalosides are the most abundant triterpene saponin glycosides. .

Furthermore, flavonoids mostly isoflavones have been isolated from various *Astragalus* plants. In

recent years, pharmacological investigations have been extensively conducted to understand the effectiveness of the secondary metabolites particularly saponins and flavonoids of *Astragalus* plants.

Table 2. ^{1}H and ^{13}C NMR data of the aglycon of compound **4** in CD_3OD; (δ in ppm, J in Hz)

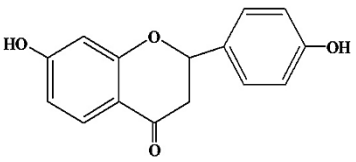
	4		
Position	**δH**	**δC**	**DEPT**
1	1.43 *m*	31.4	CH2
2	1.58,1.69 *m*	28.82	CH2
3	3.19	88.93	CH
4	-	41.42	*Q
5	1.66 *t*	51.71	CH3
6	3.54 *m*	78.71	CH
7	1.94 *m*	33.77	CH2
8	1.88	45.35	CH
9	-	20.74	*Q
10	-	28.47	*Q
11	1.32	25.60	CH2
12	1.63	32.74	CH2
13	-	44.59	*Q
14	-	45.69	*Q
15	2.10	44. 75	CH2
16	4.68 *ddd* (J=2.8, 4, 7.8)	73.27	CH
17	2.39 *d* (J=8)	57.54	CH
18	1.27 *s*	19.98	CH3
19	0.6 *d*, 0.29 *d* (J=4.4)	28.3	CH2
20	-	87.06	*Q
21	1.23 *s*	26.23	CH3
22	2.64 *q* (J=10.4)	34.07	CH2
23	2	25.42	CH2
24	3.79 *t* (J=6)	81.19	CH
25	-	71.08	*Q
26	1.28 *s*	26.96	CH3
27	1.22 *s*	27.12	CH3
28	1.152 *s*	18.82	CH3
29	0.923 *s*	15.17	CH3
30	1.042 *s*	25.25	CH3

*Quaternary carbon

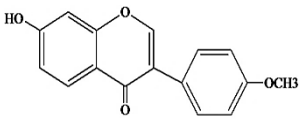
The results have clearly revealed their connections to the biological activities and have shown that flavonoids and saponins were the major contributors in pharmacological properties of *Astragalus* species. It has been shown that flavonoids together with saponins obtained from *Astragalus* species have demonstrated antioxidant, radioprotective, anti-inflammatory, hypotensive and immunoregulatory activities [39]. Accordingly, saponins and flavonoids have been used as marker compounds for quality control of *Astragalus* preparations.

Table 3. ^{1}H and ^{13}C NMR data of sugars moieties at C-3 and C-6 of the aglycon from compound **4** in CD_3OD; (δ in ppm, J in Hz)

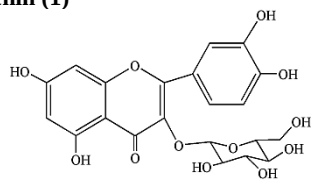
Position	**δ_H**	**δ_C**
3-*O*-xylopyranosyl		
1′	4.44 *d* (J=8.4)	103.55
2′	4.8	74.25
3′	5	77.23
4′	3.74	67.95
5′	3.94, 3.21	65.14
2′-$CH_3\underline{C}O$	-	170.07
2′-$\underline{C}H_3CO$	2.06	19.58
3′-$CH_3\underline{C}O$	-	170.75
3′-$\underline{C}H_3CO$	2.04	19.43
6-*O*-glucopyranosyl		
1″	4.34	103.29
2″	3.29	72.25
3″	3.31	75.54
4″	3.2, 3.6	70.16
5″	3.32	76.34
6″	3.87, 3.69	61.54



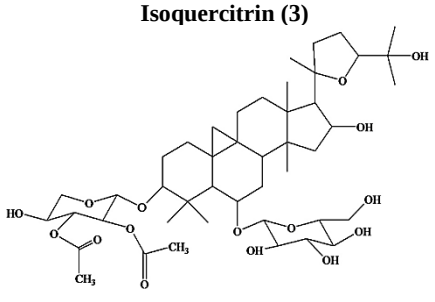
Liquiritigenin (1)



Formononetin (2)



Isoquercitrin (3)



Astragaloside I (4)

Figure 1. Structures of liquiritigenin, formononetin, isoquercitrin and astragaloside I

In the present study, formononetin (isoflavone), isoquercitrin (flavonol), liquiritigenin (flavanone) and astragaloside I (cycloartane saponin) were isolated from the EtOAc extract of *A. maximus* roots. These results were in line with the data that have been previously reported, where the isoflavones, flavonols and saponins were the prevailing metabolites in *Astragalus* genus [40].
Since there has been no systematic researches on *Astragalus* genus from Iran, we aimed to investigate the chemical components of *A. maximus* from East Azarbijan for the first time. With regard to our results, it is promising for designing further phytochemical and pharmacological studies with the goal of introducing the medicinally effective *Astragalus* species endemic to Iran.
In general, such a study can not only be helpful for researchers to identify potential new lead candidates from *Astragalus* genus for future pharmacological studies but also may serve information to rationalize the use of Iranian endemic plants as herbal drugs in traditional medicine.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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