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Article

Flavonoid Glycosides from Endemic Bulgarian *Astragalus aitosensis* (Ivanisch.)

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Abstract: *Astragalus* is a very interesting plant genus well-known for content of flavonoids, triterpenes and polysaccharides. Its secondary metabolites are described as biologically active compounds showing a number of activities, like immunomodulating, antibacterial, antiviral and hepatoprotective. This inspired us to analyze the Bulgarian endemic *A. aitosensis* (Ivanisch.) to obtain deeper information about its phenolic components. We used extensive chromatographic separation of *A. aitosensis* extract to obtain seven phenolic compounds (**1**-**7**), which were identified using combined LC-MS and NMR spectral studies. The 1D and 2D NMR analysis and HR-MS allowed us to resolve the structures of known compounds **5**-**7** as isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-(2,6-di-*O*-α-rhamno-pyranosyl-β-galactopyranoside), and alangiflavoside, respectively, and further comparison of these spectral data with available literature helped us with structural analysis of newly described flavonoid glycosides **1**-**4**. These were described in plant source for the first time.

Keywords: *Astragalus aitosensis*; flavonoid; glycoside.

1. Introduction

Genus *Astragalus* comprises from more than 2500 species, which makes it the largest genus in the family Fabaceae. *Astragalus* species are widely distributed, mainly in the temperate regions of the Northern hemisphere [1], and they are well-known and widely used as remedies in the traditional folk medicine of different countries. The pharmacological activity of flavonoids, saponins and polysaccharides – found in species from genus *Astragalus* [2] – endue these plants with immunomodulating, antibacterial, antiviral, hepatoprotective and other protective effects [3-5]. 31 species represent genus *Astragalus* in Bulgarian flora [4], and many of them are yet unstudied. In the recent years Bulgarian researchers have isolated and reported new tri- and tetraglycosides of flavonols, including some new compounds from the rarely-met group of flavo-alkaloids [3-5]. Many species of *Astragalus* possess in nature the widely-distributed aglycones – kaempferol, quercetin and methylquercetines in their free and glycosidic forms [6].

The object of this research - *A. aitosensis* (Ivanisch.) (syn. *Astracantha aitosensis* (Ivanisch.) Podlech, *Astracantha arnacantha* (M. Bieb.) Podlech subsp. *aitosensis* (Ivanisch.) Réer & Podlech), is a Bulgarian endemic plant and yet phytochemically studied very scarcely [6,7]. *A. aitosensis* is a low,

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spiny, tussock-forming shrub with strongly branched stems (30–50 cm in height) [8]. The plant grows on dry stony places (90–550 m alt) with neutral to alkaline soil. It is distributed in the suburbs of the small Bulgarian town Aytos, which gives rise to its name.

Aim of this study was to isolate compounds from the methanolic extract from aerial parts of *A. aitosensis* and preform their structural elucidation using ^{1}H, ^{13}C, COSY, HSQC, HMBC, NOESY and TOCSY NMR experiments. HR-ESI-MS was used for additional confirmation of the structures revealed by NMR. We report here the isolation and structural elucidation of six isorhamnetin and one kaempferol glycosides **1**–**7**, and four of them are new natural glycosides: three tetra- (**1**-**3**) and one triglycoside (**4**), as well, another three already known structures were determined for the first time in *A. aitosensis*: with two (**5**), three (**6**) and four (**7**) sugar units, respectively.

2. Results

2.1. Isolation of compounds

Aerial parts of *A. aitosensis* were extracted with 80% methanol under reflux. The preliminary chromatographic analysis showed a bunch of signals of flavonoid compounds, with retention times predicting a high degree of glycosylation.

The crude extract was therefore defatted by liquid–liquid partitioning with chloroform and further fractionated *via* successive column chromatography with final step of semi-preparative HPLC purification of the isolated compounds (Figure 1). Their nature (UV spectral properties) and the behavior of these compounds during separation on reversed phase – polar character of compounds - gave us the idea of flavonoid glycosides with a high glycosylation pattern.

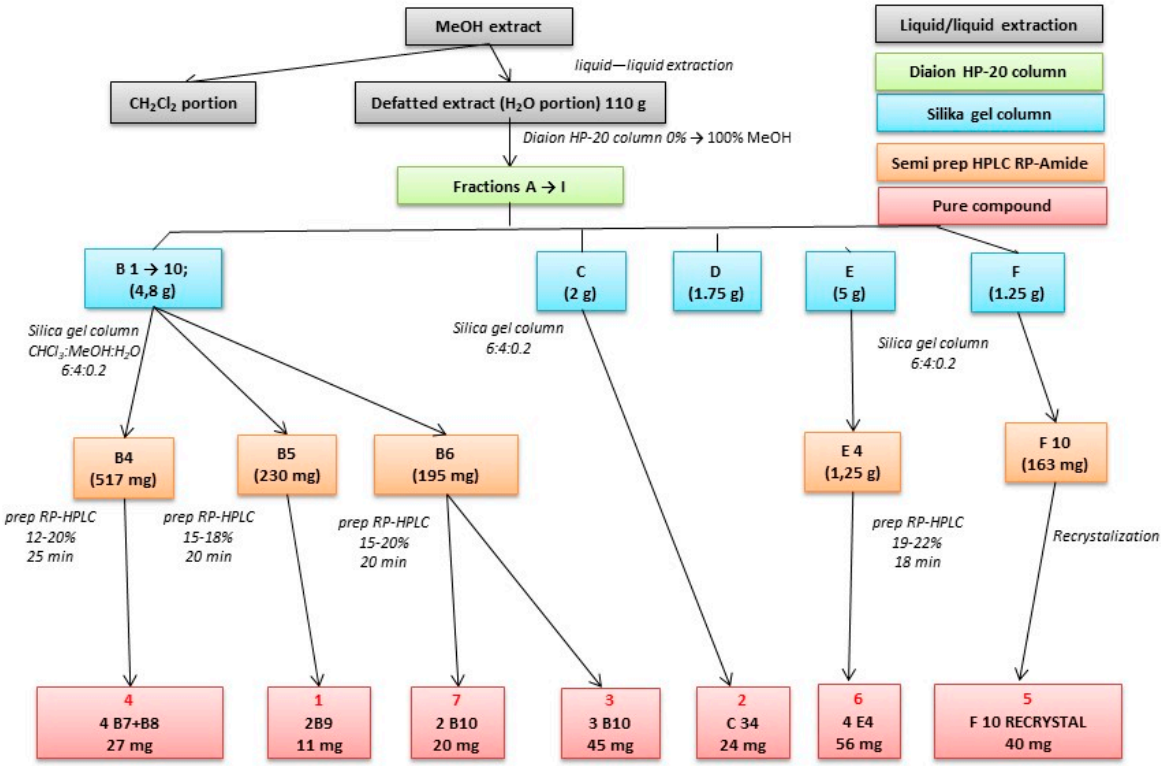

Figure 1. Simplified scheme of separation.

Our separation procedures resulted in isolation of seven pure compounds (**1**-**7**) (Figure 2).

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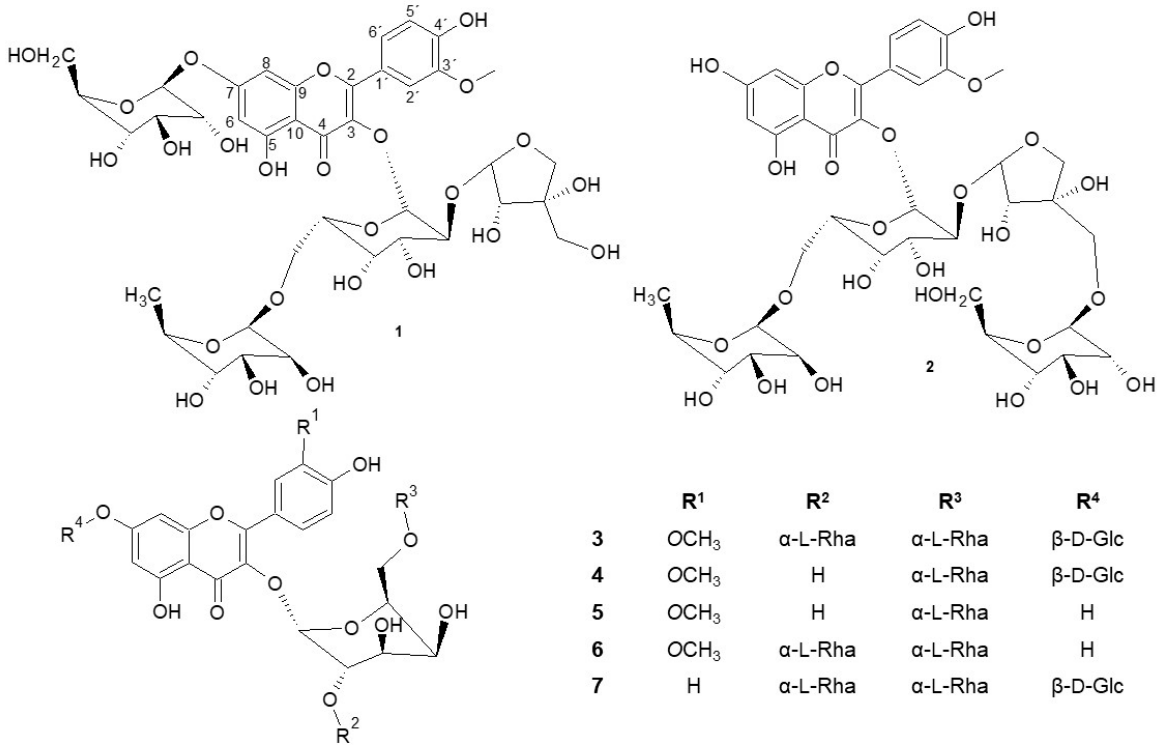

	R¹	R²	R³	R⁴
3	OCH_3	α-L-Rha	α-L-Rha	β-D-Glc
4	OCH_3	H	α-L-Rha	β-D-Glc
5	OCH_3	H	α-L-Rha	H
6	OCH_3	α-L-Rha	α-L-Rha	H
7	H	α-L-Rha	α-L-Rha	β-D-Glc

Figure 2. Structures of isolated compounds **1**-**7**.

2.2. Structural analysis

To the best of our knowledge, spectral data of four of isolated compounds (**1**-**4**) did not correspond to the data of compounds previously published in literature. Because the 1D and 2D NMR analysis, HR-MS and comparison with literature allowed us to resolve the structures of compounds **5**-**7** as isorhamnetin-3-*O*-robinobioside [9], isorhamnetin-3-*O*-(2,6-di-*O*-*α*-rhamno-pyranosyl-*β*-galactopyranoside) [10,11], and alangiflavoside [12], respectively, further comparison of these spectral data with available literature helped us with structural analysis of newly described flavonoid glycosides **1**-**4**.

The aglycones for compounds (**1**-**6**) were determined based on HR-ESI-MS and NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC) spectral analysis. ^{1}H and ^{13}C spectra are shown in Table 1. HMBC spectra showed the following significant correlations: proton at C-2′ was a doublet with *meta* coupling and in the HMBC it showed strong correlation to C-4′ and weak to C-3′. The proton at C-6′ was observed as doublet of doublet (*ortho* and *meta* coupling) and showed strong correlation in the HMBC to C-4′. The proton at C-5′ was a doublet with *ortho* coupling, displaying in the HMBC the strong correlation to C-3′ and weak to C-4′. The methoxy group showed correlation to C-3′, and therefore, the position of the methoxy is at C-3′ and the aglycone of compounds **1**-**6** was finally identified as 3′-*O*-methylquercetin, syn. isorhamnetin [13].

Because of the identification procedure, we describe the elucidation of structures of known compounds prior to the new compounds. Detailed ^{1}H and ^{13}C chemical shifts for compound **5** are listed in Table 2 and Table 3, respectively. The LC-MS analysis showed single peak (t_R 7.95 min) chromatogram with a signal of a deprotonated molecule at *m*/*z* [M-H]$^-$ 623.16205, which showed a good correlation with the calculated value of *m*/*z* 623.16176 for $C_{28}H_{31}O_{16}^-$ (Δ= 0.00029). The (+)HRMS supported the idea of this molecular formula, the glycosylation pattern was predicted to be the rhamnose and a hexose from MS/MS analysis of *m*/*z* [M+H]$^+$ 625.1175 and observed fragments *m*/*z* [M+H-rhamnose]$^+$ 479.1184, *m*/*z* [M+H-rhamnose-oxygen]$^+$ 463.1213, and finally *m*/*z* 317.0645 showing the aglycone. The molecular formula $C_{28}H_{32}O_{16}$ (calcd 624.54408) plays accord with the NMR and further HR-ESI-MS spectral data and data of compound **5** are corresponding to the reported data for isorhamnetin-3-*O*-robinobioside [9].

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101 **Table 1**. ^{13}C NMR δ_C (ppm) (100 MHz); ^{1}H NMR (600 MHz for **1** and 500 MHz for **2**). δ_H (ppm),
102 multiplicity (*J* in Hz)

	1		**2**	
	δ_C, type	**δ_H (*J* in Hz)**	**δ_C, type**	**δ_H (*J* in Hz)**
2	157.8, C		157.2, C	
3	133.6, C		133.3, C	
4	178.0, C		177.8, C	
5	161.3, C		161.6, C	
6	99.4, CH	6.45, d (2.02)	98.5, CH	6.19, d (2.00)
7	163.0, C		164.4, C	
8	94.4, CH	6.75, d (2.02)	93.4, CH	6.38, d (2.00)
9	156.5, C		157.0, C	
10	106.3, C		104.5, C	
1′	121.8, C		121.9, C	
2′	113.3, CH	8.05, m (1.94)	113.7, CH	8.04, d (1.97)
3′	147.0, C	-OCH_3	147.0, C	-OCH_3
4′	149.4, C	-OH	149.2, C	-OH
5′	114.5, CH	6.91, m (8.39)	114.5, CH	6.90, d (8.40)
6′	122.3, CH	7.62, m (1.92; 8.45)	122.1, CH	7.57, dd (1.90; 8.43)
-OCH_3	55.7, CH	4.00 s	55.7, CH3	3.98, s
3-*O*-gal				
1	99.8, CH	5.57, d (7.84)	99.9, CH	5.58, d (7.76)
2	75.1, CH	3.96, dd	74.8, CH	3.97, dd
3	73.9, CH	3.73 dd	73.9, CH	3.74 dd
4	69.0, CH	3.77 dd	69.0, CH	3.77 dd
5	74.1, CH	3.67 dt	74.0, CH	3.64 dt
6	66.0, CH_2	3.47/3.68 dd	65.8, CH_2	3.47/3.71 dd
Api (1→2)				
1	109.2, CH	5.43, d (1.55)	109.1, CH	5.44, d (1.73)
2	76.6, CH	4.01, d	76.9, CH	4.07, d
3	79.5, C	-OH	78.8, C	-OH
4	74.2, CH_2	3.64, d (9.58) 3.75, d (9.59)	74.3, CH_2	3.66, d (n/a) 4.07, d (9.70)
5	65.1, CH_2	3.64, d (11.45) 3.75 d (11.56)	61.3, CH_2	3.67, d (11.95) 3.85 d (11.90)
Rha (1→6)				
1	100.5, CH	4.50, d (1.56)	100.5, CH	4.52, d (1.53)
2	70.7, CH	3.51, dd	70.7, CH	3.57, dd
3	70.9, CH	3.46, dd	70.9, CH	3.49, dd
4	72.4, CH	3.25, pt	72.4, CH	3.26, pt

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5	68.3, CH	3.49, dq	68.3, CH	3.51, dq
6	16.5, CH_3	1.15, d (6.17)	16.5, CH_3	1.16, d (6.23)
Glc	**7-O-Glc**		**Api (5→1) Glc**	
1	100.1, CH	5.06, d (7.53)	103.4, CH	4.25, d (7.54)
2	73.3, CH	3.49, dd	73.6, CH	3.17, dd
3	77.0, CH	3.55, dd	76.3, CH	3.21, dd
4	69.9, CH	3.38, dt	70.4, CH	3.27, dd
5	76.4, CH	3.49, dq	76.3, CH	3.27, dt
6	61.1, CH_2	3.69/3.94 dd	72.9, CH_2	3.68/4.15, dd

ESI$^-$ HRAM spectrum of compound **6** showed presence of signal of deprotonated molecule *m/z* [M-H]$^-$ 769.21948 (calcd for $C_{34}H_{41}O_{20}^-$ 769.219667 m/z; Δ= 0.00019). The MS/MS analysis (a minimal fragmentation using 20 eV) in a positive mode showed the presence of a protonated parental ion *m/z* [M+H]$^+$ 787.2284 and fragments corresponding to loss of two rhamnose units and a hexose *m/z* 641.1708, 479.1180 and 317.0653. The molecular formula $C_{34}H_{42}O_{20}$ (Mr 770.68528) is in accordance with the NMR and HR-ESI-MS spectral data and ^1H and ^{13}C chemical shifts for compound **6** are listed in Table 2 and 3, respectively. Compound **6** was therefore identified as isorhamnetin-3-*O*-(2,6-di-*O*-*α*-rhamno-pyranosyl-*β*-galactopyranoside) [10,11].

The molecular formula $C_{39}H_{50}O_{24}$ (Mr 902.7999) is in accordance the NMR and HR-ESI-MS spectral data, where the mass of deprotonated molecule was found at *m/z* [M-H]$^-$ 901.2624 (calcd 901.2619; Δ=0.0005). The detailed MS analysis of fragments observed in spectrum showed signal *m/z* [M+H]$^+$ 903.2759, *m/z* [M+H-rhamnose]$^+$ 757.2178, *m/z* [M+H-rhamnose-rhamnose]$^+$ 611.1601, *m/z* [M+H-rhamnose-rhamnose-hexose]$^+$ 449.1078, and aglycone *m/z* 287.0549, with pairs of fragments showing loos of water 18 *m/z* [M+H-rhamnose-H_2O]$^+$ 741.2245, *m/z* [M+H-rhamnose-rhamnose-H_2O]$^+$ 595.1660. Spectrum showed also fragments of sugars *m/z* 309.1186 (hexose-rhamnose). ^1H and ^{13}C chemical shifts for compound **7** are listed in table 2 and 3 respectively. NMR data of compound **7** and mass spectral analysis results show a good accordance with the data already reported for alangiflavoside [12].

Based on the previously described analysis of known compounds, we tried to identify the other isolated compounds, which showed differences from that previously described in literature. Some of the fragmentation MS/MS results used for the identification of compounds **1**-**4** are depicted in Figure 3.

Table 2: ^{13}C NMR assignments (100 MHz) for compounds **3**-**7**, δ (ppm)

Compound/ position	3	4	5	6	7
	δc, type	**δc, type**	**δc, type**	**δc, type**	**δc, type**
2	157.7, C	157.4, C	156.8, C	157.0, C	158.0, C
3	133.3, C	133.8, C	133.5, C	133.0, C	133.4, C
4	177.9, C	178.0, C	177.8, C	177.8, C	177.1, C
5	161.3, C	161.3, C	161.6, C	161.7, C	161.4, C
6	99.3, CH	99.8, CH	99.2, CH	98.4, CH	99.4, CH
7	163.0, C	163.4, C	164.7, C	164.4, C	163.0, C
8	94.4, CH	95.1, CH	94.2, CH	93.3, CH	94.3, CH

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9	156.5, C	156.4, C	156.8, C	157.0, C	156.6, C
10	106.3, C	106.1, C	104.4, C	104.5, C	106.2, C
1′	121.8, C	121.3, C	121.5, C	122.0, C	121.5, C
2′	113.3, CH	113.9, CH	113.9, CH	113.3, CH	131.0, CH
3′	147.1, CH	147.5, CH	147.4, C	147.0, C	114.8, CH
4′	149.3, C	150.1, C	149.9, C	149.1, C	160.1, C
5′	114.6, CH	115.6, CH	114.6, CH	114.5, CH	114.8, CH
6'	122.1, CH	122.6, CH	122.4, CH	121.8, CH	131.0, CH
***O*CH$_3$**	56.37, CH3	56.39, CH	56.37, CH	55.79, CH3	
3-*O*-Gal					
1	99.3, CH	102.1, CH	102.3, CH	99.4, CH	99.4, CH
2	76.4, CH	74.1, CH	74.0, CH	76.4, CH	76.1, CH
3	74.2, CH	71.6, CH	71.5, CH	74.2, CH	74.3, CH
4	69.2, CH	68.4, CH	68.4, CH	69.1, CH	69.4, CH
5	74.1, CH	73.4, CH	73.4, CH	73.9, CH	74.1, CH
6	65.9, CH$_2$	65.6, CH$_2$	65.6, CH$_2$	65.7, CH$_2$	66.0, CH$_2$
Rha (1→2)					
1	101.4, CH			101.3, CH	101.2, CH
2	71.0, CH			71.0, CH	71.0, CH
3	71.0, CH			71.0, CH	70.9, CH
4	72.5, CH			72.5, CH	72.7, CH
5	68.4, CH			68.4, CH	68.4, CH
6	16.0, CH$_3$			16.0, CH$_3$	16.1, CH$_3$
Rha (1→6)					
1	100.6, CH	100.5, CH	100.5, CH	100.5, CH	100.5, CH
2	70.7, CH	70.9, CH	70.9, CH	70.7, CH	70.7, CH
3	70.9, CH	71.1, CH	71.1, CH	70.9, CH	70.9, CH
4	72.4, CH	72.2, CH	72.3, CH	72.6, CH	72.5, CH
5	68.3, CH	68.7, CH	68.7, CH	68.3, CH	68.3, CH
6	16.6, CH$_3$	18.3, CH$_3$	18.3, CH$_3$	16.6, CH$_3$	16.6, CH$_3$
7-*O*-Glc					
1	100.1, CH	100.3, CH			100.1, CH
2	73.3, CH	73. 6, CH			73.3, CH
3	77.0, CH	76.9, CH			77.0, CH
4	69.9, CH	70.0, CH			69.9, CH
5	76.4, CH	77.7, CH			76.4, CH
6	61.1, CH$_2$	61.2, CH$_2$			61.1, CH$_2$

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Table 3: ^{1}H NMR assignments (600 MHz) of compounds **3-7** δH (ppm), multiplicity (*J* in Hz)

	3	**4**	**5**	**6**	**7**
	δH (*J* in Hz)	**δH (*J* in Hz)**	**δH (*J* in Hz)**	**δH (*J* in Hz)**	**δH (*J* in Hz)**
2					
3					
4					
5					
6	6.44, d (2.07)	6.44, d (1.91)	6.19, d (1.97)	6.15, d (1.97)	6.46, d (2.15)
7					
8	6.75, d (2.09)	6.77, d (1.97)	6.42, d (1.97)	6.37, d (1.97)	6.75, d (2.14)
9					
10					
1′					
2′	8.09, m (1.93)	8.00, d (1.84)	7.98, d (1.97)	8.07, d (1.94)	8.09, m (8.95)
3′					6.90, m (8.90)
4′					
5′	6.91, m (8.44)	6.90, d (8.39)	6.88, d (8.44)	6.90, d (8.42)	6.90, m (8.90)
6'	7.57, m (1.91; 8.42)	7.54, dd (1.82; 8.48)	7.49, dd (2.03; 8.39)	7.52, dd (1.99; 8.43)	8.09, m (8.95)
***O*CH3**	3.83, s	3.84, s	3.83, s	4.00, s	-
3-*O*-Gal					
1	5.79, d (7.84)	5.47, d (7.73)	5.45, d (7.67)	5.59, d (7.83)	5.59, d (7.74)
2	3.97, dd	3.60, dd	3.57, dd	3.96, dd	3.95, dd
3	3.78, dd	3.57, dd	3.57, dd	3.76, dd	3.70, dd
4	3.80, dd	3.62, dd	3.63, dd	3.81, dd	3.75, dd
5	3.74, dt	3.42, dt	3.41, dt	3.71, dt	3.64, dt
6	3.54/3.73dd	3.30/3.60 dd	3.31/3.61 dd	3.52/3.72 dd	3.46/3.69 dd
Rha (1→2)					
1	5.16, d (1.52)			5.16, d (1.53)	5.21, d (1.32)
2	4.00, dd			4.00, dd	4.00, dd
3	3.75, dd			3.76, dd	3.78, dd
4	3.33, pt			3.32, pt	3.33, pt
5	4.04, dq			4.03, dq	4.06, dq
6	0.89, d (6.25)			0.89, d (6.23)	0.98, d (6.23)
Rha (1→6)					
1	4.53, d (1.53)	4.41, d (1.53)	4.41, d (1.53)	4.55, d (1.55)	4.49, d (1.24)
2	3.54, dd	3.37, dd	3.38, dd	3.59, dd	3.48, dd
3	3.49, dd	3.28, dd	3.28, dd	3.51, dd	3.46, dd
4	3.25, pt	3.06, pt	3.07, pt	3.26, pt	3.25, pt
5	3.52, dq	3.36, dq	3.34, dq	3.54, dq	3.49, dq
6	1.16, d (6.08)	1.04, d (6.18)	1.03, d (6.25)	1.17, d (6.22)	1.16, d (6.21)

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7-*O*-Glc			
1	5.06, d (7.24)	5.05, d (7.89)	5.07, d (7.45)
2	3.48, dd	3.24, dd	3.48, dd
3	3.53, dd	3.27, dd	3.53, dd
4	3.39, dd	3.15, dd	3.38, dd
5	3.50, dt	3.43, dt	3.49, dt
6	3.69/3.92, dd	3.44/3.68, dd	3.69/3.91, dd

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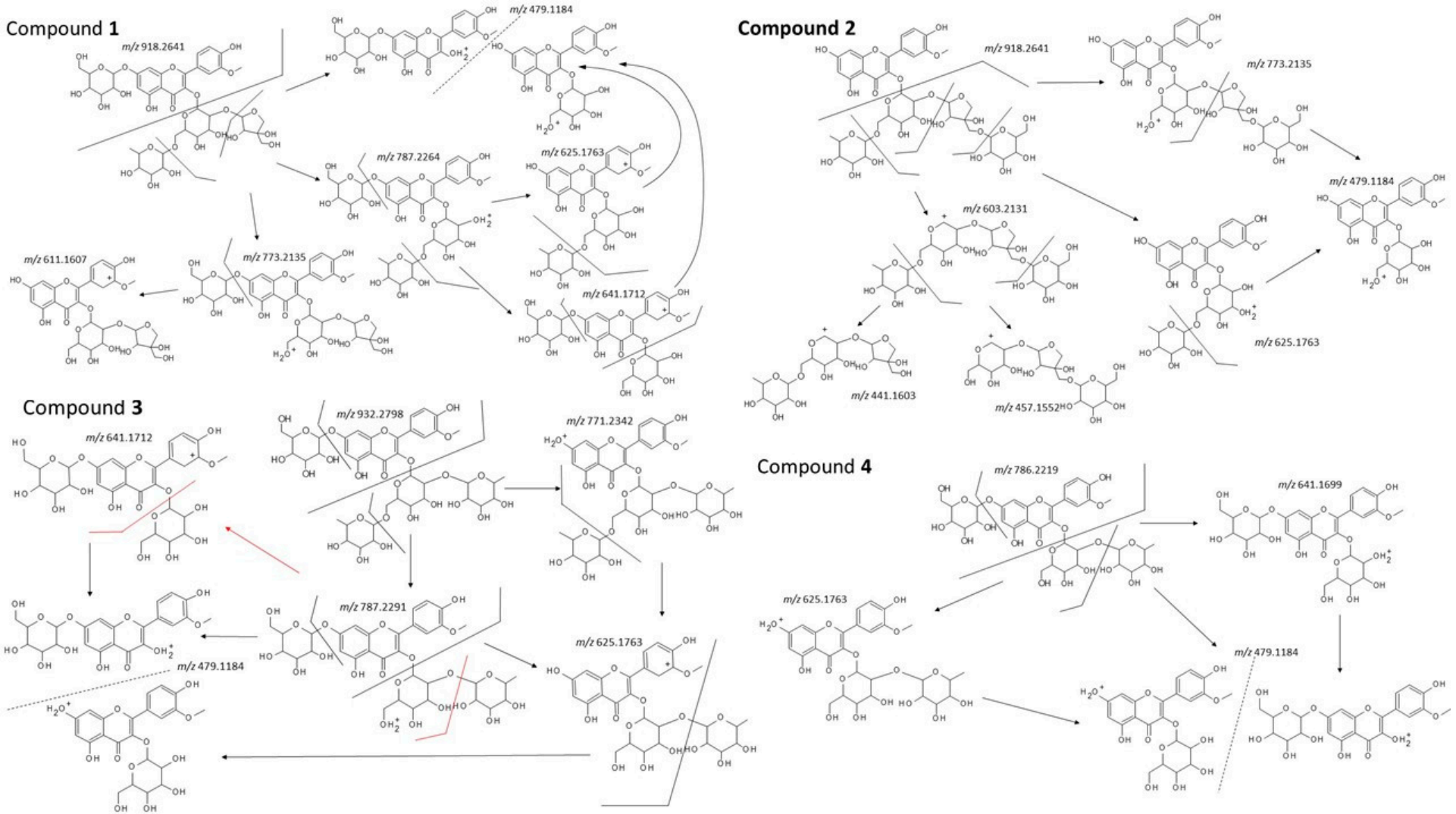

Figure 3. A fragmentation pattern of flavonoid glycosides **1**-**4** (shown *m*/*z* are calculated values according to molecular formulas of the ions).

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Compound **1** was isolated as a yellow amorphous powder. The UV spectra analysis showed a typical flavonoid course, with the retention time typical for glycosylated substances. The HR-ESI-MS in a negative mode displayed a molecular ion *m/z* [M-H]$^-$ 917.2572 (calc. for [M-H]$^-$ 917.256841), confirming the molecular formula $C_{39}H_{50}O_{25}$ with calculated molecular mass 918.7993 Da. HRESIMS showed an adduct formation *m/z* [M+HCOO]$^-$ 963.26269 (confirming the analyte), and a hexose (*m/z* 162.0235) loss leading to *m/z* 755.21208 (possible representing cleavage of glucose from 7-*O* position or galactose from 3-*O*- position of expected flavonol skeleton). Furthermore, the MS in a positive mode confirmed this loss showing precursor ion *m/z* [M+H]$^+$ 919.2705 with product ions formed either by subsequent loses of a pentose (*m/z* 787.2264), a rhamnose (*m/z* 625.1750), and a hexose (479.1170) unit, either loss of rhamnose (*m/z* 773.2136) followed by hexose (*m/z* 611.1613) moieties (see Figure 3). After lining up interpretation of NMR spectra (Table 1) with MS analysis, the structure of the compound **1** was elucidated to be the isorhamnetin substituted by four sugars. One of them is a 6-deoxyhexose (a methyl group as doublet), two are hexoses (a glucose, a galactose), and one is a pentose. DEPT spectrum of compound **1** showed 4 methylene groups. Altogether, two of them belong to each hexose (the glucose and galactose), respectively, and since a 6-deoxyhesose (expected rhamnose) does not have a methylene moiety, the two left CH_2 moieties must belong to a pentose. Four anomeric protons are found in HMBC spectrum: δ_H 5.57 ppm, (1H, d, *J*=7.84 Hz), δ_H 5.43 ppm (1H, d, *J*=1.55 Hz), δ_H 4.50 ppm (1H, d, *J*=1.56 Hz) and δ_H 5.06 ppm (1H, d, *J*=7.53 Hz), corresponding to carbon atoms with δ_C 99.8 ppm, δ_C 109.2 ppm, δ_C 100.5 ppm and δ_C 100.1 ppm from the HSQC spectrum. After complete resonance assignments, analyses of coupling constants, intensities, interpretation of cross-peaks in the COSY spectrum, and ^{13}C NMR chemical shift values, one hexose moiety was identified as a *β*-glucosyl unit, the second as a *β*-galactosyl moiety, the 6-deoxysugar was identified to be the *α*-rhamnose, and the pentose was recognized as the *β*-apiose, which contains two of the above-mentioned methylene groups. ^{13}C values of C-6 indicate that *β*-glucose residue is not connected to other sugar unit (δ_C 61.1 ppm), while the β-galactose moiety is connected at C-6 position (δ_C 66.0 ppm – shifted to a higher field). HMBC correlations allowed us to elucidate the precise structure of the sugar chains and the positions of their attachment to the aglycone. The anomeric proton (δ_H 5.06 ppm) of the β-glucose moiety showed a three-bond correlation to C-7 (δ_C 163.0 ppm) of the aglycone, while a HMBC correlation between the anomeric proton of the galactose (δ_H 5.57 ppm) and the carbon at δ_C 133.6 ppm indicated that the galactosyl unit is connected at C-3 towards the aglycone. The anomeric proton of *α*-rhamnose correlated to C-6 in the *β*-galactose (δ_C 66.0 ppm). The anomeric atom of the last pentose sugar (apiose) δ_H 5.43 ppm bonded to δ_C 109.2 ppm showed a HMBC correlation with C-2 of the *β*-galactose molecule (δ_C 75.1 ppm). The remaining two methylene groups (δ_C 74.2 ppm and δ_C 65.1 ppm) were recognized as carbons C-4 and C-5 in the apiosyl moiety. Hence, according to all interpreted spectra, we identified the compound **1** as the new natural product named isorhamnetin-3-*O*-[*β*-D-apiofuranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl]-7-*O*-*β*-D-glucopyranoside.

Compound **2** was obtained as a yellow amorphous powder. The spectral analysis showed data very similar to that observed for compound **1** (Table 1), and the only difference found was the presence of a *β*-glucosyl residue connected to the sugar chain, attached to the 3-*O* position (particularly C-5 of apiosyl residue), a connection to the 7-*O* position of the aglycone was not found. Further interpretation of HRESIMS supported this suggestion by observing a lacking of the fragment of a hexose loss (*m/z* 162.0235), which can be observed in spectra of all compounds possessing glucose attached at 7-*O* position (compounds **1**, **3**, **4** and **7**). The HR-ESI-MS in negative mode displayed a molecular ion *m/z* [M-H]$^-$ 917.25696 (calculated for *m/z* [M-H]$^-$ 917.256841), confirming molecular formula $C_{39}H_{50}O_{25}$ with calculated molecular mass 918.7993 Da. Adduct formation [M+HCOO]$^-$ (*m/z* 953.2336) and [M+Cl]$^-$ (*m/z* 963.2621) was observed in a negative ESI mode. Furthermore, MS in a positive mode confirmed this by [M+H]$^+$ with *m/z* 919.2709, and a series of corresponding losses of four sugar units at a minimal fragmentation of 20 eV: *m/z* 773.2136 as loss of rhamnosyl unit, *m/z* 625.1759 loss of rhamnose and hexose, and 479.1185 corresponding to isorhamnetin hexoside after loss of three sugar units of rhamnose, hexose and pentose. Except them, also the aglycone signal *m/z*

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317.0655 and the fragment ion *m/z* 603.2144 interpreted as the chain of all sugar moieties with corresponding fragments *m/z* 441.1610 and *m/z* 457.1552 after losses of terminal hexose and rhamnose, respectively, were detected. According to differences in chemical shifts in NMR spectral data (compared with **1**), the glucosyl moiety changed its bonding position from 7-*O* position of the aglycone to C-5 position of the *β*-apiosyl residue (see Table 3). The compound was therefore identified as isorhamnetin-3-*O*-[*β*-D-glucopyranosyl-(1→5)-*β*-D-apiofuranosyl]-(1→2)-robinobioside or [*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-galactopyranosyl], a newly described flavonoid glycoside.

Compound **3** was isolated as a yellow amorphous powder. Rt of compound **3** was 4.61 minutes, again slightly different from other isolates. The HR-ESI-MS in a negative mode displayed a molecular ion *m/z* $[M-H]^-$ 931.27352 (calcd $[M-H]^-$ 931.272491), confirming molecular formula $C_{40}H_{52}O_{25}$ with calculated molecular mass 932.82588 Da. HRESIMS showed additional adduct formation at *m/z* $[M+HCOO]^-$ 977.27862, and a hexose loss (*m/z* 162.05387) leading to *m/z* 769.21965 (representing supposed cleavage of glucose from 7-*O* position). In the HRAM ESI positive mode, $[M+H]^+$ ion with *m/z* 933.2810 (calcd $[M+H]^+$ 933.2876) was obtained. Fragmentation with collision energy of 20 eV gave ions with *m/z* 787.2229 and *m/z* 771.2324 formed by loss of hexose and rhamnose, respectively. Further subsequent loses of the hexosyl and rhamnosyl moieties resulted fragments with *m/z* 625.1679 (aglycone-rhamnosyl-hexoside) and *m/z* 641.1652 (aglycone-hexosyl-hexoside). Finally, ions of *m/z* 479.1082 (hexosylated aglycone) and aglycone *m/z* 317.0526 (supposed isorhamnetin) were found. Together with an interpretation of NMR spectra (Table 2 and 3), the structure was predicted to be composed of isorhamentin and four sugar moieties. Two of them were recognized as 6-deoxyhexoses (two methyl groups in the form of the doublet), and the other two were hexoses (possible a glucose, or a galactose). DEPT spectrum showed their two methylene groups (δ_C 61.1 ppm and 65.9 ppm) that belonged to the galactose and the glucose, respectively. We observed four anomeric protons in HSQC spectrum: δ_H 5.79 ppm, (1H, d, *J*= 7.84 Hz), δ_H 5.16 ppm (1H, d, *J*= 1.52 Hz), δ_H 4.53 ppm (1H, d, *J*= 1.53 Hz) and δ_H 5.06 ppm (1H, d, *J*= 7.24 Hz), corresponding to carbon atoms δ_C 99.3 ppm, 101.4 ppm, 100.6 ppm and 100.1 ppm, respectively. After complete resonance assignments and analyses of coupling constants, intensities of cross-peaks in the COSY spectrum, and ^{13}C NMR chemical shift values, one hexose moiety was identified as a *β*-glucosyl unit, the other as a *β*-galactosyl moiety, and the 6-deoxy sugars were found to be *α*-rhamnosyl moieties. ^{13}C values indicated that *β*-glucosyl residue is free at C-6 (δ_C 61.1 ppm), while the *β*-galactose is connected to C-6 (δ_C 65.9 ppm – shifted to a higher field). According to HMBC correlations, structure of the side chains and their attachment to the aglycone were established. Anomeric proton (δ_H 5.06 ppm) of the glucose moiety showed a three-bond correlation to C-7 (δ_C 163.0 ppm) of the aglycone, while an HMBC correlation between the anomeric proton of the *β*-galactosyl moiety (δ_H 5.79 ppm) and the carbon at δ_C 133.3 ppm indicated that the *β*-galactosyl unit is bonded at C-3 toward the aglycone. The anomeric proton of one of the *α*-rhamnose residues (δ_H 5.16 ppm) was correlated to position C-2 of the *β*-galactose (δ_C 76.4 ppm), while the anomeric proton of the other *α*-rhamnose showed correlation to C-6 of the *β*-galactose (δ_C 65.9 ppm). Methyl residues of *α*-rhamnopyranosyl residues were located in the low-field region of ^1H spectrum at δ_H 0.89 ppm, (3H, d, *J*= 6.25 Hz) for the *α*-rhamnosyl residue attached to C-2 of the β-galactose and at δ_H 1.16 ppm (3H, d, *J*= 6.08 Hz) for the *α*-rhamnosyl residue attached to C-6 of the β-galactose, respectively. Hence, we identified the compound **3** as the new natural product isorhamnetin-3-*O*-(2,6-di-*O*-*α*-rhamnopyranosyl-*β*-D-galactopyranoside)-7-*O*-*β*-D-glucopyranoside. The compound is similar to compound **7**, the difference lies in the absence of *β*-D-glucopyranosyl moiety at 7-*O* position in compound **7**.

Compound **4** was isolated as a yellow amorphous powder. Chromatographic analysis showed Rt 5.40 minutes. The HR-ESI-MS in negative mode displayed a molecular ion *m/z* $[M-H]^-$ 785.21509 (calcd for $[M-H]^-$ 785.21403), confirming molecular formula $C_{34}H_{42}O_{21}$ with calculated molecular mass 786.68468 Da. HRESIMS in negative mode showed additional adduct formation *m/z* $[M+HCOO]^-$ 831.22089, and a hexose (*m/z* 162.05404) loss leading to *m/z* 623.16174 (representing cleavage of glucose from 7-*O* position or a galactose from 3-*O*). The moderate fragmentation in positive ESI (20 eV) showed the presence of *m/z* 787.2284 for parental ion, and then the corresponding fragment with a cleavage of deoxyhexose *m/z* 641.1714, hexose *m/z* 625.1753, two sugar (rhamnosyl and hexosyl)

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238 units *m/z* 479.1178 and an aglycone at *m/z* 317.0658, possible isorhamnetin. After the interpretation of
239 NMR spectra (Table 2 and 3), we confirmed the presence of isorhamnetin and three sugar moieties.
240 Compound **4** possesses similar structure as compound **3**, with absence of the *α*-rhamnopyranosyl
241 moiety connected to C-2 of *β*-galactosyl residue, and we identified compound **4** as isorhamnetin-3-
242 *O*-robinobioside-7-*O*-glucoside, a new flavonoid glycoside.
243

244 **3. Discussion**

245 As visible from above described, we isolated 7 flavonoid compounds from *A. aitosensis* extract.
246 Their structures were elucidated by 1D (^{1}H, ^{13}C) and 2D NMR experiments (COSY, HSQC, HMBC,
247 NOESY and TOCSY) and confirmed by HR-ESI-MS. We report the structures of six isorhamnetin and
248 one kaempferol glycosides (**1**–**7**), out of which three new tetra- (**1**, **2**, **3**), one new tri-glycoside (**4**) and
249 three already known compounds with two (**5**), three (**6**) and four (**7**) sugar units, respectively.
250 Genus *Astragalus* is one of the largest genera of Fabaceae family. As mentioned, bioactivity of
251 *Astragalus* plants are connected with a presence of flavonoids, saponins and polysaccharides. The use
252 of *Astragalus* spp. is mainly connected with immunomodulation, antibacterial and antiviral activity,
253 and hepatoprotection [3-6]. The reviews of Gorai et al. [14], Bratkov et al. [5], and Li et al. [15] showed
254 an overview of *Astragalus* genera and flavonoids isolated, showing the presence of flavones,
255 flavonols, flavanones, flavan-4-ols, isoflavones, isoflavans, pterocarpans and others in 60 different
256 *Astragalus* species. Their review included also isorhamnetin and kaempferol glycosides. Another
257 comprehensive review of Bulgarian *Astragalus* species was published in 2016, similarly describing
258 the presence of several kaempferol and isorhamnetin glycosides, including alangiflavoside from *A.*
259 *monspessulanus* ssp. *monspessulanus [16, 17].*
260 The recent years Bulgarian researchers have isolated and reported new tri- and tetraglycosides
261 of flavonols, including some new compounds from the rarely-met group of flavo-alkaloids [3,5].
262 Many species of *Astragalus* possess in nature the widely-distributed aglycones – kaempferol,
263 quercetin and methylquercetines in their free and glycosidic forms [3]. *A. aitosensis* showed
264 previously presence of rutin, quercetin-3-*O*-*β*-D-glucoside and astragalin [7]. As visible from
265 comparison of glycosides isolated from *A. aitosensis* with literature, similar compounds – glycosides
266 were obtained for example from A. *monspessulanus* ssp. *monspessulanus, A. cicer and A. centralpinus*
267 [16-18]. This could from chemotaxonomic point of view confirm their close relationships.
268

269 **4. Materials and Methods**

270 *4.1. General Experimental Procedures.*
271 Optical rotations were measured on a JASCO P-2000 spectropolarimeter (Easton, MD, USA) at 20 °C
272 in MeOH with Spectramanager software.
273 HPLC used configuration of analytical system by Agilent 1100 Series (Degasser G1322A, Quaternary
274 Pump G1311A, Autosampler ALS G1313A, Column Compartment G1316, DAD G1315B, Loop 20 µl,
275 UV spectrum 200-900 nm) with column Kinetex® PFP 100 A, 250 x 4,6 mm I.D., 5 µm (Phenomenex,
276 USA), flow rate: 1 ml/min. Semi-preparative HPLC was carried out using Dionex UltiMate 3000
277 system (Pump Dionex UltiMate 3000 UPLC+ Focused, Dionex UltiMate 3000 RS Variable Wavelenght
278 Detector, fraction collector Dionex UltiMate 3000 with 6 positions, LCO 101 ECOM column oven,
279 constant temperature 40°C, autosampler Dionex UltiMate 3000, loop 100 µl), column: Ascentis® RP-
280 AMIDE, 250 mm × 10 mm, 5 µm (Supelco, USA), flow rate 5 ml/min. TLC was carried out on
281 precoated silica gel plates (Supelco Kieselgel G, F254, 60, Merck, Darmstadt, Germany) with the
282 solvent systems EtOAc:MeOH:H_2O (100:13.5:10, v/v/v). Spots were visualized under UV light (365
283 nm) after spraying with NTS/PEG reagent. Column chromatography (CC) was performed using
284 Diaion HP-20 (Supelco, USA), Ø=80 mm, height 70 cm ~ 700 g and Silica gel (40–63 µm, Sigma-
285 Aldrich®, USA) Ø=35 mm, height 60 cm.
286
287 *4.2. Plant material*

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Aerial parts of *A. aitosensis* (Ivan.) Podl (Fabaceae) (syn. *Astracantha aitosensis* (Ivan.) Podl.) was collected and identified by Hristo Vasilev in June 2015 in the suburbs of town Aytos, Bulgaria (coordinates Google maps: 42.702191 N, 27.266976 E, UTM: NH22), voucher specimen has been deposited in Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences with Ref № SOM001362.

4.3. Extraction and isolation

Dried aerial parts (3600 g) of *A. aitosensis* were extracted under reflux with 80% MeOH (20 × 1.25 L, 40 min each) at 70 °C. The total methanol extract was evaporated to dryness (128 g, 3.5 %), and it was re-suspended in H_2O (800 ml) to remove unpolar compounds by liquid/liquid extraction with chloroform (200 ml × 5), giving 18 g of $CHCl_3$ fraction and 110 g of MeOH soluble material. This defatted methanol portion was subjected to Diaion HP-20 column, and eluted with gradient system of H_2O and MeOH (100:0 to 0:100, v/v), giving 10 combined fractions assigned A-J. Fraction B, C, E, and F were further purified *via* open column chromatography (Silica gel, eluted with $CHCl_3$:MeOH:H_2O 60:40:2, v/v/v). Fraction B was re-chromatographed on silica gel ($CHCl_3$:MeOH:H_2O 6:4:0.2, v/v/v, as mobile phase,) and resulted in 6 combined fractions (B_1-B_6). Fraction B_4, was further purified using a semipreparative HPLC (gradient of acetonitrile in 0.2% HCOOH from 12 to 20 % in 25th minute), and this purification gave compound **4** (27 mg). Fraction B_5, after semipreparative HPLC (gradient of acetonitrile in 0.2% HCOOH from 15 to 18 % in 20th minute), gave compound **1** (11 mg). Fraction B_6, was purified on semipreparative HPLC (gradient of acetonitrile in 0.2% HCOOH from 15 to 20 % in 20th minute), gave compounds 7 (20 mg) and **3** (45 mg), respectively. Fraction C was re-chromatographed on silica gel (mobile phase 6:4:0.2 $CHCl_3$:MeOH:H_2O, v/v/v) and this separation resulted in 45 fractions. Pure compound **2** (24 mg) precipitated from fraction C_{34}. 300 mg of fraction E was purified with semipreparative HPLC (gradient of acetonitrile in 0.2% HCOOH from 19 to 22 % in 18th minute), and gave compounds **6** (56 mg). Fraction F was re-chromatographed on silica gel (mobile phase 6:4:0.2 $CHCl_3$:MeOH:H_2O, v/v/v), what resulted in 35 fractions 100 ml each. Pure compound **5** (40 mg) yielded from fraction F_{10} after re-crystallization from MeOH.

4.4. Identification of isolated compounds

NMR spectra were recorded on a NMR Agilent DD2 600 MHz (compounds **1**-**7**) and a NMR Agilent VNMRS 500 MHz (compound **2**) – equipped with four and three channels, respectively, and structure elucidation was carried out using modern 1D and 2D pulse sequences, following 1D and 2D experiments were carried out: ^{1}H, ^{13}C, COSY, HMBC, HSQC, TOCSY, and NOESY. The spectra were processed with MestReNova version 12.0.0. Mass spectra were recorded using a Thermo Scientific Q Exactive Plus quadrupole − Orbitrap mass spectrometer coupled with a UPLC Dionex Ultimate 3000 RSLC system equipped with an RP-18 Kinetex column (2.10 mm × 100 mm, 2.6 µm, Phenomenex Corporation, Torrence, CA, USA). Full-scan data were recorded in negative ESI mode from *m/z* 100 to 1500 at a resolution of 70 000 (at *m/z* 200). Full-scan dd-MS^2 (top 5) was performed at a resolution of 17 500 (at *m/z* 200), AGC target 1^{e5} with maximum IT 30 ms. For HR-MS in positive mode Q-TOF mass spectrometer with ultra-high resolution and high mass accuracy (HRAM) Impact II (Bruker Daltonik, Bremen, Germany). UHPLC Dionex UltiMate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for LC, with mobile phases: 0.1% formic acid (A) and MeOH (B), flow rate: 0.2 mL/min, gradient elution: 0. min 5% of B, 3.6 min 10% of B, 10th min 100% of B. Column block temperature was 35°C, injection volume 5 uL. Kinetex P5, 100A, 1.7um, 100 × 2.1 mm (Phenomenex, Torrance, CA, USA) was used as column. Solution of natrium formate clusters was used as calibration mixture for accurate mass calibration, with MS source settings: end plate offset: 500 V, capillary 4500 V, nebulizer pressure 0.3 Bar, dry gas: 4.0 L/min, dry temperature: 250°C. MS/MS spectra were collected at three collision energy levels of 20, 40, 60 eV per each peak. Data acquisition were carried out by Control 4.0 and HyStar 3.2 software and the results were processed using Compass DataAnalysis 4.3 (all SW of Bruker Daltonik, Bremen, Germany). Chromatography was controlled by Chromeleon Xpress link (Thermo Fisher Scientific, Waltham, MA, USA). For fragmentation analysis

Mass Frontier 7.0.5.9 SR3 (High Chem Ltd., Bratislava, Slovakia) and Thermo Excalibur 3.0.63 (Thermo Fisher Scientific, Waltham, MA, USA) software was used.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1-S77: NMR spectra of compounds **1**-**7**, HRMS of compounds **1**-**7**, HPLC chromatograms of compounds **1**-**7**.

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Sample Availability: Samples of the compounds are not available from the authors.