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Research Article

Liquid chromatography – high resolution mass spectrometry screening of *Astragalus hamosus* and *Astragalus corniculatus*

Aleksandar Shkondrov[1], Ilina Krasteva[1]

1 *Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000 Sofia, Bulgaria*

Corresponding author: Ilina Krasteva (krasteva.ilina@abv.bg)

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Abstract

Astragalus hamosus and *Astragalus corniculatus* were examined for the presence of flavoalkaloids, acylated and highly glycosylated flavonoids. Non-purified extracts of the overground parts of the species were subjected to ultra-high performance liquid chromatography – high resolution electrospray ionisation mass spectrometry (UHPLC-HRESIMS) analysis and the results were compared to authentic reference substances. A flavoalkaloid of kaempferol was newly identified in an extract of *A. hamosus*. In addition, three compounds – quercetin and kaempferol flavonoids, acylated with hydroxymethylglutaric acid and alcesefoliside, were found in extracts of *A. hamosus* and *A. corniculatus* for the first time.

Keywords

Astragalus, qualitative analysis, flavoalkaloids, flavonoids, UHPLC-MS

Introduction

Astragalus hamosus L. (Fabaceae) is a prostrate or ascending annual or biennial herbaceous plant, distributed in Southern Europe, the Mediterranean, Caucasus, Central and Southwest Asia. In Bulgaria it is spread on the Black Sea coast, North-eastern Bulgaria, in Stara Planina Mt., Thracian lowland, the Rhodope Mt., Tundzha hilly plain (Asyov et al. 2012). The flavonols rhamnocitrin-3-*O*-glucoside (Toaima 2002), rhamnocitrin-4′-*β*-D-galactopyranoside, hyperoside, isoquercitrin, astragalin, rhamnocitrin-3-*O*-neohesperidoside (Krasteva et al. 2007; Krasteva 2013) were isolated from the aboveground parts. In callus and suspension cultures of the species rutin, astragalin and isoquercitrin (Ionkova and Alfermann 1990; Ionkova 1995) and later in introduced samples of the plant hyperoside, astragalin and isoquercitrin (Krasteva et al. 2007) were identified. When rhamnocitrin-4′-*β*-D-galactopyranoside was administered with Cisplatin or Gentamicin, a protection of human kidney cells HEK-293T against the cytotoxic effects of nephrotoxic drugs was observed (Krasteva et al. 2008). The antiproliferative activity of a saponin mixture obtained from the herbs of *A. hamosus*, comprised of two saponins, was examined on human malignant cell lines (HL-60, HL-60/Dox, SKW-3, RPMI-8226, U-266 and OPM-2) using the MTT test (Krasteva et al. 2008). It has been found that the saponin mixture caused concentration-dependent suppression of the proliferative activity of malignantly transformed cells, confirmed by ELISA test to assess apoptosis-specific DNA fragmentation (Dineva et al. 2010). Two oleanane-type saponins – peregrinozide I and azukisaponin V from *A. hamosus* did not show cytotoxic activity against various human tumour cells, but a

dose-dependent modulation of lymphocyte proliferation was observed (Verotta et al. 2002). Volatile compounds from the species were tested for cytotoxic activity *in vitro*. A well-defined concentration-dependent antiproliferative activity has been established on REH cells (acute human lymphoid leukaemia) (Momekov et al. 2007). Rhamnocitrin-4′-*β*-D-galactopyranoside exhibited hepatoprotective activity against N-diethylnitrosamine (DENA)-induced liver cancer in Wistar rats (Saleem et al. 2013). A hydro alcoholic extract, as well as hexane and ethyl acetate fractions obtained from the fruits of the plant exhibited anti-inflammatory and analgesic activity in various animal models (Shojaii et al. 2015).

Astragalus corniculatus Bieb. (Fabaceae) is a perennial herbaceous plant with well-developed roots. In Europe the species is distributed in Romania, Ukraine, and Moldova. It is novel to Bulgarian flora and found in the Danube Plain (Pavlova 1988). From the aerial part of the plant tree pentacyclic triterpene saponins were isolated: 3-O-*β*-[4-oxo-*β*-pentopyranosyl-(1→2)-*β*-D-glucopyranosyl]-21*α*-hydroxy oleanoic acid, 3-O-*β*-4-oxo-pentopyranosyl-21*α*-hydroxy oleanolic acid and 3-*O*-*β*-D-xylopyranosyl-3*β*,19*α*-dihydroxy-olean-12-ene-28,21*β*-olide (Krasteva et al. 2006; Krasteva et al. 2007). Flavonoids identified are vitexin, orientin, eryodictiol-7-*O*-glucoside, isorhamnetin-3-*O*-glucoside, rutin, hyperoside, isoquercitrin, isorhamnetin-3-*O*-rutinoside, quercetin, kaempferol, isoramnetin, eriodictiol-7-*O*-rutinoside, quercitrin, floridzin and homoerydictiol (Krasteva 2013). A standardised ethyl acetate extract from *A. corniculatus* was found practically non-toxic (LD_{50} > 5000 mg/kg), showed an antihypoxic effect in a model of chemical hypoxia, and particularly favourably affected circulatory hypoxia (Krasteva et al. 2004). Volatile fractions from the aboveground parts were cytotoxic against a panel of human malignant cells (Krasteva et al. 2008). In series of studies, a purified saponin mixture (PSM) had protective effects against the invasiveness of bone marrow cancer in hamsters. Treatment of animals with implanted tumours with the saponin mixture resulted in an increase in the number, migration, and phagocytic index of peritoneal macrophages and blood polymorphonuclear leukocytes and there was an evidence of an immunostimulatory effect (Toshkova et al. 2007; Toshkova et al. 2008). Statistically significant antioxidant and hepatoprotective activity has been established *in vitro* for the same PSM on enzyme- and non-enzyme-induced lipid peroxidation in liver microsomes derived from spontaneously hypertensive rats (Simeonova et al. 2010). These effects have been confirmed in other *in vivo* and *in vitro* models of hepatic impairment (Vitcheva et al. 2013).

In continuation of our efforts to gain knowledge on the chemical composition of Bulgarian *Astragalus* species, an ultrahigh performance liquid chromatography – electrospray ionisation mass spectrometry (UHPLC-HRESIMS) screening for the presence of rare flavoalkaloids, acylated and highly glycosylated flavonoids in *A. corniculatus* and *A. hamosus* was performed.

Materials and methods

Plant material and extraction

The overground parts of *A. hamosus* were collected in July 2018 from Sofia. The above ground parts of *A. corniculatus* were harvested form Gorna Studena in June 2019. Both plants were in flowering. The identity of the species was confirmed by us (A. S. and I. K.). Voucher specimens were deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences: SOM 1398 (*A. hamosus*) and SOM 1399 (*A. corniculatus*). The samples were dried at room temperature and then 200 mg of each were extracted twice with 2.5 mL 80% MeOH on a water bath for 30 min each (in reflux). The extracts obtained from each sample were filtered, combined in a volumetric flask and the volume adjusted to 10.0 mL with 80% MeOH. After filtration through a membrane PVDF syringe filter (0.22 μm) an aliquot of 2 μL was injected to the UHPLC system.

Ultra high performance liquid chromatography-high resolution electrospray ionization mass spectrometry (UHPLC-HRESIMS)

A Q Exactive Plus Orbitrap mass spectrometer with a heated electrospray ionisation (HESI) ion source (ThermoFisher Scientific, Bremen, Germany) coupled with a UHPLC system (Dionex UltiMate 3000 RSLC, ThermoFisher Scientific, Bremen, Germany) was used. The full scan MS was set at: resolution 70000 (at *m/z* 200), AGC target 3e6, max IT 100 ms, scan range 250 to 1700 *m/z*. The MS^2 conditions were: resolution 17500 (at *m/z* 200), AGC target 1e5, max IT 50 ms, mass range *m/z* 200 to 2000, isolation window 2.0 *m/z* and (N)CE 20. The ionization device (HESI source) was operating at: +3.5 or -2.5 kV spray voltage and 320 °C capillary and probe temperature, 38 arbitrary units (a.u., as set by the Extactive Tune software) of sheath gas and 12 a.u. of auxiliary gas (both Nitrogen); S-Lens RF level 50.0. UHPLC separations were performed on a Kromasil C_{18} column (1.9 μm, 2.1 × 50 mm, Akzo Nobel, Sweden) at 40 °C. The mobile phase was H_2O + 0.1% HCOOH (A) and MeCN + 0.1% HCOOH (B) with a flow rate of 0.3 mL/min. Elution was as follows: 10% B for 0.5 min, increase to 30% B for 7 min, isocratic with 30% B for 1.5 min, increase to 95% B for 3.5 min, isocratic with 95% B for 2 min, return to 10% B for 0.1 min. Detection of the compounds in plant samples was performed in both the positive and the negative ionisation mode by a set range of *m/z* of the corresponding protonated or deprotonated molecule with a time filter, adjusted to the retention time of each standard. Identification was supported by MS^2 experiments which revealed the aglycone part of the molecule as well as the successive loss of monosaccharides of the sugar moiety. The fragmentation pattern was compared to that of the reference substances. The software Xcalibur, Version 4.2 (Thermo Scientific) was used for data collection and processing.

Reference substances

From *A. monspesulanus* subsp. *monspesulanus* *N*-(8-methylquercetin-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidine-2-one (**1**), *N*-(8-methylkaempferol-3-O-[α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranosyl])-3-hydroxypiperidine-2-one(**2**), quercetin-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside] (**3**), kaempferol-3-O-α-L-rhamnopyranosyl-(1→2)-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-D-galactopyranoside] (**4**), quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside (alcesefoliside, **5**) were obtained by a previously described procedure (purity more than 95%); from *A. glycyphyllos* kaempferol-3-*O*-[2-*O*-β-D-galactopyranosyl-6-*O*-α-L-rhamnopyranosyl]-β-D-glucopyranoside (camelliaside A, **6**) (purity 98%) was isolated as reported before. The compounds were identified by extensive MS and NMR analyses (Krasteva et al. 2015; Shkondrov et al. 2020). Rutin (**7**) was purchased form Sigma Aldrich (Germany) and used to confirm the accuracy of the analysis. Standard solutions of each reference substance were prepared in MeOH (1000 ng/mL). Two μL of each solution were injected in the UHPLC-HRESIMS system three times to obtain the mean retention time.

Results and discussion

Non-purified extracts of the aerial parts of both species were investigated by LC-MS for the presence of flavoalkaloids, acylated and highly glycosylated flavonoids, using comparison with selected reference substances (both the retention time and the fragmentation pattern). The results are presented in Table 1.

Table 1. Compounds identified in the samples.

Compound*	*A. hamosus*	*A. cornicilatus*
1	not found	not found
2	present	not found
3	present	present
4	present	present
5	present	present
6	not found	not found
7	present	not found

*see *Reference substances.*

Compounds in *A. hamosus*

Six compounds were identified in the extract (Fig. 1) for the first time. The quercetin flavoalkaloid (**1**) was not present in the sample – neither a peak with t_R corresponding to the reference (2.47 min) was found, nor a protonated or a deprotonated molecule with the corresponding *m/z* of that of the reference (*m/z* 884, $[M+H]^+$ or *m/z* 882, $[M-H]^-$) was identified. The kaempferol-derived flavoalkaloid **2** was found and had a t_R of 3.00 min and matching fragmentation patterns in both ionization modes to that of the reference compound. In the negative mode a chlorine adduct (*m/z* 901.2643, $[M+Cl]^-$) and the kaempferol moiety in the MS^2 (*m/z* 284.0328) were proved. The protonated molecule was in a very low abundance with *m/z* 868.2852. The hydroxymethyl acylated flavonol **3** was proved in the sample with t_R of 5.79 min and its fragmentation coincided with the reference in both ionization modes. In the negative mode an ion, corresponding to the deprotonated molecule $[M-H]^-$ (*m/z* 753.1900) and in the MS^2 a quercetin fragment (*m/z* 301.0336), also a characteristic fragment for flavonols with *m/z* 151, after retro Diels-Adler reaction (RDA) were recorded. In the positive mode, the protonated molecular ion (*m/z* 755.2036) and in the MS^2 again a quercetin fragment (*m/z* 303.0501) were observed. The kaempferol flavonoid acylated with hydroxymethylglutaric acid (**4**) had a t_R

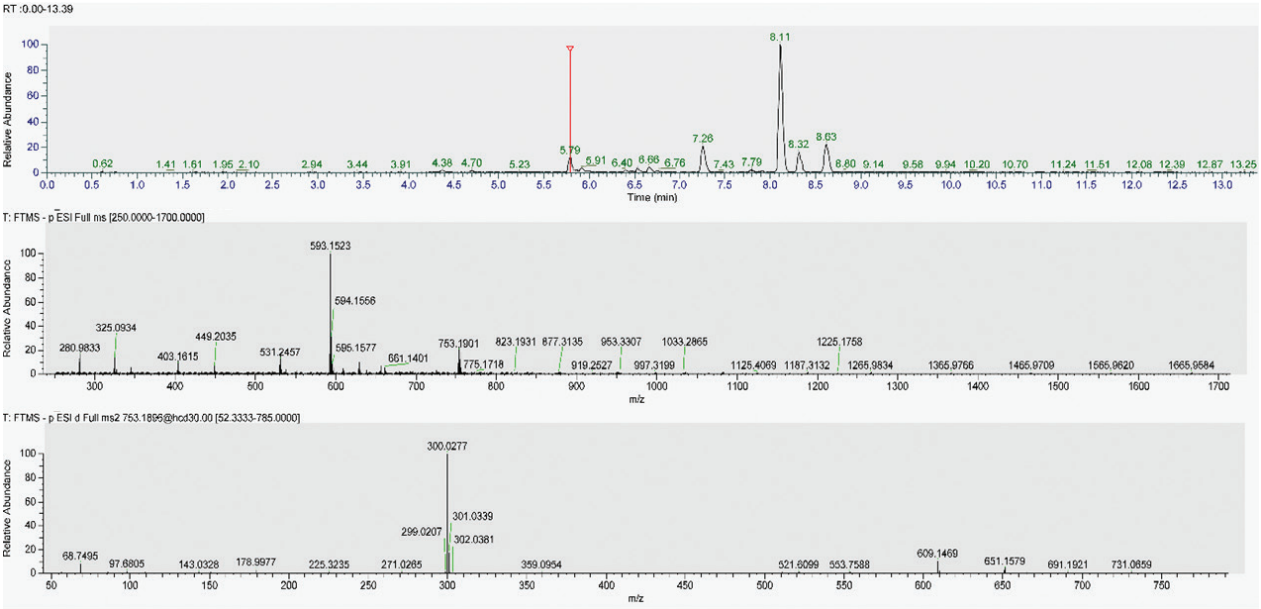

Figure 1. A base peak chromatogram of the extract of *A. hamosus* with identification of **3** (*m/z* 753 $[M-H]^-$) and MS^2 spectrum displaying quercetin fragment (*m/z* 301).

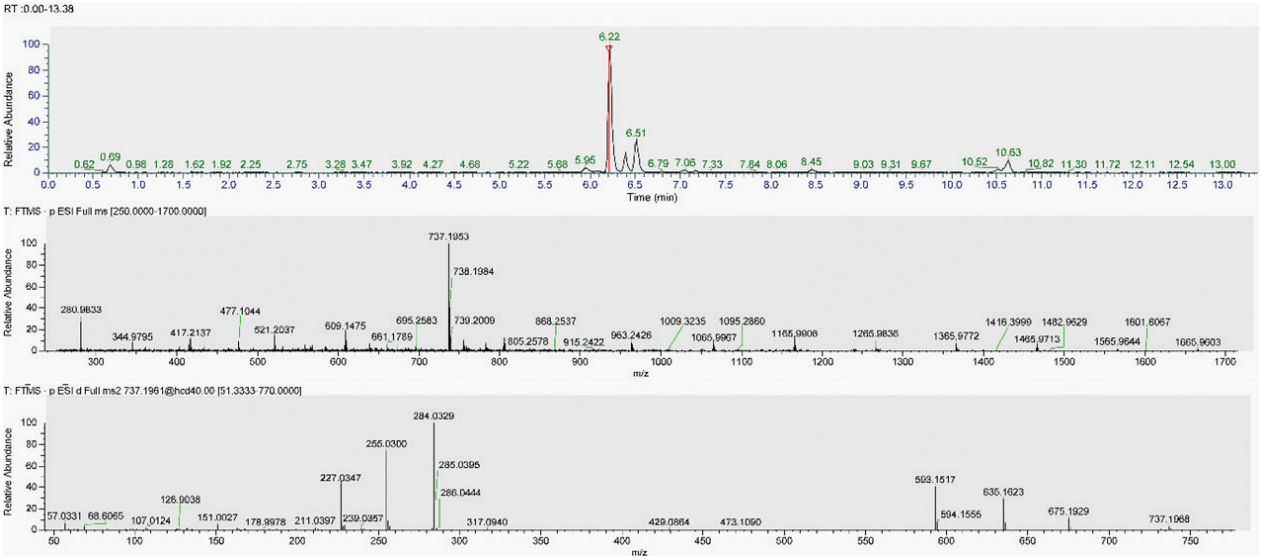

Figure 2. A base peak chromatogram of the extract of *A. corniculatus* with identification of **4** (*m/z* 737 $[M-H]^-$) and MS^2 spectrum displaying kaempferol fragment (*m/z* 284).

of 6.21 min, with fragmentation identical to the reference substance in both polarities. In the negative mode an ion $[M-H]^-$ with *m/z* 737.1953 and in the MS^2 fragment ions of kaempferol (*m/z* 284.0329) and a flavonol-specific ion (*m/z* 151, RDA) were found. In the positive mode the compound was protonated (*m/z* 739.2087) with the cleavage of kaempferol moiety (*m/z* 287.0551) in the MS^2. In the sample alcesefoliside (**5**) was identified as well. The compound had a t_R of 4.94 min and the same fragmentation to that of the reference substance. In the negative mode an ion $[M-H]^-$ (*m/z* 755.2062) was observed which in the MS^2 gave quercetin (*m/z* 300.0279) and a flavonol-specific ion after RDA (*m/z* 151); whilst in the positive mode, a protonated molecule $[M+H]^+$ (*m/z* 757.2195) and quercetin fragment (*m/z* 303.0050) were registered. Camelliaside A (**6**) was not identified in the sample. There was no peak with t_R of **6** (4.99 min) and no ions with *m/z* corresponding to the protonated or deprotonated molecule (*m/z* 757, $[M+H]^+$ or *m/z* 755, $[M-H]^-$). In the sample **7** had a retention time of 5.69 min, which in the negative mode gave $[M-H]^-$, (*m/z* 609.1474), a quercetin fragment *m/z* 301.0347 and the RDA related ion (*m/z* 151), while in the positive mode it was registered as a protonated molecule (*m/z* 611.1601), which fragmented to the same aglycone (*m/z* 303.0496). Rutin was previously found only in suspension cultures of *A. hamosus* (Ionkova and Alfermann 1990).

Compounds in *A. corniculatus*

Only three of the investigated compounds were found in the extract for the first time (fig. 2). The acylated flavonoids (**3** and **4**) were both present with t_R and fragmentation patterns corresponding to the standards (see compounds in *A. hamosus*). In addition, alcesefoliside **5** was discovered in the sample as well. Again, its fragmentation pattern and t_R coincided to the reference substance (see above). The compounds **1**, **2**, **6** and **7** were not found in the sample.

Conclusion

Using a highly sensitive UHPLC-HRESIMS method a flavoalkaloid of kaempferol, quercetin and kaempferol flavonoids, acylated with hydroxymethylglutaric acid and alcesefoliside, were newly identified in extracts of *A. hamosus* and *A. corniculatus*. The presence of the quercetin flavoalkaloid and camelliaside A was not proved in any of the samples.

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