



Phenolic Compounds in Four *Astragalus* Species

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Author's contribution

The only author performed the whole research work. Author INK wrote the first draft of the paper. Author INK read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To investigate the phenolic compounds in four *Astragalus* species (*A. hamosus*, *A. ponticus*, *A. corniculatus* and *A. cicer*) distributed in Bulgarian flora.

Study Design: Using LC-MS, HPLC, UV, NMR and HRESIMS for identification of the compounds.

Place and Duration of Study: Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, Bulgaria, between May 2009 and December 2012.

Methodology: LC/MS/MS analysis was performed using Agilent 1100 and API 365 tripequadrupole mass spectrometer. HPLC was carried on a Shimadzu LC-10 Advp chromatographic system included UV-VIS detector SPD. The structure of the flavonoid isolated from *A. hamosus* was determined by acid hydrolysis, UV, MS and NMR.

Results: Seven phenolic compounds were identified in *A. ponticus*, four in *A. corniculatus* and three in *A. cicer* by LC/MS/MS and HPLC. The structure of one flavonoid was established on the basis of UV, NMR and HRMS data as rhamnocitrin-3-O-neohesperidoside.

All identified compounds are new for the species and rhamnocitrin-3-O-neohesperidoside – for the genus *Astragalus*.

Keywords: *Astragalus corniculatus*; *Astragalus ponticus*; *Astragalus cicer*; *Astragalus hamosus*; phenolic compounds.

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1. INTRODUCTION

Astragalus L., the largest genus in the family Fabaceae, is represented by 29 species in the flora of Bulgaria [1]. Many *Astragalus* plants are used in traditional medicine as diuretic, tonic, emollient, antiperspirant, laxative, carminative, antihypertensive, in diabetes treatment, etc. [2,3]. Some of Bulgarian *Astragalus* species have been studied for their saponin and phenolic content, and their biological activity [4,5,6,7].

Chemical study of *A. hamosus* has indicated the presence of peregrinoside I, azukisaponin V [8] and rhamnocitrin-3-O-glucoside [9]. Rutin, astragaloside, and isoquercitrin have been identified in callus and suspension cultures of the plant by HPLC [10]. Our previous investigation led to the isolation of a new flavonoid rhamnocitrin 4'- β -D-galactopyranoside together with hyperoside, isoquercitrin, and astragaloside from the introduced samples of the species [11]. The cytotoxicity of this flavonoid and saponin mixture from *A. hamosus* was also determined [12].

A. corniculatus is a new species for Bulgarian flora [13]. Our earlier investigations of the ethyl acetate extract obtained from the species resulted in a low acute oral toxicity and a remarkable antihypoxic activity, especially in a model of circulatory hypoxia [14]. Later nine flavonoids were identified in the extract [15]. In addition three new saponins were isolated from the butanol extract of aerial parts of *A. corniculatus* [16]. It was found that purified saponin mixture, containing these saponins has immunostimulating and immunorestorating activity on the T- and B-spleen cells in Graffi tumor bearing and healthy hamsters [17]. Nine known flavonoids and D-pinitol were identified in the aerial parts of *A. ponticus* [18,19]. One flavon, two flavonols, four isoflavonoids and one pterocarpan were isolated from *Astragalus cicer* [6,7].

In a continuation of our phytochemical studies on Bulgarian *Astragalus* species, this work describes the identification of phenolic compounds in four *Astragalus* species - *A. ponticus*, *A. corniculatus*, *A. cicer* and *A. hamosus*.

2. MATERIALS AND METHODS

2.1 General Experimental Procedures

Liquid chromatography coupled with ion spray mass spectrometry in the tandem mode (LC/MS/MS): LC analysis was performed using Agilent 1100 (Hewlett Packard). An Aqua C₁₈ 125 A (150x3.0 mm i.d., 5 μ l) (Phenomenex, Torrance, CA, USA) column was used. Gradient elution was carried out with water-0.1% formic acid v/v and water-acetonitrile-0.1% formic acid v/v at a constant flow rate of 400 μ l/min-1. The MS and MS/MS data were obtained using an API 365 tripe-quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, ON, Canada). All the analyses were performed by a Turbo Ionspray source. The operating parameters as follows: capillary voltage – 3500 V, nebulizer gas (N₂; 10 arbitrary units), curtain gas (N₂; 8 arbitrary units), drying gas (N₂; 7000 cm³ /min-1), collision gas (N₂; 5 arbitrary units), focusing potential – 240 V and entrance potential 10 V. The collision energy (CE) and declustering potential (DP) were optimized for each standard.

HPLC was performed on Shimadzu 10 Advp (Japan) chromatographic system including UV-VIS detector SPD with wavelength set at 254 nm. A Tracel Excel RP-C₁₈ ODS-2, 5 μ m (250 x 4.6 mm) column was used. The mobile phase was composed of methanol-water in

different proportions (Table 2). Isocratic elution was carried out at a constant flow rate of 1 ml/ min⁻¹.

UV spectra were recorded on a "WPA-LIGHTWAVE" spectrometer with diagnostic shift reagents. NMR data were obtained on Bruker DRX 500 or Bruker AVANCE 700 spectrometers (Bruker, Germany). Chemical shifts were referenced to solvent peaks. ESI-TOF was carried out on an Agilent 6210 ESI-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

TLC study was performed on silica gel plates (Kieselgel 60 F₂₅₄, Merck). The spots were visualized by spraying with 1% methanolic solution of diphenylboric acid aminoethyl ester (NST). Column chromatography (CC) was carried out with Polyamid S (Riedel de Haën, Germany), Sephadex LH-20 (Pharmacia, Sweden), Silica gel and flash CC over Silica gel 60 C18 (40–63 mm, Merck, Darmstadt, Germany).

2.2 Plant Materials

The aerial parts of *A. cicer*, *A. ponticus* and *A. corniculatus* were collected during the flowering period in Southern parts of Sofia region and North Bulgaria, near Pleven. The plant material from introduced seeds samples of *A. hamosus* was collected at the Experimental field, Institute of Botany, BAS Sofia.

The plant materials were identified by Dr D. Pavlova, Department of Botany, Faculty of Biology, Sofia University. The voucher specimens have been deposited in Herbarium of Sofia University - *A. cicer* (SO 102681), *A. ponticus* (SO 95177), *A. corniculatus* (SO95265) and *A. hamosus* (SO 102680).

2.3 Sample Preparation for LC/MS/MS and HPLC Analysis

The aqueous/alcoholic extracts obtained from the overground parts of *A. ponticus* (600 g) and *A. corniculatus* (800 g) were treated successively with CHCl₃ and EtOAc. The EtOAc extract was further purified by repeated column chromatography over Polyamide using H₂O–EtOH gradient (0–90% EtOH, v/v) and Sephadex LH-20 with MeOH. After TLC analysis some of purified fractions, rich of phenolic compounds, were analysed by LC/MS/MS.

The aerial parts of *Astragalus cicer* (840 g) was exhaustively extracted with 80% MeOH. After partial evaporation the aqueous solutions were extracted with CH₂Cl₂ and EtOAc successively. The residue from the EtOAc layer was separated on Sephadex LH-20 column eluting with MeOH. Two main purified fractions were obtained and analysed by HPLC.

2.4 Isolation of Compound 1 from *A. hamosus*

Air-dried powdered aerial parts of *A. hamosus* (500 g) were defatted with *n*-hexane and extracted with MeOH/H₂O (9:1) and (1:1). The extracts were filtrated, concentrated under reduced pressure, and successively partitioned with CHCl₃, EtOAc, and *n*-BuOH. The *n*-BuOH extract was submitted to CC on Sephadex LH-20 and gave three main fractions (I-III). Fraction II was further purified by flash CC over Silica gel and RP-18, followed by preparative TLC (ethyl acetate-methyl ethyl ketone-formic acid-water, 5:3:1:1) to afford compound 1 (18 mg).

2.4.1 Rhamnocitrin-3-O-neohesperidoside (1)

Yellow powder; UV (MeOH) λ_{max} : 268, 352; (+NaOMe) 268, 385; (+NaOAc): 268, 352; (+NaOAc+H₃BO₃): 268, 352; (+AlCl₃): 275, 354, 404; (+AlCl₃+HCl): 278, 354, 404; HRESIMS m/z 631.1621[M+Na]⁺ (calcd 631.1503 for C₂₈H₃₂O₁₅Na); ¹H- and ¹³C NMR (¹H: 500 MHz; ¹³C: 100.6 MHz, methanol-d₄) data as reported in ref. 25 and 26.

2.4.2 Acid hydrolysis

A methanolic solution of the compound **1** (2 mg) was refluxed with 2 N HCl (5 mL) for 1 h. The MeOH was evaporated, the mixture was diluted with H₂O, and the hydrolysate was partitioned between EtOAc and H₂O. The aglycone-containing organic phase and aqueous layer were concentrated and analysed by co-TLC with authentic samples. The aglycone was found to be identical with rhamnocitrin. Two sugars were identified as glucose and rhamnose.

3. RESULTS AND DISCUSSION

Purification of the ethanol extract from *A. corniculatus* and *A. ponticus* by repeated column chromatography yielded fractions rich in phenolic compounds, which were studied by LC/MS/MS. The optimum conditions were applied to the identification of the compounds [15]. After MRM analysis kaempferol, isorhamnetin, isorhamnetin-3-O-glucoside, isorhamnetin-3-O-rutinoside, vitexin, eriodyctiol-7-O-rutinoside and phloridzin were determined in *A. ponticus* and quercetin-3-O-rhamnoside (quercitrin), homoeriodyctiol, eriodyctiol-7-O-rutinoside and phloridzin in *A. corniculatus* (Table 1). Fragmentation of aglycones provided characteristic ions for each family of flavonoids [15,20,21]. In the spectra of the flavonol and flavanone glycosides present both ions – the deprotonated molecule [M-H]⁻ of the glycosides and the ion corresponding to the deprotonated aglycone [A-H]⁻. Apigenin-8-C-glucoside (vitexin) was identified on the bases of the product ion spectrum and comparison with literature data [20]. For flavone-C-glycosides the characteristic ions are at m/z 431 (deprotonated molecule), m/z 341 (loss of 90 u) and m/z 311 (loss of 120 u) [21].

Table 1. Phenolic compounds identified in *A. corniculatus* and *A. ponticus* by LC/MS/MS

Compounds	t _r (min)	Molecular ion [M-H] ⁻ (m/z)	Characteristic Product ions (m/z)	Plants
Eriodyctiol-7-O-rutinoside	13.74	595	287,151	<i>A. ponticus</i> and <i>A. corniculatus</i>
Isorhamnetin-3-O-rutinoside	14.10	623	315,151	<i>A. ponticus</i>
Isorhamnetin-3-O-glucoside	14.74	477	315,151	<i>A. ponticus</i>
Quercetin-3-O-rhamnoside (quercitrin)	14.97	447	301	<i>A. corniculatus</i>
Phloridzin	15.74	435	273	<i>A. ponticus</i> and <i>A. corniculatus</i>
Vitexin	17.75	431	341,311,269	<i>A. ponticus</i>
Homoeriodyctiol	21.00	301	151	<i>A. corniculatus</i>
Kaempferol	21.20	285	151	<i>A. ponticus</i>
Isorhamnetin	21.50	315	300,151	<i>A. ponticus</i>

Two purified fractions were obtained from ethyl acetate extract of *Astragalus cicer* and analysed by HPLC. The prescribed method is based on literature with some modifying elements – changed mobile phases and column [22]. In order to achieve an assuredly results the reference substances were tested in different mobile phases. As usual, acetonitrile-water based phase was chosen at first. It can be concluded that the compounds showed very short and inappropriate retention times (acetonitrile-water, 70:30 v/v) t_R (Rutin) = 2.16 min; t_R (Hyperoside) = 2.16 min). Obviously, methanol-water mobile phases gave sufficient and adequate separation of the examined compounds. Results showed retention times which confirm that the HPLC method is suitable for routine analysis. Mobile phase methanol-water (60:40 v/v) was the most suitable for studying fractions containing rutin and hyperoside. For the fraction containing umbelliferone the most suitable is mobile phase methanol-water (70:30 v/v). Rutin was identified using both mobile phases as described in Table 2. The conditions used in this experiment are very suitable for simultaneous determination of both compounds without preliminary separation. This is another proof that the above described method could easily be used in every day analysis of plant based products. After HPLC analyses by comparison of their retention time with those of the standards three compounds were identified as umbelliferone, hyperoside and rutin.

Table 2. Identified compounds in *Astragalus cicer* by HPLC

Compound	Mobile phase	Rt (min)
Rutin	Methanol-Water (60:40 v/v)	4.40
Hyperoside	Methanol-Water (60:40 v/v)	4.73
Rutin	Methanol-Water (70:30 v/v)	6.30
Umbelliferone	Methanol-Water (70:30 v/v)	7.10

One flavonoid **1** was isolated from ethyl acetate extract of *A. hamosus* by repeated column chromatography over different sorbents and preparative TLC. UV spectral data of compound **1** with diagnostic shift reagents suggested the likely presence of 3,7-disubstituted flavonoid glycoside with free hydroxyl groups at 5 and 4 positions [23,24]. Acid hydrolysis of **1** gave rhamnocitrin, glucose and rhamnose. The compound **1** exhibited in HRESIMS (positive-ion mode) a pseudo-molecular ion peak at m/z 631.1621 $[M+Na]^+$ (calcd 631.1503), consistent with molecular formula of $C_{28}H_{32}O_{15}Na$. Other fragment ion peak was observed at m/z 301.0711 indicating the loss of rhamnose and glucose. The obtained 1H and ^{13}C NMR data of **1** were comparable with those reported for rhamnocitrin-3-O-neohesperidoside (Fig. 1) [25,26].

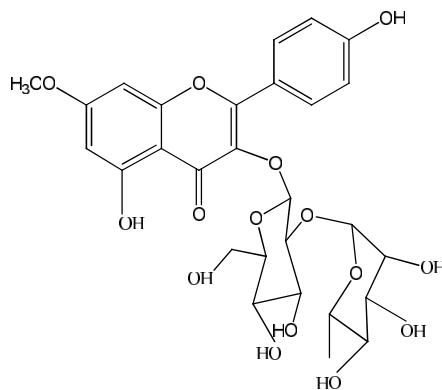


Fig. 1. Structure of 1

As a part of our ongoing project on Bulgarian *Astragalus* plants, four species (*A. ponticus*, *A. corniculatus*, *A. cicer* and *A. hamosus*), which belong to different subgenus, were studied for phenolic compounds. Up to now 11 *Astragalus* species, growing in Bulgaria have been investigated mostly for saponins and phenolic compounds [5-8,11,15-16,18]. The obtained results were compared with data from previous phytochemical studies of *A. ponticus*, *A. corniculatus* and *A. cicer* [6,11,15,18,19]. The analysis showed that all compounds were identified for the first time in the species. In the literature there are data of isolated rhamnocitrin and some rhamnocitrin glycosides from *Astragalus* species [5,7,15]. However this is the first report for isolation of rhamnocitrin-3-O-neohesperidoside from the genus *Astragalus*.

4. CONCLUSION

LC/MS/MS analysis of *A. ponticus* and *A. corniculatus* led to the identification of nine flavonoids. Two flavonoids and one coumarin were determined in *A. cicer* by HPLC. The structure of one flavonoid from *A. hamosus* was established on the basis from UV, NMR and MS.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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